BACTERIAL VAGINOSIS, A MODEL OF TRUE POLYMICROBIAL INFECTIONS: GENETICS, EVOLUTION, CLINICAL AND SOCIO-CLINICAL IMPLICATIONS

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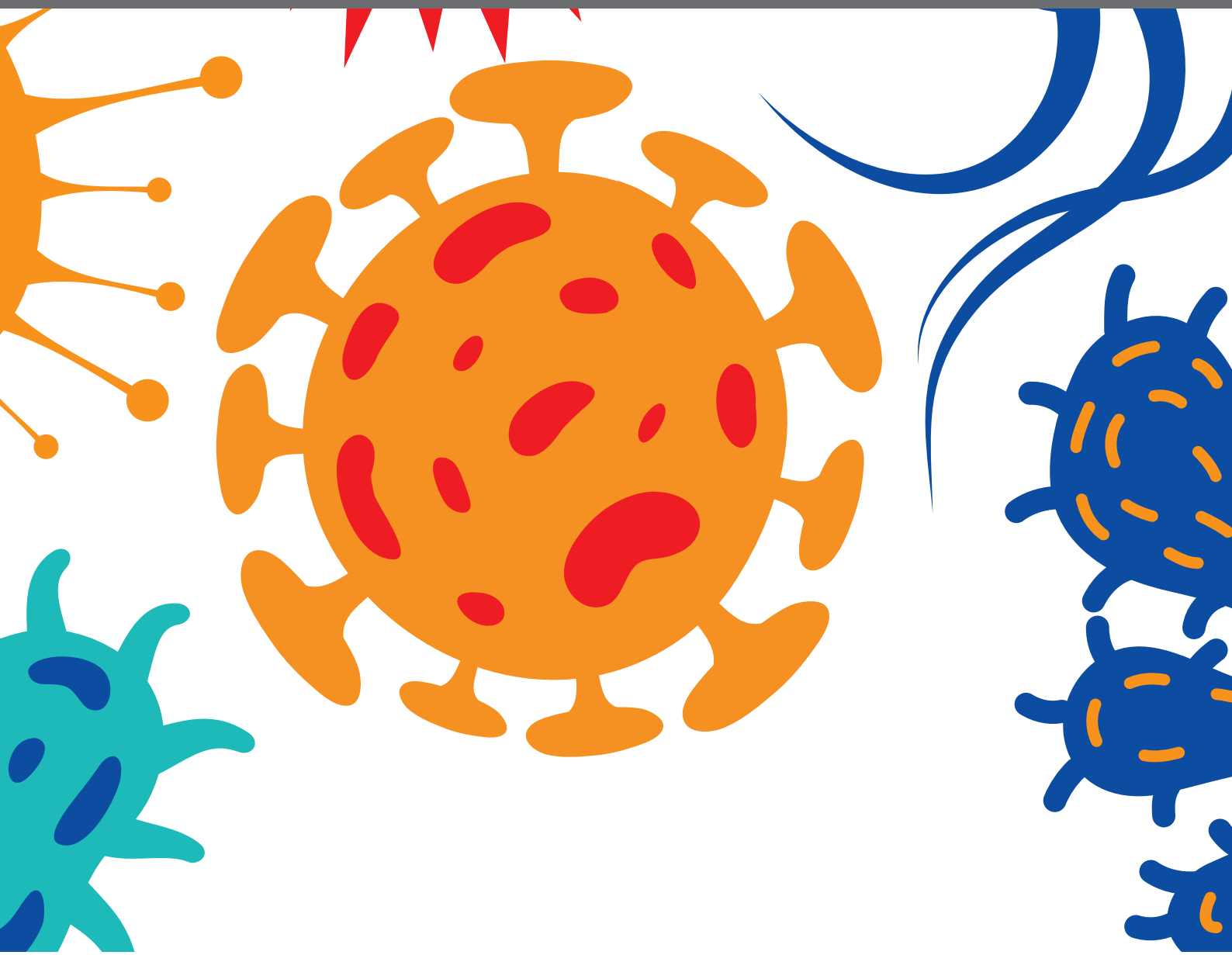

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BACTERIAL VAGINOSIS, A MODEL OF TRUE POLYMICROBIAL INFECTIONS: GENETICS, EVOLUTION, CLINICAL AND SOCIO-CLINICAL IMPLICATIONS

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PERSPECTIVE
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The Need to Focus on Therapy Instead of Associations

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Molecular analyses of the vaginal microbiota have uncovered a vast array of organisms in this niche, but not so far changed what has been known for a long time: lactobacilli are dominant in health, and the diagnosis and treatment of symptomatic bacterial vaginosis is sub-optimal, and has not changed for over 40 years. While the lowering cost of DNA sequencing has attracted more researchers to the field, and bioinformatics, and statistical tools have made it possible to produce large datasets, it is functional and actionable studies that are more urgently needed, not more microbial abundance, and health or disease-associative data. The triggers of dysbiosis remain to be identified, but ultimately treatment will require disrupting biofilms of primarily anaerobic bacteria and replacing them with the host's own lactobacilli, or health-promoting organisms. The options of using probiotic strains to displace the biofilms and for prebiotics to encourage resurgence of the indigenous lactobacilli hold great promise, but more researchers need to develop, and test these concepts in humans. The enormity of the problem of vaginal dysbiosis cannot be understated. It should not take another 40 years to offer better management options.

Keywords: vaginal dysbiosis, microbiota, *Lactobacillus*, probiotics, prebiotics

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With the advent of less expensive microbial sequencing methodologies, the volume of data being produced on which microbes inhabit the vagina, bladder, and other human body sites increases exponentially. But are these leading us closer to improved treatment or are they just delaying this long-awaited step?

A recent study of vaginal samples from 45 pregnant women who delivered preterm and 90 who delivered at term (Fettweis et al., 2019) concluded an association between *Sneathia amni* and a few other bacteria along with depleted *Lactobacillus crispatus*. As intensive as the study was in terms of human-power and techniques used, the impressive processing of over 200,000 samples from women and neonates, and the enormity of the expense, the end result is an unproven cause and effect with no path to practical action.

If indeed anaerobic bacteria such as *Sneathia*, are responsible for preterm birth, why have studies using antibiotics failed to prevent this outcome? If adequate clinical trials have not fully tested this theory, what agents are available to specifically target, and eradicate anaerobes such as *Sneathia*? The answer is none. Indeed, the pharmaceutical armamentarium for treating vaginal dysbiosis or symptomatic bacterial vaginosis (BV) has been inadequate for 40 or more years. There lies the real problem.

For all the vast number of characterizations of the vaginal microbiota, few surprises have emerged. Indeed, culture analysis from decades ago have informed us that lactobacilli are dominant in health in most women, and depleted and replaced by anaerobes or other pathogens in "disease" (Pfau and Sacks, 1977; Hill et al., 1984).

In a recent report, the "disease" of BV was questioned as being a catch-all for several conditions, one of which may be infection (Reid, 2018). This therefore counters the very topic of this collection of papers and states that BV is not in fact a true polymicrobial infection. Rather, one component of the term includes an infectious state, but many other conditions that are not infectious or symptomatic are still referred to as BV. A call to re-define BV was made. One sub-group certainly fits as being an infection requiring treatment, but this relies on appropriate diagnosis which is currently far from effective.

FAILURE OF DIAGNOSTIC TECHNIQUES

Indeed, credit to Fettweis et al. for not using the Nugent score (Gram stain assessment of bacteria on vaginal cells) or Amsel criteria (discharge, microscopic assessment of cells, whiff test and pH) to diagnose BV as they depend on the experience of the reader and even then cannot distinguish bacterial types effectively. Instead, they use the term "dysbiosis" as we proposed 4 years ago (McMillan et al., 2015). The result was that changes in bacterial diversity and taxa abundance were reported.

The net effect of this for clinical practice, however, is that assessment of patients would require analysis of their vaginal swab using a system that presently is not rapid, easily available, or affordable. This again speaks to the actionable outcomes of such studies. In the not so distant future, rapid "diagnostic" tools will be developed to identify the microbes present on vaginal swabs, and more importantly the metabolic profile. The identification of molecules like gamma hydroxybutyrate or amines (McMillan et al., 2015) could well-prove more useful for clinical assessment. But as noted above, given the lack of targeted therapies, this would still be insufficient to optimally manage the patient.

TOO MUCH EMPHASIS ON COMMUNITY STATE TYPES AND NOT ENOUGH ON RESTORATION AND MAINTENANCE OF A HEALTHY VAGINA

One of the outcomes of microbiota studies has been an effort to try and create community state types (Ravel et al., 2011). The original five types are now up to 13 and could increase further once more samples are processed. The problem is these are based on statistical associations rather than a direct means to identify patients who require intervention. Given the therapeutic options available, essentially metronidazole and clindamycin, and the lack of targeted drugs to only kill *Sneathia, Atopobium, Prevotella,* or whatever other etiological agent falls into one of the state types, then the groupings are not informative.

Rather, the issue that has existed for decades is why do these organisms primarily anaerobes, propagate and form biofilms (Swidsinski et al., 2013), and how can this be prevented or better treated so that ecological equilibrium is restored? The trigger for a *L. crispatus*-dominated vaginal microbiota to be displaced and replaced by *Gardnerella* and these other anaerobes is completely unknown. Douching, sexual intercourse, spermicides, stress, urinary tract infections and numerous other "causes" have been proposed (Hickey et al., 2012) but again these are associations and not molecularly proven triggers. Research is needed to answer this important question.

The emphasis on *L. crispatus* as being "protective" because it is most associated with a healthy vagina and therefore the optimal choice as a probiotic intervention, is not particularly based on properties of the species. Indeed, selection of candidate probiotic strains seems to be their ability to adhere to vaginal cells and produce lactic acid and hydrogen peroxide (Hemmerling et al., 2009). Genomic and metabolomic studies have shown that there are significant differences between *L. crispatus* strains (van der Veer et al., 2019; Watson et al., unpublished), so some selection criterion is needed before expecting a strain to be effective. If the species is so protective, why is it apparently readily displaced by anaerobes? Arguably strains with anti-pathogen properties capable of disrupting BV biofilms would be a better choice, irrespective of their species (McMillan et al., 2011). This was the rationale for developing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 for urogenital application with positive clinical outcomes reported (Reid, 2017). At the very least, it was an attempt at intervention, prevention and even treatment based upon strain properties with a rationale (Reid, 2017; Petrova et al., 2018).

THE OPTIONS ARE NOT FUTURISTIC

Some researchers are reluctant to go to human studies until the ultimate "magic bullet" has been found. I would argue that this discovery will never happen, but in the meantime hundreds of millions of women with continue to suffer from malodour, discharge, and the other signs, and symptoms that occur with dysbiosis.

This is not to suggest the testing of products with no plausible documentation as to why they would work. Safety and ethics are paramount.

But, if after all this time we know the problem (malodour, discharge, discomfort) that takes women to seek medical assistance, and we know the causative agents are anaerobic bacteria, plus we know that "normal" is a lactobacilli-dominated vagina, why have more therapies not been developed?

Part of the answer lies within the regulatory system that in Europe and the United States takes the outdated view that only drugs can treat, prevent, cure or mitigate disease. Thus, even if a probiotic food or supplement was to show disease efficacy, it would not be allowed to make that claim until it had been registered and processed as a drug. This almost always requires animal sacrificies, and with no animal model for vaginal dysbiosis, this becomes a problem for developing therapies. The very nature of regulatory agencies means that the assessment tends to fall under biological agents as if lactobacilli generally regarded as safe were somehow equivalent to *Salmonella*. The end result is a process of drug approval that is enormously expensive and time-consuming. As the profit margins of food and supplement companies is invariably significantly smaller than for drug companies, this acts a deterrent.

Clearly pharmaceutical companies have so far avoided developing new vaginal therapies, in part because no suitable classes of antibiotics have emerged, and because there is a

perception that probiotic strains and products cannot be patent protected to allow market exclusivity. The latter is of course incorrect and companies like BioGaia have been operating for decades based upon intellectual property for *L. reuteri* strains. In terms of return on investment, the enormity of the clinical problem and lack of suitable treatments should be sufficient for companies, and the profitability of clotrimazole, and miconazole nitrate over-the-counter treatments for vulvovaginal candidiasis serves as a precedent.

Some probiotic strains are available as food or supplements with the intent of either reaching the vagina via ascension from the rectum or reducing pathogen re-seeding, or through a systemic immune, or other mechanism. Unfortunately, a consequence of current regulations is that non-drug products, even those highly documented in human studies, cannot be differentiated on the label from those with no evidence of benefit. This makes it difficult for women and physicians to know the studies that support treatment, prevention or mitigation of disease. As physicians are trained to prescribe drugs and there is oversight in manufacturing and an expectation of safety and effectiveness with them, they may dismiss probiotics *per se* rather than considering some for their potential value to the patient. A clinical guide has been prepared by a group of experts who reviewed only products available in Canada (and another guide for the U.S.) that have been tested in humans. The list gives the level of evidence and citations are provided to help consumers and healthcare providers select documented probiotics (Clinical Guide to Probiotic Products Available in Canada, 2019).

In Canada at least, three ovule products for vaginal insertion have been approved. These comprise *L. acidophilus* A-212, with *L. rhamnosus* A-119, and *S. thermophilus* A-336; *L. rhamnosus* Lcr35; and *L. rhamnosus* PBO1, *L. gasseri* EN-153471 (EB01). The clinical evidence in support of their approval by Health Canada for use in BV is not readily available on sources such as PubMed, so it is unclear how the review panel that prepares the Clinical Guide had sufficient data to include them in the list. The exception is a study of Lcr35 administered daily for 7 days as a suppository following clindamycin treatment of BV (Petricevic and Witt, 2008). The outcome was only assessed at 4 weeks with 69 of 83 women (83%) having at least a five-grade reduction of the Nugent score compared to 31 of the 88 women (35%) in the control group. The selection of the strain appeared to be primarily based on its intestinal probiotic activity plus the possession of a bacteriocin against *Gardnerella vaginalis* (Turovskiy et al., 2009). It is unusual for a Gram-positive bacterium to produce a bacteriocin against a Gram-negative species, so it is unfortunate that the killing of *G. vaginalis* following Lcr35 application was not verified in the human study. Such mechanistic evidence and a longer follow-up would be more convincing in supporting the use of this product to treat BV without antibiotics, or to prove it allows recovery of healthy microbiota to equilibrium after drug therapy.

Only one strain combination, *L. rhamnosus* GR-1, and *L. reuteri* RC-14, has been tested to actually cure symptomatic BV without the use of antibiotics. In a randomized trial, 40 women diagnosed with BV by discharge, fishy odor, sialidase positive test, and Nugent Gram stain scoring, were randomized to receive the probiotic in capsules daily for 5 days, or 0.75% metronidazole gel, applied vaginally twice a day (in the morning and evening) (Anukam et al., 2006). The 90% cure rate with probiotics was superior to the antibiotic. If this result was to be confirmed in a second, larger study, it would provide strong evidence that probiotics can be an alternative to antibiotics.

In terms of issues of safety, these of course cannot be overlooked no matter the regulatory path. The administration of live organisms to humans is never completely without risk, and the ease of eradication of lactobacilli, and bifidobacteria by antibiotics reduces the risk of serious complications. Some risk can be mitigated by carefully testing the strains for virulence factors or drug resistance before their application to at-risk patients. Nevertheless, there are cases of both these genera being associated with infection (Omar et al., 2019; Pruccoli et al., 2019). Thus, recommendations as to how to deal with higher risk recipients of probiotic strains should be considered (Sanders et al., 2016). The potential to use prebiotic compounds (a substrate that is selectively utilized by host microorganisms conferring a health benefit) (Gibson et al., 2017) has been explored to a much lesser extent. The challenge is the degree to which lactobacilli have been depleted in dysbiosis. If there are sufficient present to be resurrected by prebiotics such as lactulose stimulating their growth (Collins et al., 2018), then this could potentially be a useful therapeutic approach. It would likely not be considered a drug unless disease claims were made, but it is also not considered a food since it is being administered to the vagina and the European Food Safety Authority have stated that the vagina does not require nutrition (Reid, 2011).

ULTIMATELY

Microbiome studies have informed us of the organisms that are present in the vagina, and to a lesser degree what they are doing. The same is true for multiple studies of the gut microbiota where the failure to translate to clinical interventions is all too apparent (Brussow, 2019). Metabolomic studies may be more informative than microbiota profiles to "diagnose" an aberration that requires treatment or to show that a treatment has succeeded. But the complexity of the reproductive tract with the menstrual cycle, hormonal changes, exposure to sexual partners and the environment, presents many challenges that will require a variety of options for health restoration and maintenance over the lifespan.

This does not mean a single *Lactobacillus* or a cocktail of strains or a mixture with prebiotics, cannot make a significant dent in the symptomatic dysbiotic conditions. There is sufficient evidence to already indicate this is possible before, during and after pregnancy (Reid et al., 2016).

Going forward, the onus is on the research community to select candidate strains/products, pilot test them in humans, and understand how they succeed or why they fail. The methods of selection have advanced with metabolomic and transcriptomic

techniques (Macklaim et al., 2013; McMillan et al., 2015) which can differentiate lactobacilli strains more suited to the vagina (Watson et al. unpublished) and may one day benefit from tissues on a chip technology (Maurer et al., 2019). Funding from industry and government agencies should make this a priority given the extent of the problem and the failure to adequately manage it for so long. If only to make an impact in quality of life and to reduce episodes of dysbiosis and any symptoms and signs associated with it, then current and future products will have made an important contribution to women's health.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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ORIGINAL RESEARCH
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Competition Among *Gardnerella* Subgroups From the Human Vaginal Microbiome

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Gardnerella spp. are hallmarks of bacterial vaginosis, a clinically significant dysbiosis of the vaginal microbiome. *Gardnerella* has four subgroups (A, B, C, and D) based on cpn60 sequences. Multiple subgroups are often detected in individual women, and interactions between these subgroups are expected to influence their population dynamics and associated clinical signs and symptoms of bacterial vaginosis. In the present study, contact-independent and contact-dependent interactions between the four *Gardnerella* subgroups were investigated *in vitro*. The cell free supernatants of mono- and co-cultures had no effect on growth rates of the *Gardnerella* subgroups suggesting that there are no contact-independent interactions (and no contest competition). For contact-dependent interactions, mixed communities of 2, 3, or 4 subgroups were created and the initial (0 h) and final population sizes (48 h) were quantified using subgroup-specific PCR. Compared to the null hypothesis of neutral interactions, most (69.3%) of the mixed communities exhibited competition. Competition reduced the growth rates of subgroups A, B, and C. In contrast, the growth rate of subgroup D increased in the presence of the other subgroups. All subgroups were able to form biofilm alone and in mixed communities. Our study suggests that there is scramble competition among *Gardnerella* subgroups, which likely contributes to the observed distributions of *Gardnerella* spp. in vaginal microbiomes and the formation of the multispecies biofilms characteristic of bacterial vaginosis.

Keywords: *Gardnerella*, vaginal microbiome, interaction, bacterial vaginosis (BV), competition, microbial ecology, biofilm

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INTRODUCTION

Gardnerella vaginalis is considered a hallmark of bacterial vaginosis, a dysbiosis of the vaginal microbiome, although it is also commonly detected in women who do not meet the clinical criteria for vaginosis. *Gardnerella* comprises four sub-groups (A, B, C, and D), based on cpn60 barcode sequences and whole-genome sequences (Paramel Jayaprakash et al., 2012; Schellenberg et al., 2016). These subgroups have also been classified as clades 1–4 (Ahmed et al., 2012), with subgroups A, B, C, and D corresponding to clades 4, 2, 1, and 3, respectively. More recently, Vaneechoutte et al. amended the description of *Gardnerella* and defined three new species within the genus: *G. leopoldii, G. swidsinskii*, and *G. piotti* (Vaneechoutte et al., 2019). These species correspond to three of the previously defined subgroups: *G. vaginalis* (subgroup C/clade 1), *G. leopoldii* and *G. swidsinskii* (subgroup A/clade 4), and *G. piotii* (subgroup B/clade 2), while subgroup D/clade 3 encompasses several unnamed "genome species."

Phenotypic differences between the subgroups have been identified that could influence the role of *Gardnerella* spp. in the vaginal microbiome and their contributions to establishment and maintenance of vaginal dysbiosis (Schellenberg et al., 2016; Janulaitiene et al., 2018; Vaneechoutte et al., 2019). Furthermore, it has been shown that clades 4, 1, and 3 (Subgroups A, C, and D) are more often associated with bacterial vaginosis as defined by a high Nugent score or Amsel's criteria (Albert et al., 2015; Hilbert et al., 2017). Subgroup B or Clade 2 has been reported to be more abundant in women with an intermediate Nugent score (Balashov et al., 2014; Albert et al., 2015; Hilbert et al., 2017). Taken together, these observations highlight the potential clinical significance of the composition of the *Gardnerella* community within the vaginal microbiome.

Gardnerella clades or subgroups can be reliably distinguished in vaginal microbiome profiles using cpn60 barcode sequences (Hill et al., 2005; Paramel Jayaprakash et al., 2012), whereas 16S rRNA gene sequencing does not provide sufficient resolution (Vaneechoutte et al., 2019). Profiling of vaginal microbiomes using cpn60 barcode sequencing, and application of clade-specific PCR has shown that the vagina is often colonized by multiple subgroups simultaneously (Albert et al., 2015; Hilbert et al., 2017). The relative abundances of these subgroups, however, are not equal, and one subgroup usually dominates. The combinations and relative abundances of cpn60-defined subgroups of *Gardnerella* have been used to define previously undescribed population structures called community state types (CST) in the human vaginal microbiome (Albert et al., 2015). Given the observed phenotypic diversity within *Gardnerella*, an understanding of the factors that determine *Gardnerella* population structure in the vaginal microbiome is critical.

Potential factors contributing to the relative abundance patterns of *Gardnerella* subgroups in the vaginal microbiome include differences among subgroups in terms of biofilm formation, adhesion, overall fitness, and resistance to anti-bacterial factors (either produced by other microbiota or delivered as a medical intervention). Interactions between the subgroups may influence the population dynamics of the *Gardnerella* subgroups (Czárán et al., 2002; Hibbing et al., 2010; Faust and Raes, 2012). When the vaginal microbiome is dominated by *Gardnerella,* interactions between subgroups would be more frequent than with other bacterial species because they are closely related and therefore more likely to occupy the same niche (Darwin, 1859). *Gardnerella* can form biofilm in isolation and can also be incorporated in multispecies biofilms in the vagina (Hardy et al., 2015, 2016). Inter- and intraspecies interactions are ubiquitous within such multispecies biofilms (Narisawa et al., 2008; Elias and Banin, 2012; Burmølle et al., 2014), and such interactions may lead to competitive exclusion (Kerr et al., 2002; Oliveira et al., 2015). Thus, it is possible that competition between *Gardnerella* subgroups within the biofilm shapes the microbial population structure in the vaginal microbiome.

Competition between subgroups could take the form of a contest where two subgroups interact directly in either a contact-dependent manner or a contact-independent manner involving the secretion of effectors that reduce the fitness of competitors (Stubbendieck and Straight, 2016). Direct interactions can either inhibit the growth of one or more competitor(s) (Hayes et al., 2014; Willett et al., 2015), or trigger an enhanced biofilm response (Oliveira et al., 2015; Ren et al., 2015). In either case, competition could result in the exclusion of one or more competitor(s). Alternatively, competition between closely related taxa may take the form of a scramble (Hibbing et al., 2010), where they do not interact directly, but one competitor outgrows the others through its superior ability to use shared resources, such as nutrients. In a scramble mode of competition, all competitors have to share finite resources, which can reduce the fitness of competing organisms. This type of competition is often referred to as non-interfering exploitative competition (Russel et al., 2017).

The objective of our study was to seek evidence of contact-independent or contact-dependent interactions between *Gardnerella* subgroups that affect growth *in vitro*. Our results demonstrate that strains representing *Gardnerella* subgroups A, B, C, and D can coexist in biofilms but that mixing of subgroups does not enhance biofilm formation. Our findings also suggest the presence of a non-interfering, exploitative competition in mixed subgroup communities of *Gardnerella*.

METHODS

Gardnerella Isolates

Isolates of *Gardnerella* spp. used in this study were drawn from a previously described culture collection kept at −80°C (Schellenberg et al., 2016) (**Table S1**). The subgroup affiliations of all isolates were determined by cpn60 barcode sequencing (Links et al., 2012). Selected isolates were revived from −80°C on Columbia agar plates with 5% sheep blood and incubated under anaerobic conditions (BD GasPak EZ Anaerobe Gas Generating Pouch System, NJ, USA) at 37°C for 48 h.

Measurement of the Effect of *Gardnerella* Culture Supernatant on Growth

The purpose of this part of the study was to test whether the *Gardnerella* subgroups produce molecules that inhibit the growth of the other subgroups (i.e., contact-independent interactions). Specifically, we wanted to test the effect of cell-free supernatant from *Gardnerella* subgroups on the growth and biofilm formation of the other subgroups. To detect contact-independent interactions, we tested a total of 56 combinations using 14 isolates of subgroups A ($n = 4$), B ($n = 4$), C ($n = 3$), and D ($n = 3$) (**Table 1**). Isolates that were used to derive cell-free supernatant (CFS) were called producer strains, and strains on which the effect of prepared CFS was tested were called focal strains. Selected focal strains of all four subgroups were grown in medium containing 10% CFS from producers belonging to other subgroups and their own subgroup ("self-CFS"). Experiments were performed in two culture media: NYC III broth, which is recommended by the American Type Culture Collection (ATCC) for *Gardnerella* culture, and BHI + 1% glucose broth, to determine if culture media influenced growth of *Gardnerella* subgroups in the presence or absence of CFS.

To produce the CFS, colonies from Columbia blood agar plates were harvested using sterile swabs, resuspended in 5 ml

TABLE 1 | Numbers of combinations of CFS producers and focal subgroups tested to detect contact-independent interactions.

Focal subgroup	CFS producer subgroup			
	A	B	C	D
A	3	3	3	3
B	4	4	4	4
C	4	4	4	4
D	3	3	3	3

of NYC III broth and incubated anaerobically for 72 h at 37°C to reach stationary phase. CFS was generated by centrifuging the broth culture at 3,000 × g for 30 min (Jung et al., 2014). The supernatant was filter-sterilized using 0.22 μm filters and was used on the same day. Filter-sterilized CFS was streaked on Columbia blood agar plates to confirm sterility.

To test the effect of CFS on the focal strains, colonies from blood agar plates were harvested using sterile swabs, resuspended in 0.85% saline and adjusted to McFarland turbidity standard 1. Fifteen microliters of each test strain suspension were added to 135 μl of NYC III or BHI + 1% (v/v) glucose and 10% (v/v) CFS in individual wells of a flat bottom 96-well plate (Corning Costar, NY, USA). The focal strains were also grown in control wells with media containing no CFS. Negative controls consisted of 15 μl of 0.85% saline added to 135 μl of culture media (NYC III or BHI + 1% glucose) and sterile culture media alone. To confirm the viability of the inocula, focal strain suspensions were streaked on to Columbia blood agar. Each combination of CFS and the focal strain was performed in three technical replicates.

Quantification of Total Growth, Planktonic Growth, and Biofilm Growth

Total growth, planktonic growth, and biofilm formation were determined for each combination at 48 h (**Figures S1–S14**). Total growth was calculated as the difference in optical density measured at 595 nm between the 48 h and the 0 h time points. The OD_{595} was measured using a VMax Kinetic ELISA Absorbance Microplate Reader (Molecular Devices, CA, USA). Planktonic growth was measured by transferring 150 μl of supernatant from each well to a fresh 96-well plate and determining the OD_{595}. To measure the biofilm formation, a crystal violet (CV) staining assay was performed (O'Toole, 2011; Ren et al., 2014; Oliveira et al., 2015). Briefly, after removal of the supernatant, plates were washed twice with water, biofilms were stained with 1% CV for 10 min, plates were washed twice with water and air dried. To solubilize stained biofilm, 150 μl of 33% glacial acetic acid was added to each well, and the OD_{595} was measured.

Co-culture Assays to Detect Contact-Dependent Interactions

The purpose of this part of the study was to test whether there were interactions between *Gardnerella* subgroups when they were grown together in the same culture. Four independent experiments were conducted at separate points in time with two different sets of *Gardnerella* isolates. Experiments 1A and 1B were done in March and April 2018, respectively, and subgroups A, B, C, and D were represented by isolates NR020, N170, NR038, and NR003. Experiments 2A and 2B were done in April and May 2018, respectively, and subgroups A, B, C, and D were represented by isolates VN003, VN002, NR001, and WP012. For each of the four replicate experiments, we grew the four subgroups alone ($n = 4$; A, B, C, D), and in all possible combinations of two ($n = 6$; AB, AC, AD, BC, BD, CD), three ($n = 4$; ABC, ABD, ACD, BCD), and four subgroups ($n = 1$; ABCD) for a total of 15 different combinations. Each of the 15 combinations was replicated three times in the wells of a 96-well tissue culture plate (i.e., 4 experiments * 15 combinations * 3 replicates per combination = 180 replicates). The members of each community were allowed to interact for a period of 48 h and the abundance of the constituent subgroups was estimated at the start (0 h) and the end (48 h) of this period using subgroup-specific quantitative real-time PCR (qPCR). Prior to the interaction assay, each of the four subgroups was grown alone at 37°C anaerobically in BHI with 0.25% maltose and 10% horse serum for a period of 12 h and then mixed in BHI + 0.25% maltose. Immediately prior to combining the subgroups to create the mixed communities, a sub-sample was taken from each of the four cultures to determine the abundance of each subgroup at the time point of 0 h using the subgroup-specific qPCR. To create the mixed communities, equal volumes of each isolate containing ∼5 × 10^6 genome equivalents per mL (i.e., verified by qPCR) were included in a total volume of 200 μl per well. For each of the four experiments, two plates were used: one for quantifying the number of cells in both the planktonic and biofilm fractions using qPCR and the other to quantify biofilm formation using the CV staining assay.

Quantification of *Gardnerella* Using Subgroup-Specific Quantitative Real-Time PCR

Cells from planktonic and biofilm fractions were collected at 48 h and extraction of DNA was performed using a commercial kit (DNeasy PowerBiofilm, Qiagen, Mississauga, ON) following the manufacturer's instructions with minor modifications. To collect the planktonic phase, 200 μl of culture supernatant was removed from the wells and transferred to bead tubes. To collect biofilm, 200 μl of lysis reagent MBL was pipetted directly into the wells of the 96-well plate and incubated for 30 min to solubilize the biofilm. The bottom of each well was scraped with a pipette tip and the suspension was pipetted up and down several times before transferring it to a bead tube. The biofilm solubilization step was repeated to maximize biofilm collection. To enhance lysis, 100 μl of chaotropic agent FB was added to the bead tubes. The bead tubes were incubated at 65°C for 5 min in a water bath, and then vortexed using a multitube vortexer at maximum speed for 15 min. Later steps were performed following the manufacturer's instructions.

Subgroup-specific qPCR was performed using previously published primers and probes (Balashov et al., 2014) (**Table S2**). Amplification was performed in 10 μl reactions containing 1×

iQ Supermix (BioRad, Mississauga, ON), 800 nM of each primer, 100 nM of TaqMan probe, and 2 μl of template. The qPCRs were performed using a CFX Connect (BioRad, Mississauga, ON) instrument. The qPCR results were reported as target copy number per PCR reaction (2 μl of template DNA extract). Each sample was assayed in duplicate reactions with the appropriate standard curve comprised of plasmids containing probe targets (10^2-10^9 plasmid copies per reaction). Thermocycling conditions were: initial denaturation at 95°C for 3 min, 40 cycles of 95°C for 15 s, and annealing/extension at 60°C (subgroups A, B, and D) or 63.3°C (subgroup C) for 40 s. Each plate contained a no template control, DNA extraction controls, and non-target subgroup templates as negative controls. For each qPCR reaction, the genome copy number was calculated using the standard curve. The qPCR assay was repeated for samples with a difference in Cq value > 1 between duplicate wells.

Statistical Analysis of Contact-Independent Interactions

The contact-independent interactions were analyzed using Kruskal-Wallis non-parametric one-way ANOVA with Dunn's *post-hoc* test (Prism 8, Graphpad Software).

Statistical Analysis of Contact-Dependent Interactions

To characterize the interactions between the four subgroups, the 180 replicates of 15 unique communities in four independent experiments (1A, 1B, 2A, 2B) were analyzed collectively (4 experiments * 15 combinations per experiment * 3 replicates per combination = 180 replicates; each of the 15 unique combinations was replicated 12 times). Outcomes from these co-culture experiments were interpreted as shown in **Figure 1** (Foster and Bell, 2012). First, the growth rates of all isolates grown as singletons were calculated using the following formula: $r_i = \ln(N_{t,i}/N_{0,i})/T$. Here, r_i is the observed growth rate of subgroup i when it is alone (has no competitors), $N_{0,i}$ and $N_{t,i}$ are the initial and final population sizes of subgroup i as estimated by qPCR (sum of biofilm and planktonic cells), and T is the time period of 48 h (**Table S3**). Under the null hypothesis of no competition, we used our estimates of r_i from the singletons and our estimates of $N_{0,i}$ in the mixed communities to predict the expected abundance of each subgroup ($\hat{N}_{t,i}$) in the mixed communities after 48 h of growth. The sum of predicted abundances (based on the singleton growth rates) for each subgroup in the community is the expected null abundance for the mixed communities (i.e., the expected community size in the absence of interaction). If the observed abundance of a mixed community was higher than the expected null abundance, the interaction was classified as facilitation (positive interaction). If the observed abundance of a mixed community was lower than the predicted null abundance, it was classified as competition (negative interaction) (**Figure 1**). The null hypothesis of no interaction predicts that due to random measurement error, 50% of the interactions should be positive (facilitation) and 50% of the interactions should be negative (competition). A proportion test was used to determine whether the observed prevalence of facilitation and competition were significantly different from the 50/50 expectation. This approach is a general test of the nature of interactions between *Gardnerella* subgroups and does not consider that each subgroup may be affected differently by competitors.

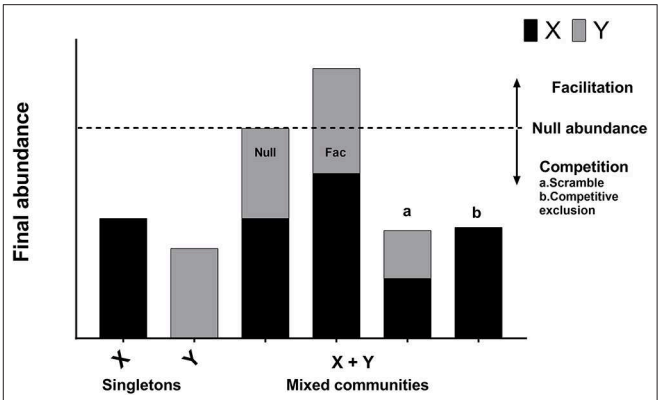

FIGURE 1 | Regime of interpretation. Interactions between bacterial species or strains can be classified as neutral, competition, or facilitation. When bacterial species X and Y are grown in isolation, their abundances correspond to bars X and Y. Under the null hypothesis of no interaction, the expected abundance of the mixed community is equal to the sum of the abundances of two organisms grown separately (Null abundance, indicated by broken line). A neutral interaction occurs when the observed abundance of the mixed community is exactly equal to the null abundance (bar "Null"). In practice, neutral interactions are almost never observed due to measurement error. Facilitation occurs when the observed abundance of the mixed community is greater than the null abundance (bar "Fac"). Competition occurs when the observed abundance of the mixed community is less than the null abundance (bars "a" and "b"). Competitive exclusion occurs when one of the species is completely eliminated in the mixed community (bar "b").

To test whether the subgroups were affected differently by the number of competitors, the growth rates of the *Gardnerella* subgroups were analyzed using linear mixed effect models (LMMs). The residuals of the growth rates were considered as normally distributed. The fixed effects were subgroup (four levels: A, B, C, and D), the number of competitors (0, 1, 2, and 3), and their interaction. The random effects were the 3 replicates of each community nested in the 4 different experiments (i.e., total of 12 replicates for each community). This approach does not consider the identity of the competitors. We used R (v 1.1-21) to analyze the data; the LMM models were run using the lmer() function in the R package lme4.

To test whether the identity of the competitors mattered, the growth rate of each subgroup was analyzed separately using LMMs. The four subgroups had to be analyzed separately, because the identity of the competitors differs for each subgroup. The fixed effects were the identities of the competitors. For example, for the growth rate of subgroup A, the competitors included B, C, D, BC, BD, CD, and BCD. The random effects structure was the same as before.

RESULTS

Effect of *Gardnerella* Culture Supernatant on Growth and Biofilm Formation

The initial optical density (OD) of all the focal strains was ∼0.05 and they grew in both NYC III and BHI + 1% glucose, except for one subgroup C strain, NR001, which did not grow in BHI

+ 1% glucose (**Figure S11**). The OD at 48 h of these strains varied from as low as 0.05 (after subtracting initial OD) to 0.80. There was no effect of CFS on overall growth or planktonic growth of focal strains, nor on biofilm formation (Kruskal-Wallis non-parametric one-way ANOVA with Dunn's *post-hoc* test, $p > 0.05$ for all comparisons) (**Figures S1–S14**). The type of medium, however, influenced mode of growth: NYC III had more planktonic growth, whereas BHI + 1% glucose had more biofilm growth. Increasing the concentration of CFS from 10 to 20% had no effect on the growth pattern of the *Gardnerella* subgroups (data not shown).

Validation of qPCR Assays

Prior to performing the co-culture experiments, the efficiency of each subgroup-specific qPCR assay and the limits of detection and quantification were determined, since these values had not been reported previously (Balashov et al., 2014). The amplification efficiency for subgroups A, B, C, and D were 99.9, 107.4, 110, and 98.2%, respectively. The lowest concentration at which all subgroups were detected was 1 target copy per qPCR reaction. However, the lower limit of quantification (LOQ) was different for each subgroup. The LOQ for subgroups A, B, C, and D were 1, 10, 100, and 1 copy per reaction, respectively.

Characterization of Contact-Dependent Interactions Between *Gardnerella* Isolates

Our null hypothesis approach of testing the type of interaction (facilitation vs. competition) between subgroups of *Gardnerella* found that competition was 2.3 times more common than facilitation. Of the 132 mixed communities, 69.7% (92/132) had negative interactions (competition), and 30.3% (40/192) had positive interactions (facilitation) (**Table S3**). A proportion test found that these percentages were significantly different ($p < 0.0001$) from the 50/50 null expectation. Competition was more frequently observed in communities with more subgroups. The prevalence of competition was 58.3% (42/72), 79.2% (38/48), and 100.0% (12/12) in communities with two, three, or four subgroups, respectively.

Alternatively, we can set an arbitrary threshold so that absolute differences <500 million cells (between the observed and expected values) are considered as neutral interactions (i.e., the difference was caused by random measurement error). Using this approach for the 132 mixed communities, there were 25.0% (33/132) neutral interactions, 75.0% (99/132) competitive interactions, and 0.0% (0/132) facilitative interactions. The probability of getting this result under the null hypothesis that competition and facilitation are equally likely (50/50) is vanishingly small ($p < 0.0001$). This alternative analysis shows that introducing an arbitrary threshold to separate measurement error from biologically interesting interactions does not change our conclusion that competitive interactions dominate between *Gardnerella* subgroups.

Competition Between Subgroups in Biofilms vs. the Supernatant

Since biofilms are a common site of interactions between species (Nadell et al., 2009; Elias and Banin, 2012; Burmølle et al., 2014; Oliveira et al., 2015), we investigated whether competition was more frequent in the biofilm fraction than the planktonic fraction. Out of 132 mixed communities, 68.9% of the biofilm fractions (91/132), and 65.9% (87/132) of the planktonic fractions exhibited competition (**Table S3**). This result indicates that competition occurs in both biofilm and planktonic populations of *Gardnerella* spp. In addition, these observations demonstrate that mixed subgroup biofilms can occur, with no subgroup excluded.

Effect of Mixed Communities on the Growth Rate of Individual Subgroups

When grown in isolation, the instantaneous growth rates (per hour) of the four subgroups ranked from lowest to highest are as follows: 0.098 for subgroup A, 0.119 for subgroup D, 0.120 for subgroup B, and 0.211 for subgroup C. The population doubling times (in hours) ranked from slowest to fastest are as follows: 7.1 for subgroup A, 5.8 for subgroup D, 5.8 for subgroup B, and 3.3 for subgroup C. The LMM found a significant interaction between subgroup and number of competitors on the growth rate ($p < 0.0001$) indicating that the effect of the number of competitors on the growth rate differs between subgroups. Growth rates of subgroups A, B, and C decreased significantly ($p < 0.0001$) in mixed communities (**Figures 2A–C**). In contrast, the growth rate of subgroup D increased significantly ($p < 0.0001$) in mixed communities (**Figure 2D**). Thus, subgroups A, B, and C experienced competition in mixed communities, whereas subgroup D experienced facilitation. Regardless of the identity of the community, subgroup C always had a higher intrinsic growth rate than the other subgroups (**Figure 2**).

Impact of Competitor Subgroups on Focal Subgroups

Next, we investigated whether subgroups differed in the magnitude of their negative (or positive) effect on the growth rate of other subgroups. Subgroup D had the most negative impact on the growth rates of the other subgroups. The presence of subgroup D in any community reduced the growth rate of the other members of the communities by 44.2% (**Figures 3A–C**; LMM, $p < 0.0001$). Subgroup A reduced the growth rates of the other members of the communities by 4.8%, whereas B and C increased the growth rates of the other members of the communities by 7.2%, and 1.6%, but none of these effects were statistically significant (**Figure 3**). In summary, subgroup D has a large and negative effect on the growth rate of all other subgroups, whereas the effects of the other subgroups are essentially neutral.

Quantification of Biofilm Formation in Monocultures and Co-cultures

Mixing of different bacterial species often leads to increased biofilm formation. We therefore investigated whether mixing of *Gardnerella* subgroups would enhance biofilm formation. If the amount of biofilm formed by a mixture of subgroups was greater than the amount formed by the best individual biofilm former of that mixture, the interaction was considered synergistic. If the amount of biofilm formed by a mixture was less than the amount

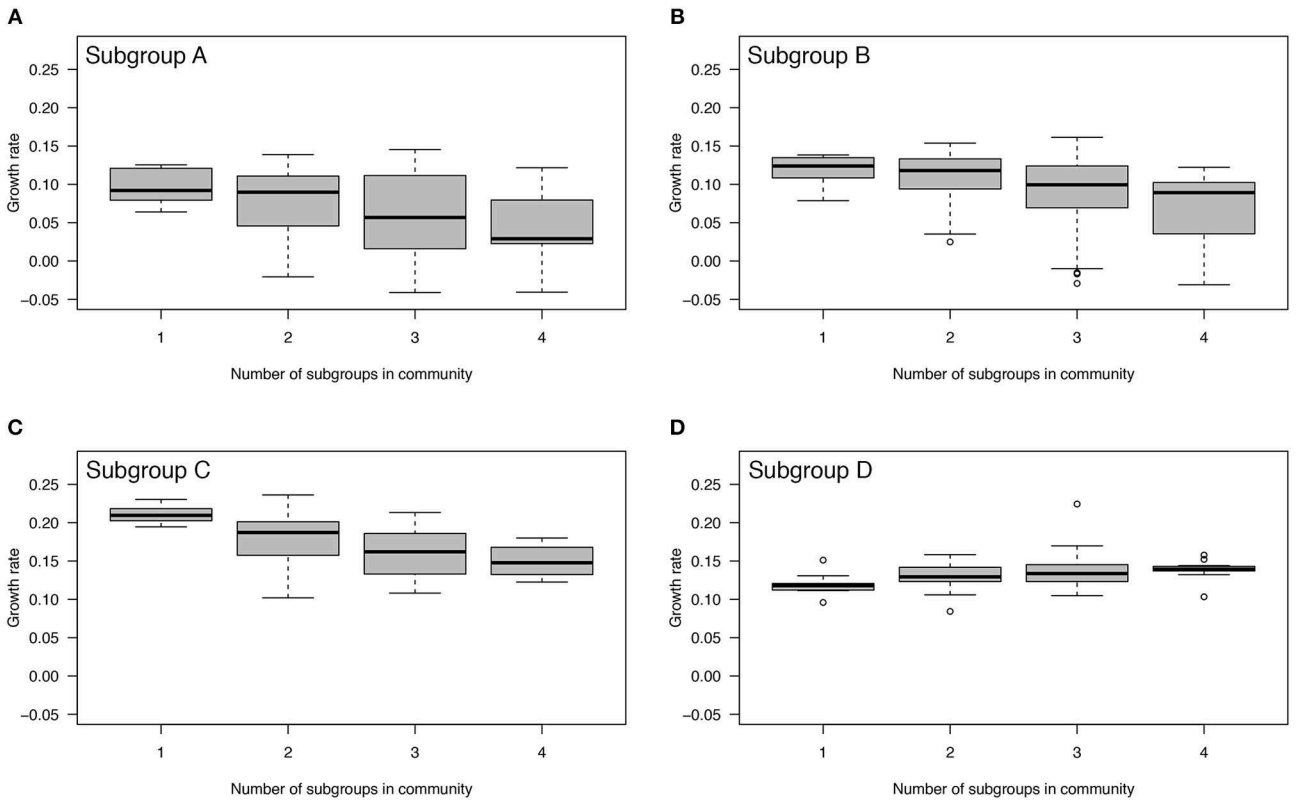

FIGURE 2 | Effect of mixed communities on growth rates of *Gardnerella* subgroups. The growth rates of subgroups A, B, and C decreased with an increasing number of competitors indicating competition **(A–C)**. In contrast, the growth rate of subgroup D increased with an increasing number of competitors indicating facilitation **(D)**. Each community was allowed to interact and grow over a period of 48 h.

formed by the worst individual biofilm former of that mixture, the interaction was considered antagonistic (Madsen et al., 2016). Here biofilm formation by mixed subgroups was almost always less than the individual biofilm formation by the best biofilm-forming subgroup but greater than the worst biofilm former in the mixture (**Figures 4A,B**). Only one co-culture of subgroups A and D was higher than the individual biofilm formation of either strain (Kruskal-Wallis, $p < 0.05$) (**Figure 4B**). The results of this experiment show that overall biofilm biomass is not enhanced by mixing of subgroups.

Effect of *Gardnerella* Co-culture Supernatant on Individual Subgroups

Our initial experiments showed that the CFS of singleton cultures had no impact on the growth of isolates from other subgroups, but negative interactions were frequently observed in co-cultures. To determine if effectors were secreted as a result of contact, we derived CFS from pairwise co-cultures and prepared media conditioned with 10% co-culture supernatant. We grew four representative isolates of all four subgroups in media with and without co-culture CFS and measured optical density to monitor growth and used a CV assay for quantification of biofilm formation (**Figure S15**). No significant differences in the amount or mode of growth were observed with exposure to co-culture CFS (Kruskal-Wallis, $p > 0.05$).

DISCUSSION

A critical step in the development of bacterial vaginosis is when *Gardnerella* spp. displace *Lactobacillus* spp. and initiate multispecies biofilm formation (Schwebke et al., 2014). The recent amendment of the genus and the "fine-tuning" of the taxonomy of *Gardnerella* (Vaneechoutte et al., 2019) re-emphasizes the clinical importance of determining the particular contributions of different *Gardnerella* spp. to vaginosis, and the degree to which different subgroups may compete or cooperate in the vaginal microbiome. The detection of multiple subgroups (or species) of *Gardnerella* in the vaginal microbiomes of individual women is common and so interactions are expected to occur frequently (Hardy et al., 2016, 2017; Castro et al., 2019). Our current study was designed to determine the types of interactions that occur between isolates from different cpn60-defined subgroups of *Gardnerella,* and to discover whether multiple subgroups can be incorporated into biofilms.

No Evidence of Contest Competition Between Subgroups of *Gardnerella*

A contest is a direct, interference competition where the secretion of small molecules (secondary metabolites or toxins) by one organism inhibits the growth of other organisms in an environment (Czárán et al., 2002; Hibbing et al., 2010; Faust and

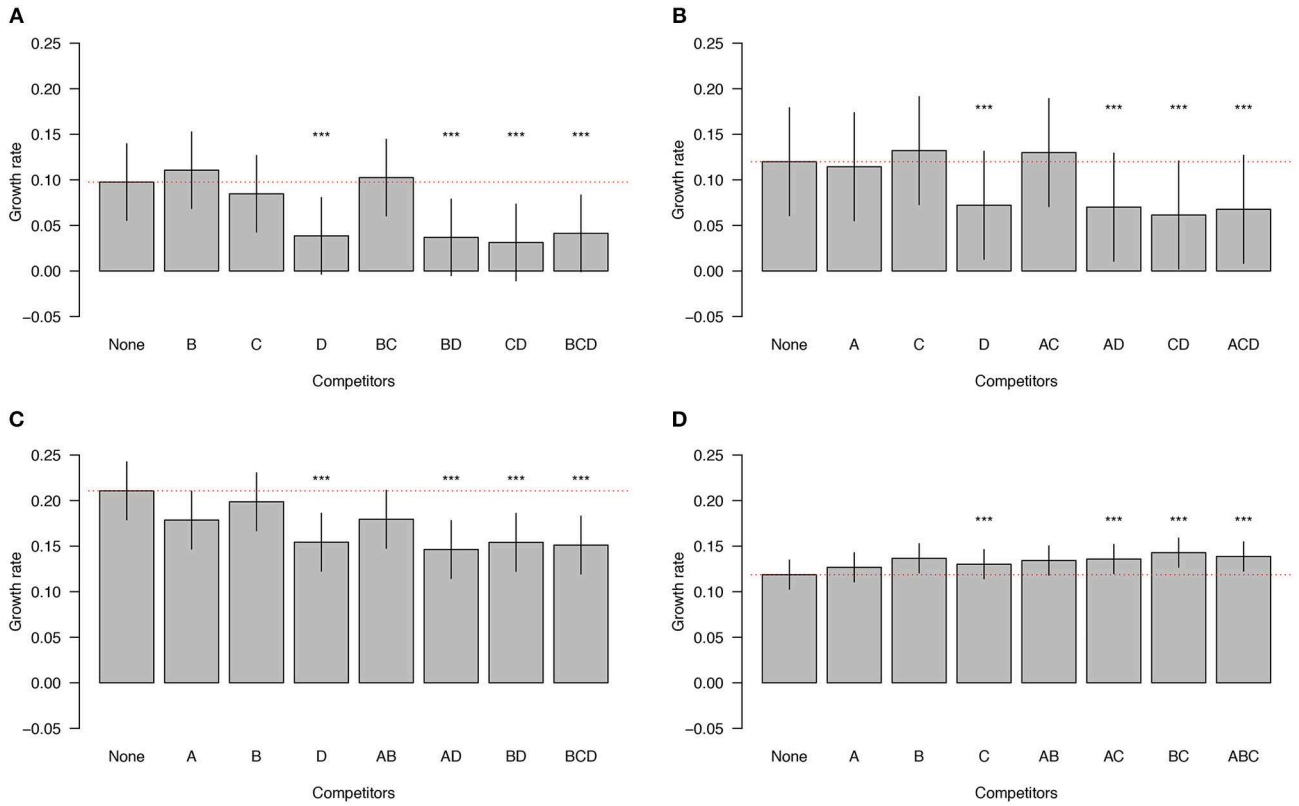

FIGURE 3 | Impact of competitor subgroups on focal subgroup. The growth rates of subgroups A, B, and C are inhibited by the presence of subgroup D **(A–C)**. In contrast, the growth rate of subgroup D is enhanced by the presence of the other subgroups **(D)**. The identities of the competitors are indicated on the X axis. The red dotted line is the expected null growth rate (growth as a singleton). Growth rates above and below the red dotted line indicate facilitation and competition, respectively. Significant effects on growth rates of the focal subgroups in the presence of competitors are denoted by asterisks (***). The vertical lines on each bar indicate the 95% confidence intervals for the means.

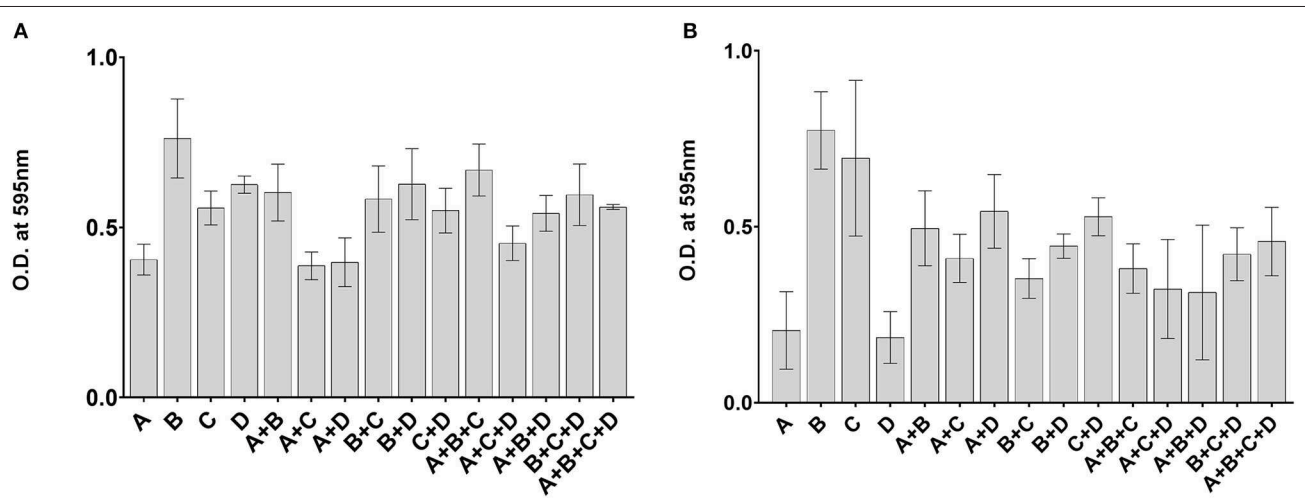

FIGURE 4 | No enhancement of biofilm formation in mixed communities. Isolates used in Experiment 1 **(A)** and Experiment 2 **(B)** were cultured alone or in combinations. Biofilm biomass was quantified by a crystal violet assay after 48 h of growth. The error bars indicate standard deviations of three replicates.

Raes, 2012; Garbeva et al., 2014; Oliveira et al., 2015; Ismail et al., 2016; Stubbendieck and Straight, 2016). Cell-free supernatant (CFS) is the first place to look for any such secreted small molecules that could affect the growth of other bacterial species or strains. *Gardnerella* isolates have been shown to inhibit the growth of some vaginal lactobacilli in a contact-independent

manner (McLean and McGroarty, 1996; Teixeira et al., 2010), and both inhibitory and stimulatory effects of *Gardnerella* CFS on the growth of a range of vaginal microbiota have been documented (Chanos and Mygind, 2016). These previous reports, however, have involved relatively few isolates and no information was provided regarding the *Gardnerella* species. In the present study, we detected no effect on the amount or mode of growth of *Gardnerella* isolates when they were exposed to the CFS from other isolates grown in isolation (**Figures S1–S14**). Since effector molecules are often only secreted when their producers are in contact with other bacterial species (Konovalova and Søgaard-Andersen, 2011; Chanos and Mygind, 2016), we also tested whether CFS from co-culture combinations (where competition had been observed in co-culture assays) affected the growth of *Gardnerella* strains. We found no effect of co-culture CFS on growth, which further supports the conclusion that there is no contest or direct interference competition between *Gardnerella* subgroups (**Figure S15**). Similarly, no enhancement of growth was observed, which would have been expected if there was nutritional synergy or cross-feeding among *Gardnerella* spp. as has been demonstrated for *G. vaginalis* and *Prevotella bivia* (Pybus and Onderdonk, 1997).

Scramble Competition Is Common in Mixed Communities of *Gardnerella*

When *Gardnerella* isolates from different subgroups were co-cultured, all of them were present in both planktonic and biofilm fractions of each tested community, indicating that no subgroup was completely dominant or excluded over the 48-h observation period. Competition between subgroups was common, with 70% of the observed interactions classified as competitive. Although intrinsic growth rates differed among the four subgroups (**Figure 2**), subgroups A, B and C all showed a reduced growth rate as the number of competitors increased (**Figures 2A–C**). Interestingly, subgroup D experienced facilitation in co-cultures because its growth rate increased with increasing numbers of competitors (**Figure 2D**). Subgroup D also had a negative effect on the growth rates of other subgroups (**Figures 3A–D**). Taken together, these co-culture observations are consistent with a non-interfering, exploitative competition, which is also called scramble competition (Nicholson, 1954; Hibbing et al., 2010). Scramble competitions result in the dominance of the competitor with the greatest ability to exploit a shared resource (e.g., nutrients), and a general reduction in the overall fitness of all members of a mixed community that share this resource (Darwin, 1859; Nicholson, 1954; Foster and Bell, 2012).

One possible explanation for the distinct behavior of subgroup D is that it has different nutritional requirements than the other subgroups and thus remains unaffected when others compete for the same nutrient resources. It might also represent a "social cheater" (Hibbing et al., 2010; Ghoul et al., 2014); an opportunistic member of the community that occupies a distinct niche and benefits from the competition of others. Subgroup D strains are rarely detected in the vaginal microbiome, and are usually at low abundance (Albert et al., 2015). Negative-frequency dependent selection can favor rare variants that are able to exploit available niches (Hibbing et al., 2010), allowing them to thrive in an otherwise highly competitive environment (Ghoul et al., 2014). These specialized variants can also make some important nutrients unavailable to the other community members, who are carrying the cost of maintaining the multispecies community (Harrison et al., 2006). This scenario could explain why, in mixed *Gardnerella* subgroup communities *in vitro*, the presence of subgroup D negatively affects the growth of other subgroups. *In vivo,* the interactions between other bacterial species in the vaginal microbiome might check the abundance of this social cheater (Hibbing et al., 2010). Since our experiments were conducted over a relatively short period of time (48 h), we were unable to determine if mixed communities of *Gardnerella* subgroups comprise a non-transitive competitive interaction network. This type of interaction is characterized by gradual replacement of dominant species by others in the consortium (Hibbing et al., 2010), but requires the presence of other contributing factors and changes in the environment happening over time that could not be captured in our *in vitro* model. A longitudinal study using a dynamic culture system might possibly demonstrate the presence of a non-transitive network in *Gardnerella* spp.

No Synergy in Mixed Subgroup Biofilm

Gardnerella species have been implicated in the initiation of vaginal biofilms by displacing lactobacilli and adhering to the epithelium. Subsequent recruitment of other bacteria results in the characteristic multispecies bacterial vaginosis biofilm (Machado and Cerca, 2015; Hardy et al., 2016; Castro et al., 2019). Multispecies biofilms are a hotspot of interactions (Burmølle et al., 2014; Liu et al., 2016) that can be antagonistic or synergistic (Ren et al., 2015; Røder et al., 2015). Synergy can result in increased biofilm biomass in co-cultures compared to the best individual biofilm former grown alone, while antagonism can lead to a reduction in the biofilm biomass of co-culture compared to the worst individual biofilm former (Madsen et al., 2016). Enhancement of biofilm formation can also be the result of competition where the end result is the exclusion of some species from the biofilm (Foster and Bell, 2012; Oliveira et al., 2015). In the current study, no enhancement of biofilm biomass was detected using a CV assay when different *Gardnerella* subgroups were co-cultured (**Figure 4**), which is consistent with the non-interfering, exploitative competition we observed in the co-cultures. Importantly, our results show that all subgroups of *Gardnerella* can participate in biofilms, and thus contribute to the formation of this defining feature of bacterial vaginosis, regardless of their individual arsenals of "virulence factors."

LIMITATIONS

The logistical advantages of *in vitro* systems for studying bacterial interactions are balanced by some limitations. We used a closed system with one set of growth conditions where nutrient depletion over the duration of the experiments may have affected interactions. In addition, the *in vitro* environment lacks the other members of the vaginal microbiota, host immune system, vaginal fluid flow, epithelial cell turnover, and other environmental cues present in the vaginal ecosystem. In the absence of an appropriate animal model, microfluidic devices, and cultured vaginal epithelial cells may offer more

realistic conditions, but also present logistical challenges for studies involving large numbers of isolates and experimental replication. In our experiments, *Gardnerella* cell numbers were estimated based on quantitative PCR, which does not distinguish viable from non-viable bacteria, but since we used growth rate as our main measurement, this factor was likely not a major influence on our results. Another potential limitation of the study is the isolates we used were chosen to represent *Gardnerella* subgroups, and were not isolated as pre-established consortia from individual women. It has been proposed that *Gardnerella* biofilms can be sexually transmitted (Swidsinski et al., 2014), and since our results demonstrate the possibility of biofilms comprised of multiple *Gardnerella* spp., it will be interesting to investigate interactions among isolates from biofilms that may have been transmitted together over long periods of time.

CONCLUSIONS

Overall, our experiments suggest that competition is common in mixed communities of *Gardnerella* subgroups and that these negative interactions are likely due to niche overlap and competition for shared resources rather than direct interference. The combined effects of scramble competition and different vaginal microbiota compositions in individual women, physiological influences, medical interventions, and sexual and hygiene practices, results in the patterns of distribution of *Gardnerella* spp. we observe in reproductive-aged women. Colonization by multiple species is common and any one of the most frequently detected subgroups (A, B, and C, corresponding to *G. swidsinskii, leopoldii, piotii,* and *vaginalis*) can dominate the microbiome. Longitudinal studies of *Gardnerella* spp. in co-culture will be critical in deciphering the contributions of both abundant and rare species in the transition to bacterial vaginosis in the vaginal microbiome.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SK and JH conceived and designed the study. SK performed the experiments and wrote the first draft of the manuscript. MV and SK conducted the statistical analyses. All authors contributed to manuscript revision, read, and approved the submitted version.

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frontiers in Cellular and Infection Microbiology

REVIEW
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Cholesterol-Dependent Cytolysins Produced by Vaginal Bacteria: Certainties and Controversies

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Bacterial vaginosis (BV) is a vaginal anaerobic dysbiosis that affects women of reproductive age worldwide. BV is microbiologically characterized by the depletion of vaginal lactobacilli and the overgrowth of anaerobic bacterial species. Accumulated evidence suggests that *Gardnerella* spp. have a pivotal role among BV-associated bacteria in the initiation and development of BV. However, *Gardnerella* spp. often colonize healthy women. *Lactobacillus iners* is considered as a prevalent constituent of healthy vaginal microbiota, and is abundant in BV. *Gardnerella* spp. and *L. iners* secrete the toxins vaginolysin (VLY) and inerolysin (INY), which have structural and activity features attributed to cholesterol-dependent cytolysins (CDCs). CDCs are produced by many pathogenic bacteria as virulence factors that participate in various stages of disease progression by forming lytic and non-lytic pores in cell membranes or via pore-independent pathways. VLY is expressed in the majority of *Gardnerella* spp. isolates; less is known about the prevalence of the gene that encodes INY. INY is a classical CDC; membrane cholesterol acts a receptor for INY. VLY uses human CD59 as its receptor, although cholesterol remains indispensable for VLY pore-forming activity. INY-induced damage of artificial membranes is directly dependent on cholesterol concentration in the bilayer, whereas VLY-induced damage occurs with high levels of membrane cholesterol (>40 mol%). VLY primarily forms membrane-embedded complete rings in the synthetic bilayer, whereas INY forms arciform structures with smaller pore sizes. VLY activity is high at elevated pH, which is characteristic of BV, whereas INY activity is high at more acidic pH, which is specific for a healthy vagina. Increased VLY levels in vaginal mucosa *in vivo* were associated with clinical indicators of BV. However, experimental evidence is lacking for the specific roles of VLY and INY in BV. The interplay between vaginal bacterial species affects the expression of the gene encoding VLY, thereby modulating the virulence of *Gardnerella* spp. This review discusses the current evidence for VLY and INY cytolysins, including their structures and activities, factors affecting their expression, and their potential impacts on the progression of anaerobic dysbiosis.

Keywords: cholesterol-dependent cytolysin, vaginolysin, inerolysin, *Gardnerella* spp., *Lactobacillus iners*, pore-forming mechanism, virulence factors, bacterial vaginosis

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INTRODUCTION

Vaginal bacterial species composition and abundance were compared in reproductive-aged women (Ravel et al., 2011; Fettweis et al., 2014), and the results revealed that *Lactobacillus*-dominated microbial communities are the hallmark of a healthy vagina (Ma et al., 2012; Vaneechoutte, 2017a). Otherwise healthy asymptomatic women harboring a polymicrobial mixture of anaerobic bacteria with few lactic acid-producing lactobacilli also represents a healthy vagina (Ma et al., 2012; Smith and Ravel, 2017), although the lower numbers of lactobacilli reduce their protection against pathogenic microorganisms (Anahtar et al., 2015). High-throughput sequencing and microscopy studies to classify vaginal species provide a better understanding of clinical conditions associated with the disturbance of healthy, *Lactobacillus*-dominated microbiota, which lead to bacterial vaginosis (BV) (Nugent et al., 1991; Srinivasan et al., 2012) and the recently identified clinical condition called aerobic vaginitis (Donders et al., 2017).

BV is the vaginal condition associated with poor reproductive and obstetric sequelae (Kenyon et al., 2013; van de Wijgert and Jespers, 2017). BV is microbiologically characterized by the depletion of most vaginal *Lactobacillus* species and the overgrowth of diverse anaerobes (Srinivasan and Fredricks, 2008; Huang et al., 2014; Onderdonk et al., 2016). Intensive DNA-based studies on vaginal microbiota did not identify the etiology of BV and suggest that BV is a complex condition that may involve several different diseases (Cerca et al., 2017). This hypothesis was proposed because the primary causative pathogen(s) of BV have not been unambiguously determined (Muzny et al., 2019). Further, epidemiological data suggest that BV may be sexually transmitted (Fethers et al., 2008; Swidsinski et al., 2014).

Deep sequencing showed that BV is associated with an array of anaerobic bacteria (Zozaya-Hinchliffe et al., 2010; Srinivasan et al., 2012). Facultative anaerobic bacterial species of the genus *Gardnerella* have been recovered from vaginal samples of almost all women with BV (Fredricks et al., 2007; Srinivasan et al., 2012). *Gardnerella* spp. have higher virulence potential than other BV-associated bacteria, thereby supporting its possible role in BV development (Patterson et al., 2010; Muzny and Schwebke, 2013; Robinson et al., 2019). The key feature of BV is the presence of a bacterial biofilm on vaginal epithelial cells, which predominantly consists of *Gardnerella* spp. and other incorporated bacterial groups (Swidsinski et al., 2005, 2014). The impact of neighboring BV-associated bacteria has been analyzed for effects on BV pathogenesis (Muzny et al., 2018; Castro et al., 2019; Gilbert et al., 2019). However, the pivotal role of *Gardnerella* in BV has been debated because of its presence in the healthy vagina. There are likely differences in virulence among *Gardnerella* strains, and the expression of virulence traits may increase under certain conditions (Hickey and Forney, 2014; Janulaitiene et al., 2018; Castro et al., 2019; Muzny et al., 2019).

Lactobacillus iners is another bacterium found at high levels in healthy and BV-positive women (Srinivasan et al., 2012; Petrova et al., 2015), although the hallmark of BV is a depletion of lactobacilli. *L. iners*-dominated microbiota are often detected at the transitional stage between normal and BV conditions (Petrova et al., 2017; Vaneechoutte, 2017b) and during menses (Santiago Lopes dos Santos et al., 2012); therefore, these microbial populations are considered to be less stable. *L. iners* predominantly produces L-lactic acid, which has lower protective capacity than the D-lactic acid released by other vaginal lactobacilli (Witkin and Linhares, 2017). The adaptation of *L. iners* to different environmental conditions may be due to the repertoire of gene expression, which ensures competitive adaptability and survival (Macklaim et al., 2011; France et al., 2016). Different *L. iners* lineages or groups with different adaptive properties may have functional roles in this adaptation (Petrova et al., 2017).

Both *Gardnerella* spp. and *L. iners* are present in the healthy human vagina and during anaerobic dysbiosis, and both secrete cholesterol-dependent cytolysins (CDCs) that belong to a common family of pore-forming toxins (PFTs) (Alouf, 2003; Christie et al., 2018). CDCs are produced by many pathogenic Gram-positive bacteria, and are recognized as virulence factors that participate in various stages of disease progression (Los et al., 2013). CDCs are secreted by a few Gram-negative bacteria that inhabit anaerobic soils and do not colonize humans or animals (Hotze et al., 2013). CDCs are cytotoxic to eukaryotic cells, including erythrocytes, and are known as cytolysins or hemolysins. *L. iners* secretes the CDC toxin inerolysin (INY) (Rampersaud et al., 2011), whereas *Gardnerella* spp. secrete the CDC toxin vaginolysin (VLY) (Rottini et al., 1990; Cauci et al., 1993; Gelber et al., 2008). VLY-mediated cell lysis is visible by cultivation of *Gardnerella* spp. on solid agar supplemented with human blood; β-hemolysis surrounding colonies indicates complete lysis of erythrocytes in the medium. The effect of these CDC toxins could be prerequisite for the survival and adaptation of *Gardnerella* and *L. iners* under diverse environmental conditions and during the development of BV. This review discussed the current knowledge, recent evidence, and implications relating to VLY and INY.

GENETIC CHARACTERISTICS

The cytolysin-coding genes *vly* and *iny* are single-copy genes in the chromosomes of *Gardnerella* spp. and *L. iners*, respectively. Both genes were cloned and sequenced (Gelber et al., 2008; Rampersaud et al., 2011). Among *Gardnerella* spp. isolates, VLY exhibits higher amino acid sequence variations, especially in the N-terminal region, than other CDCs. However, the detected amino acid substitutions did not affect the cytolytic activity of VLY (Pleckaityte et al., 2012). The VLY protein is 56 kDa, and INY protein is 57 kDa. Both toxins contain a signal sequence at the N-terminus that ensures secretion via a type II secretion pathway (Tweten, 2005). A recent study reported that VLY was detected in extracellular vesicles (EVs) produced by the *Gardnerella vaginalis* ATCC 14019 strain (Shishpal et al., 2019). EV-mediated delivery of toxins and other virulence factors has been identified for many pathogenic Gram-positive bacteria

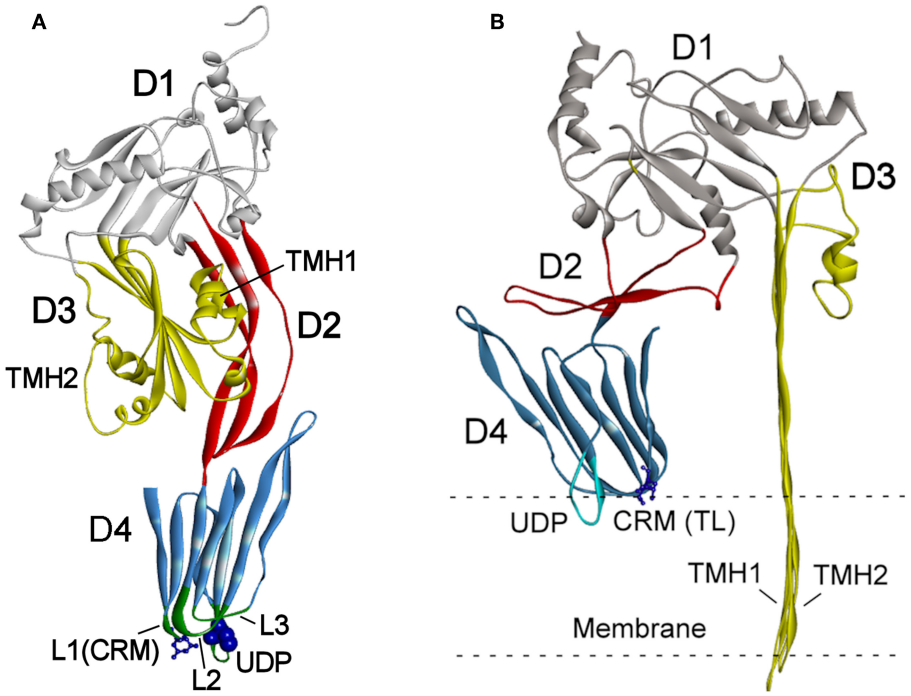

FIGURE 1 | (A) Schematic presentation of the crystal structure of PLY (PDB ID: 4QQA; Park et al., 2016). **(B)** One subunit of oligomeric PLY inserted into a membrane in the cryoEM structure (PDB ID: 5LY6; van Pee et al., 2017). The features of the structure are marked as follows: domains D1–D4 (highlighted in different colors), loops L1–L3, undecapeptide (UDP) in D4, the two-residue (Thr-Leu pair shown as sticks) cholesterol recognition motive (CRM) in loop L1, and the Cys residue in UDP (shown as blue spheres). The transmembrane hairpins TMH1 and TMH2 of D3 span the membrane in **(B)**.

(Brown et al., 2015; Liu et al., 2018). It is unclear whether the majority of VLY is secreted via EVs and how this pathway promotes *Gardnerella* spp. survival, colonization, and pathogenesis.

Genetic and phenotypic heterogeneity in the genus *Gardnerella* enabled the identification of genomic subgroups (Ahmed et al., 2012; Balashov et al., 2014; Schellenberg et al., 2016). Multiple *Gardnerella* subgroups were detected in non-cultured vaginal samples. *Gardnerella* multigroup communities were positively associated with BV (Balashov et al., 2014; Janulaitiene et al., 2017; Shipitsyna et al., 2019). A recent study identified and described 13 new species within the genus *Gardnerella*, including *Gardnerella leopoldii*, *Gardnerella swidsinskii*, *Gardnerella piotii*, and *G. vaginalis* (Vaneechoutte et al., 2019). It is likely that species or genomic subgroups are specifically associated with BV due to differences in their virulence potential. In our study (Janulaitiene et al., 2018), the majority of isolates of subgroup 4 that were associated with healthy microbiota produced a low amount of VLY *in vitro* compared to other groups. We identified isolates of subgroup 2 that were the *vly*-negative. Isolates with and without the *vly* gene colonized the same woman (Janulaitiene et al., 2018). By contrast, *iny*-negative isolates have not been identified, and INY was detected in all tested supernatants of cultured *L. iners* strains (Rampersaud et al., 2011).

STRUCTURAL FEATURES OF CDC PROTEINS AND AN OVERVIEW OF THE PORE-FORMING MECHANISM

The structures of CDCs from various bacterial genera were determined and revealed a four-domain structural organization (Rossjohn et al., 1997; Polekhina et al., 2004; Lawrence et al., 2015, 2016) (**Figure 1A**). The key sequence motifs involved in the recognition of cholesterol and membrane binding are highly conserved across all known CDCs.

The mechanisms of CDC interaction with membranes and pore formation have been extensively studied for perfringolysin (PFO) from *Clostridium perfringens* (Rossjohn et al., 1997; Ramachandran et al., 2002) and pneumolysin (PLY) from *Streptococcus pneumoniae* (Gilbert et al., 1998; Vögele et al., 2019). Cytolysins are secreted as water-soluble monomers (Tweten, 2005), which recognize membrane cholesterol via the cholesterol recognition motive (CRM) composed of the Thr-Leu pair in the L1 loop of domain D4 (Farrand et al., 2010) (**Figure 1A**). Recognition of cholesterol triggers the insertion of D4 structural components, an 11 amino acid undecapeptide (UDP) and the nearby L2–L3 loops, into a membrane that provides anchorage and stability of the monomer (Dowd et al., 2012; Christie et al., 2018). The conservative UDP sequence in the majority CDCs, including INY, contains a Cys residue (**Figure 2**) that, in the reduced state, ensures maximal activity

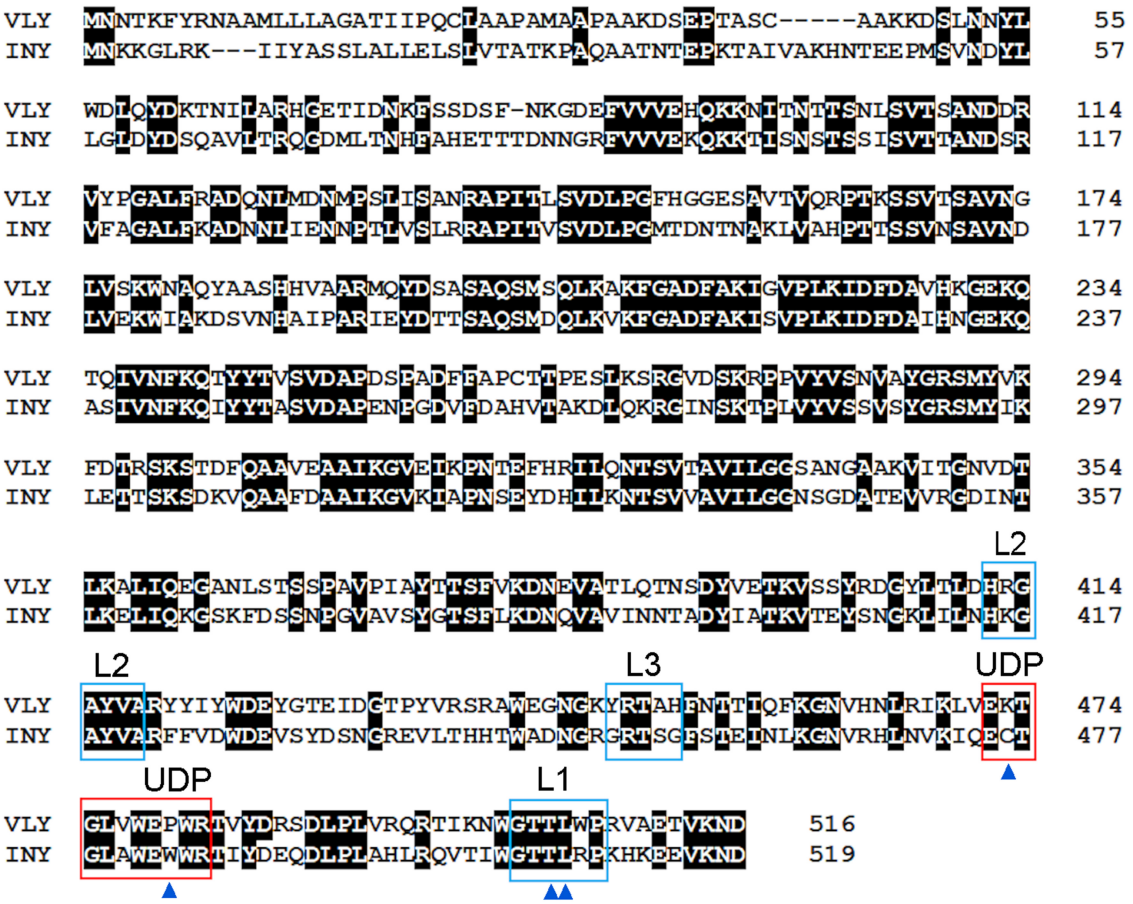

FIGURE 2 | Amino acid sequence alignment of VLY (accession no. ACD63042) and INY (accession no. WP_006730404). The sequences were aligned using Clustal W (Aiyar, 2000). Strictly conserved residues are indicated with a black background. Loops L1–L3 and the undecapeptide (UDP) are framed in blue and red, respectively. Cys residue in UDP of INY, the Pro/Trp in UDP, and the Thr-Leu pair (CMR) in L1 are indicated by blue triangles.

of toxins (Tweten, 2005; Rampersaud et al., 2011). Therefore, CDCs are also known as thiol-activated cytolysins. After membrane binding, structural changes occur in the protein that enable monomer-monomer interactions. The monomers oligomerize into rings and form a prepore structure on the membrane surface. The prepore is an SDS-resistant oligomeric complex characterized by a dense structure of ordinated and correctly oriented monomers. The prepore does not perforate the membrane (Hotze et al., 2002; Ramachandran et al., 2005). The transition from prepore to pore is accompanied by dramatic structural changes in domain D3, leading to the conversion of two helix bundles of each monomer to transmembrane β-hairpins (TMH) (Hotze and Tweten, 2012). Bilayer perforation is mediated by insertion of the TMH regions, and requires the helices to closely approach a membrane (**Figure 1B**). This is achieved by rotation of domain D2 following disruption of the D3 interface (Czajkowsky et al., 2004; Leung et al., 2014; van Pee et al., 2017). The β-barrel of the membrane-embedded pore complex (**Figure 3A**) is hydrophobic on the outside and hydrophilic inside (van Pee et al., 2017; Christie et al., 2018). This structure favors the displacement of membrane lipids and stimulates water influx, which finalizes the formation of a water-filled pore (Vögele et al., 2019).

CHARACTERISTICS OF VAGINOLYSIN AND INEROLYSIN ACTIVITIES

Cholesterol Is Not a Single CDCs' Receptor: The Vaginolysin Case

Cholesterol embedded into a cell membrane has been identified as a common and indispensable binding target for the majority CDCs (Hotze and Tweten, 2012). A distinct group of CDCs primarily bind to human complement glycoprotein CD59 (hCD59) rather than to cholesterol. Three members of this distinct group include VLY, *Streptococcus intermedius* intermedilysin (ILY), and *Streptococcus mitis* lectinolysin (Giddings et al., 2004; Gelber et al., 2008; Wickham et al., 2011). The glycosylphosphatidylinositol (GPI)-anchored human cell-surface receptor hCD59 interacts with the membrane attack complex (MAC) components C8α and C9, thereby blocking the accidental activity of MAC (Iacovache and van der Goot,

2004; Huang et al., 2007). Thus, hCD59 which ensures the unwanted lysis of host cells due to the activity of complement, serves as a receptor for some bacterial CDCs. Wickham et al. (2011) investigated ILY and MAC binding to hCD59; mutation of amino acid residues in hCD59 that modulate binding to ILY affects its protection against MAC-mediated lysis. The authors proposed that the existence of hCD59-responsive toxins may indicate microbial coevolution with humans, as the primary role of hCD59 is to block unwanted lysis of host cells.

The pore-forming mechanism of hCD59-dependent cytolysins is best-studied for ILY (LaChapelle et al., 2009; Farrand et al., 2010). When encountering host cells, ILY binds to hCD59 via the β-tongue located in D4 (**Figure 3B**). This event triggers the same structural changes as that of non-hCD59-dependent CDCs, but a pore does not form due to the lack of cholesterol. Contact between ILY and hCD59 allows the CRM-cholesterol interaction that triggers insertion of the L1–L3 loops into the bilayer (Farrand et al., 2010; Hotze and Tweten, 2012). After ILY is anchored to the membrane via the inserted loops, it disengages from hCD59 during the transition from a prepore to a pore. The release of hCD59 favors structural changes that are necessary for pore formation, as the increased binding affinity between ILY and hCD59 slows the rate of the prepore to pore transition and reduces cell lysis (Wickham et al., 2011). Boyd et al. (2016) recently reported that ILY interaction with hCD59, rather than with cholesterol, induced structural changes leading to collapse of the prepore structure, whereas cholesterol was required only for the final membrane perforation stage.

VLY-mediated cytotoxic activity was detected on cells that expressed hCD59 on their surface (Gelber et al., 2008; Zilnyte et al., 2015). Antibodies against VLY inhibited its cytolytic activity, demonstrating the specificity of VLY interaction with the cells (Zvirbliene et al., 2010; Pleckaityte et al., 2011). Membrane cholesterol is indispensable for the pore-forming activity of VLY and other known hCD59-dependent CDCs (Giddings et al., 2004; Gelber et al., 2008). ILY is a human-specific cytolysin (Giddings et al., 2004). By contrast, the cells lacking hCD59 were susceptible to VLY-mediated lysis, albeit at the elevated VLY concentrations (Zilnyte et al., 2015). Analysis of the crystal structure of VLY complexed to hCD59 (**Figure 3B**) revealed novel information regarding the dualistic activity of VLY (Lawrence et al., 2016). The stable VLY-hCD59 complex used for structural studies was obtained only with mutant VLY, which has limited capacity to oligomerize but retains its ability to interact with the receptor. The attempts of Lawrence et al. (2016) and our group (unpublished observations) to crystalize VLY alone were not successful.

The conformation of the L1 loop, which contains the CRM, is critical for cytolysin binding to cholesterol (Farrand et al., 2010; Lawrence et al., 2016). The structure of the UDP loop affects cholesterol-mediated membrane binding and D3 structural changes, which are the principal steps in the allosteric pore-formation pathway (Dowd et al., 2012). Superimposition of available CDC structures indicates that amino acids in the UPD loop of non-hCD59-dependent cytolysins interact with the L1 loop via hydrogen bonds, which leads to the proper conformation of L1 for initial binding with membrane

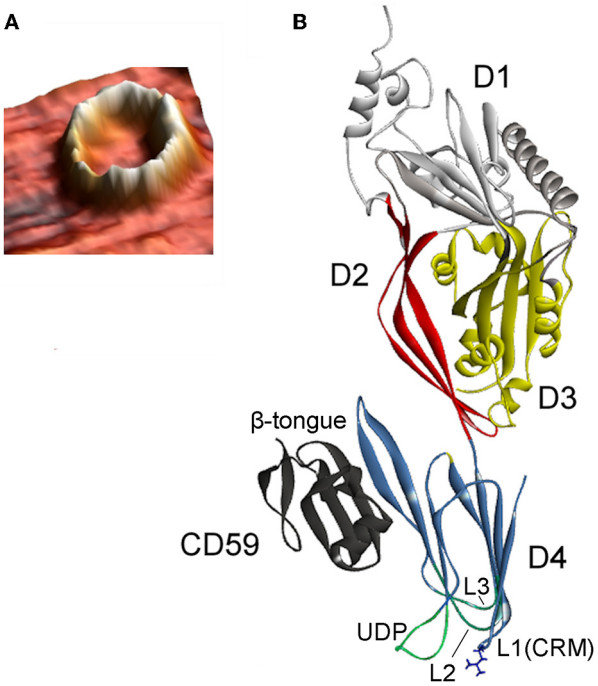

FIGURE 3 | (A) Atomic force microscopy three-dimensional topography image of the oligomeric structure obtained after reconstitution of VLY into a cholesterol-rich artificial bilayer. Calculated protrusion of the ring-shaped defect above the membrane is consistent with the height of inserted pore (reproduced with permission from Ragaliauskas et al., 2019). **(B)** Crystal structure of VLY in complex with hCD59 (PDB ID: 5IMY; Lawrence et al., 2016). VLY interacts with hCD59 via the β-tongue located in D4, similarly as ILY. Loop L1 contains the Thr-Leu (TL) pair of CRM.

cholesterol (Hotze and Tweten, 2012; Lawrence et al., 2016). The UDP loop in hCD59-dependent cytolysins contains Pro instead of Trp (**Figure 2**), which does not promote interaction of the neighboring amino acids with the L1 loop to ensure the proper orientation for cholesterol binding (Lawrence et al., 2016). Thus, hCD59-dependent cytolysins bind hCD59 instead of membrane cholesterol. Analysis of the two monomers in the VLY-hCD59 complex revealed that the UDP loop can adopt two conformations that are characteristic of either classical non-hCD59-dependent CDCs (e.g., PFO) or strictly hCD59-dependent ILY. The adopted UDP conformations predict which primary binding target, hCD59 or cholesterol, is selected in the host membrane to execute VLY pore-forming activity. It is likely that hCD59 is a more effective binding target than cholesterol for VLY interaction with the host membrane (Lawrence et al., 2016). INY is a classical non-hCD59-dependent cytolysin, although its primary amino acid sequence shows the greatest similarity to the CD59-dependent CDCs ILY (sequence identity 48.8%, Rampersaud et al., 2011) and VLY (sequence identity 50.8%, **Figure 2**).

Membrane Binding

CDCs bind and affect membranes with high cholesterol levels, which contain a cholesterol threshold of >30 mol% of total

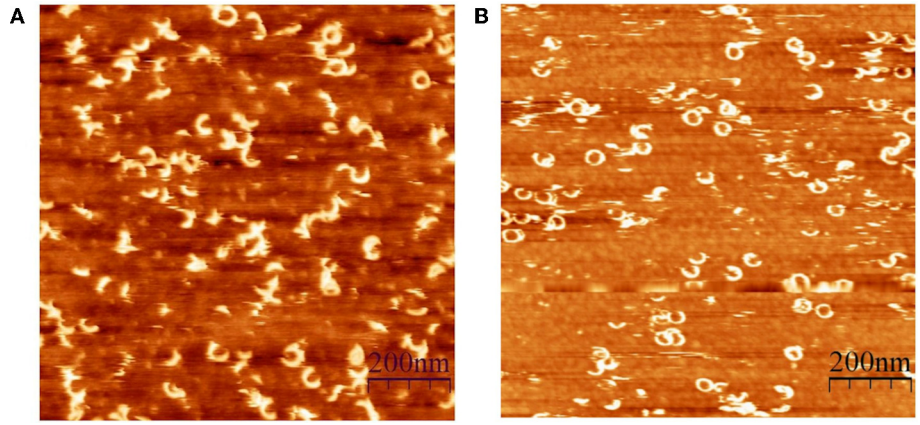

FIGURE 4 | Atomic force microscopy topography images of the ring and arciform-shaped structures obtained after reconstitution of INY **(A)** and VLY **(B)** into tBLMs (reproduced with permission from Ragaliauskas et al., 2019).

membrane lipid (Bavdek et al., 2007; Johnson et al., 2012; Zilnyte et al., 2015). The accessibility of membrane cholesterol to CDCs has been a subject of debate. CDCs were proposed to bind to microdomains called lipid rafts, which are enriched with sphingolipids, cholesterol, and proteins (Waheed et al., 2001). However, sphingomyelin, which is an essential component of lipid rafts, inhibited PFO binding to a membrane (Flanagan et al., 2009). Subsequent studies showed that CDC binding required membrane conditions with surface-exposed cholesterol (Nelson et al., 2008). Cholesterol accessibility is modulated by the structures of phospholipid headgroups and acyl chains. Tightly packed phospholipids reduced cholesterol exposure, whereas loosely packed phospholipids enhanced cholesterol availability to CDCs (Rojko and Anderluh, 2015; Chakrabarti et al., 2017; Morton et al., 2019).

The results of electrochemical studies revealed that VLY binds to tethered lipid bilayer membranes (tBLMs) and impacts membrane integrity when cholesterol content was 50 mol% (Ragaliauskas et al., 2019), whereas other methods detected SDS-resistant VLY oligomeric complexes on liposomes containing cholesterol contents of 40–55 mol% (Zilnyte et al., 2015). The fact that VLY did not permeabilize cells in the apical side of a three-dimensional model of vaginal epithelium can be explained by the differential abundance of surface-associated hCD59 and the diverse cholesterol contents in the cell membranes (Garcia et al., 2019). By contrast, INY was active on synthetic membranes with low cholesterol contents of 20–30 mol%. INY binding to tBLMs and the extent of INY-induced membrane damage were directly dependent on membrane cholesterol concentration (Ragaliauskas et al., 2019). INY is likely to be the first CDC with activity on synthetic membranes that does not require a certain cholesterol threshold. However, INY-induced membrane damage was measured in bilayers with specific phospholipid compositions (Ragaliauskas et al., 2019).

Measuring the effects of the lipid environment on the activity of VLY and INY requires detailed investigations. PFO and streptolysin (SLO) have different binding specificities to the same natural and synthetic membranes (Farrand et al., 2015; Johnson et al., 2017). Altering the structure of the L3 loop by mutating critical residues changed the membrane binding parameters of PFO and SLO. CDCs may have evolved the capacity to select lipid environments by manipulating the L3 structure, and thereby display individual specificities for cholesterol (Farrand et al., 2015; Christie et al., 2018).

Pore Size and Membrane Damage

CDCs belong to the β-PFT family because the β-sheet structures cross the host membrane (**Figure 1**). The distinguishing feature of the CDCs among β-PFTs is the large pore size; more than 30 monomers constitute the full rings, and pore diameters measure up to 30 nm (Hotze and Tweten, 2012). However, PLY (Sonnen et al., 2014), suilysin (Leung et al., 2014), ILY (Boyd et al., 2016), listeriolysin O (LLO) (Mulvihill et al., 2015), VLY, and INY (Ragaliauskas et al., 2019) also form incomplete rings, slits, and arcs that perforate synthetic membranes (**Figure 4**). Atomic force microscopy results indicated that arciform structures were predominate after reconstitution of INY into cholesterol-rich tBLMs (**Figure 4A**), whereas membrane-embedded complete rings with a diameter of 26 nm were predominate for VLY (**Figure 4B**) (Ragaliauskas et al., 2019). The majority of arciform structures produced by VLY and INY are inserted into the lipid bilayer and form a water-filled pore (Ragaliauskas et al., 2019). The VLY-generated pore geometries in tBLMs may differ from those in hCD59-containing membranes.

The arcs and slits that perforate membranes generate smaller pore sizes than those of complete rings. Pore size controls the molecules that can pass through the cell membrane, including ions, small molecules, and proteins (Dal Peraro and van der Goot, 2016). Presumably, pore size depends on the CDC function in the cell. LLO exhibits diverse activities in *Listeria monocytogenes* infection and triggers various host cell responses (Hamon et al., 2012). LLO generated small membrane perforations that leaked Ca^{2+} ions and protons from a eukaryotic cell (Shaughnessy et al., 2006), whereas other toxin activities in the host may generate extensive membrane damage (Osborne and Brumell, 2017). Another study concluded that pore size is not completely

correlated with PFO- and SLO-mediated membrane damage (Ray et al., 2019). Los et al. (2013) observed differential effects of CDC-induced small and large pores *in vitro*; however, these differences were not evident in *in vivo* data. Pore-mediated membrane damage depends on CDCs and host responses to seal the pores and prolong cell survival (Cassidy and O'Riordan, 2013; Brito et al., 2019).

Effect of pH

Several CDCs display pH-dependent activity, which is well-studied for LLO (Schuerch et al., 2005; Bavdek et al., 2012; Podobnik et al., 2015). LLO remains active at acidic pH, whereas it is irreversibly inactivated due to unfolding at neutral pH (Schuerch et al., 2005; Bavdek et al., 2012). The unfolding occurs in the D3 domain, and three acidic residues participate in the denaturation process (Schuerch et al., 2005). Podobnik et al. (2015) suggested that LLO forms pores irrespective of pH, but ionic conductance of the pores is regulated by a His residue that is pH-dependent. The pH-dependent activity of LLO may have biological significance. *L. monocytogenes* replicates within phagocytic host cells; therefore, the bacterium needs to escape from a vacuole that formed around it during entry to reach the host cell cytosol. *L. monocytogenes* acidifies the lumen of the vacuole, which activates LLO so that the cytolysin can destroy the vacuole membrane. LLO is not active at neutral pH, thereby preventing host cell destruction (Hamon et al., 2012; Osborne and Brumell, 2017).

INY and VLY effects on human erythrocytes and cholesterol-rich synthetic membranes is pH-sensitive. INY is active at acidic pH and inactive at neutral pH, whereas VLY activity peaks at neutral pH and is marginal at acidic pH (Rampersaud et al., 2011; Ragaliauskas et al., 2019). The pH- and temperature-dependent loss of activity is not related to protein denaturation, which is distinct from the dependence of LLO (Rampersaud et al., 2011; Ragaliauskas et al., 2019). INY regains activity after a pH shift from neutral to acidic (Rampersaud et al., 2011). Ratner et al. found that INY binding to membrane, including the oligomerization step, is pH-independent. The final step, INY membrane insertion, is impaired at neutral pH (Rampersaud et al., 2018). Ragaliauskas et al. (2019) reported that binding of INY and VLY to tBLMs is pH-sensitive. INY demonstrated weak or no binding activity to artificial lipid bilayer at neutral pH. Different INY and VLY activity profiles are consistent with vaginal pH under certain physiological conditions. INY is more active in the pH range characteristic of the healthy vagina, whereas VLY is active at higher pH specific for BV (Donders, 2007). The fact that INY is active in the pH range of 4.5–6.0 suggests that this toxin may be involved in the adaptation and survival of *L. iners* during transitional stages and dysbiosis.

Cytolysin Concentration *in vivo*

There are some limitations in interpreting the results of mechanistic and functional studies of PFTs (Los et al., 2013), and these limitations should be considered for interpreting VLY and INY data in various models. Specifically, high doses of purified toxins used *in vivo* might not be relevant under physiological conditions (Los et al., 2013). The concentration of CDCs may be related to the ability to form pores in host membranes. High CDC doses, which are called lytic concentrations, are linked with the formation of lytic pores that lead to cell death. CDC doses at low, sublytic levels due to expression downregulation or lower bacterial densities is likely to produce non-lytic pores that lead to the modulation of cell signaling cascades (Ratner et al., 2006; Aguilar et al., 2009). CDCs at sublytic concentrations may affect host cells indirectly through pore-independent functions (Meehl and Caparon, 2004; Osborne and Brumell, 2017). However, CDC quantities under physiological conditions are unknown, except for PLY. Unexpectedly high median concentrations of PLY up to 30 μg/mL were detected in the cerebrospinal fluid (CSF) of patients who survived pneumococcal meningitis (Wall et al., 2012). Another study reported PLY concentrations up to 0.18 μg/mL in CSF of patients with meningitis (Spreer et al., 2003). Cell lysis *in vitro* was not visible at 0.5 μg/mL PLY, although the *in vivo* lytic capacity of PLY was enhanced by other factors (Spreer et al., 2003; Wippel et al., 2011).

In previous reports, VLY content was measured solely in planktonic cultures of various *Gardnerella* spp. isolates (Randis et al., 2009; Pleckaityte et al., 2012; Tankovic et al., 2017; Janulaitiene et al., 2018). Recently, VLY contents were measured in vaginal samples of women with healthy vaginal microbiota or anaerobic dysbiosis (Nowak et al., 2018). The median VLY concentrations were highest (3 ng/mL) in vaginal samples with deficient *Lactobacillus* (Nowak et al., 2018), intermediate concentrations in the presence of primarily *L. iners*, and lowest concentrations in the presence of primarily *L. crispatus*. The authors proposed that VLY might represent a marker of BV, as other characteristics of the vaginal samples also were associated with VLY concentrations, including *Gardnerella* spp. abundance, pH, and Nugent score (Nowak et al., 2018). The VLY concentrations in these vaginal samples (Nowak et al., 2018) were high enough to lyse human erythrocytes *in vitro*. VLY-mediated cytotoxicity to vaginal epithelial cells *in vitro* was detected at substantially higher concentrations (Zilnyte et al., 2015).

INTERPRETATIONS OF THE PHYSIOLOGICAL ROLES OF INEROLYSIN AND VAGINOLYSIN

There is a general concern that studies on the pathogenesis of human vaginal dysbiosis are challenging due to many factors, including the lack of a suitable animal model, vaginal microbiota are a dynamic community, and the impact of host and interplay between neighboring microorganisms. Studies on the role of cytolysins produced by vaginal bacteria have encountered similar difficulties. There are no methods for genetic manipulation of *Gardnerella* spp. remain a limitation. Cytolytic activity, mechanisms of pore formation, and some biological parameters have been studied using human cells, cell lines, and artificial bilayers. Recently, a three-dimensional polarized human vaginal tissue model has been used to characterize the interaction between vaginal epithelium, *G. leopoldii* strain (for the strain delineation see Vaneechoutte et al., 2019), and VLY (Garcia et al., 2019).

Cytolysins from *Gardnerella* spp. and *L. iners* share features that attribute them to the CDC family, and it is believed that these toxins have a similar role as their family members during infection *in vivo*. Studies on the functions of VLY and INY in bacterial pathogenesis are still in their infancy. This section focuses on studies that demonstrate the potential role of VLY and INY in the adaptation and survival of bacteria and the progression of vaginal anaerobic dysbiosis.

Ratner et al. reviewed the role of CDCs and other PFTs in bacterial infectious diseases (Los et al., 2013), and identified two main effects of toxins during *in vivo* infection. (1) The primary effect is induced barrier dysfunction of epithelial and endothelial layers via direct and indirect effects caused by cell attack and host immune response at the site of infection, respectively. Among CDCs, these effects are best-known for PLY and SLO (Meehl and Caparon, 2004; Goldmann et al., 2009; Hupp et al., 2012; Wippel et al., 2013). (2) The second effect was disruption of the host immune response by invading host factors, cytotoxicity toward immune cells, or intracellular survival. Impairment of immune defense was studied for LLO, PLY, PFO, and SLO (Marriott et al., 2008; Domon et al., 2016; Osborne and Brumell, 2017; Bhattacharjee and Keyel, 2018). The identified toxin functions involved pathological conditions associated with a bacterial infection.

The study of Los et al. (2013) could not determine whether commensal bacteria or bacteria under non-pathogenic conditions expressed CDCs *in vivo*. Although *L. iners* is not generally considered as a common commensal (Vaneechoutte, 2017b), it is still unclear whether INY is secreted in the healthy vagina (Macklaim et al., 2013) where *L. iners* is abundant. It is hypothesized that INY activity is utilized by *L. iners* to obtain necessary growth nutrients, outcompete other bacteria, and survive under various environmental conditions (France et al., 2016; Petrova et al., 2017; Vaneechoutte, 2017b).

VLY production has been detected in BV-positive women and those with healthy vaginal microbiota (Nowak et al., 2018). The median VLY concentration (1 ng/mL) in vaginal samples with predominant *L. crispatus* is approximately three-fold lower than that in *Lactobacillus*-deficient samples. Healthy microbiota characterized by low pH express minimal VLY activity *in vitro* (Rampersaud et al., 2011). VLY concentrations approximately six-fold higher than those detected *in vivo* caused membrane blebbing but not cell lysis in epithelial cell monolayers (Randis et al., 2013). The formation of membrane blebs induced by PFT-mediated membrane injury is a mechanism of cell protection that promotes the survival of affected cells (Babiychuk et al., 2010; Brito et al., 2019).

Garcia et al. (2019) found that VLY was not essential for intensive growth of the *G. leopoldii* strain AMD on the apical side of a three-dimensional vaginal epithelium model, as VLY does not permeabilize the apical side of vaginal tissue. LaRocca et al. (2014) reported that the hCD59-dependent cytolysins VLY and ILY induced programmed necrosis in mature human erythrocytes. Although erythrocytes are not the primary targets, they may come in contact with cytolysins during menses (Santiago Lopes dos Santos et al., 2012; Schwebke et al., 2014b) and in bacteremia (McCool and DeDonato, 2012; Tankovic et al., 2017). Bacterial growth *in vitro* (including *Gardnerella* spp.) was substantially enhanced by programmed necrosis of erythrocytes. Release of the cytosolic content of erythrocytes may provide required nutrients that lead to a burst in bacterial growth (LaRocca et al., 2014). During menses, the concentrations of *Gardnerella* spp. and *L. iners* increased in the majority of women with healthy vaginal microbiota (Santiago Lopes dos Santos et al., 2012). Menses is the most disturbing factor affecting the stability of vaginal microbial communities (Santiago Lopes dos Santos et al., 2011); therefore, cytolysin-mediated release of nutrients may allow bacteria to overcome this disturbance.

A case report described a *Gardnerella* spp. infection complicated with bacteremia and a toxic-type encephalopathy in a young woman (Tankovic et al., 2017). *G. swidsinskii* strain GV37 isolated from blood cultures produced elevated amounts of VLY *in vitro* compared to that of control strains. The authors hypothesized that high concentrations of VLY *in vivo* can break the blood-brain barrier and affect brain cells.

Treatment of cervical cells with cell-free supernatants of *L. iners* and *G. vaginalis* significantly increased permeability of the cervical epithelial barrier and induced the resulting immune response (Anton et al., 2018). VLY and INY are candidates among the secreted factors that may have induced these effects. However, the authors did not analyze the presence of these toxins in the culture supernatants. The use of *Gardnerella* spp. strains lacking the *vly* gene isolated by our group might help to clarify the effect of cytolysin on cell permeability (Janulaitiene et al., 2018).

The activity of PFTs is related to the host response that defends against bacterial infections (Huffman et al., 2004; Aguilar et al., 2009). Sublytic doses of PFTs result in sublethal numbers of pores, which are perceived by epithelial cells via osmotic stress-induced activation of p38 MAPK signaling (Ratner et al., 2006). The p38 MAPK activation is a conserved response to the disruption of cell membrane integrity caused by many PFTs (Los et al., 2013). Activation of p38 MAPK induces the expression of proinflammatory cytokines that modulate the immune response *in vivo*. Thus, sublytic doses of toxins due to low bacterial density during early infection may function as a signal to epithelial cells to initiate protective immune responses (Ratner et al., 2006). Sublytic doses of INY and VLY induced p38 MAPK activation through phosphorylation in epithelial cells (Gelber et al., 2008; Rampersaud et al., 2011). The mRNA for IL-8 was upregulated after treatment of HeLa cells with VLY (Gelber et al., 2008). Garcia et al. (2019) found that IL-1β was the only cytokine induced by VLY at the basolateral side of vaginal epithelium. Specific IgA antibodies against VLY were detected in 60% of women diagnosed as BV-positive (Cauci et al., 1996); this correlated with IL-8 levels and leukocyte counts in BV-positive and BV-negative women (Cauci et al., 2002).

There have been contradictory reports on cytokine profiles in cervicovaginal lavages of BV-positive women (Mitchell and Marrazzo, 2014). Some studies showed that BV-positive lavages did not have elevated levels of IL-6 and IL-8 and increased neutrophil counts (Cauci, 2004; Donders, 2007; Nowak et al., 2018). By contrast, BV-positive lavages had higher levels of IL-6, IL-8, other proinflammatory cytokines, and chemokines (Hedges et al., 2006; Mitchell and Marrazzo, 2014). The absence

of leukocytes might be due to a lack of IL-8 induction in most women with BV, indicating that the host genotype conditions the immune response (Cauci et al., 2002; Mitchell and Marrazzo, 2014).

Several studies have tried to elucidate cytokine induction by individual bacterial species using monolayered or polarized human vaginal epithelial cells that were co-cultured with bacteria or exposed to cell-free supernatants. *L. iners* did not induce an increased production of IL-8 and IL-6 in vaginal and cervical cells (Doerflinger et al., 2014; Anton et al., 2018). However, elevated secretion of these cytokines was detected after exposure of ectocervical cells to *G. vaginalis* cell-free supernatants (Anton et al., 2018). Other studies confirmed that BV-associated bacteria induce cytokine upregulation and lead to the host response, whereas vaginal *Lactobacillus* spp. do not (Mitchell and Marrazzo, 2014). Secreted bacterial factors that modulate the immune response have not been completely determined; however, cytolysin remains as the prime suspect. The absence of inflammation in the lower genital tract is a typical clinical symptom of BV (Donders, 2007). However, BV is linked with clinical conditions that are characterized by inflammation in the upper genital tract, such as pelvic inflammatory disease and cervicitis (Mitchell and Marrazzo, 2014). Non-infectious, inflammation-related preterm delivery is associated with vaginal microbial communities that contain abundant *Gardnerella* spp. (DiGiulio et al., 2015).

FACTORS AFFECTING CYTOLYSIN EXPRESSION

The expression of cytolysin genes is affected by environmental changes in oxygen concentration, the impact of other bacteria, biofilm, and planktonic growth phenotypes. Biofilm is a favorable mode of *Gardnerella* spp. growth that confers resistance to environmental factors and contributes to survival (Patterson et al., 2007; Verstraelen and Swidsinski, 2019). The oxygen gradient within a biofilm regulates the distribution and survival of bacterial species (Monds and Toole, 2009; Castro et al., 2019). *Gardnerella* spp. colonize the vagina of many women without inducing signs of BV (Ma et al., 2012; Schwebke et al., 2014a), suggesting that *Gardnerella* spp. may adopt the planktonic style of growth by modulating the gene expression profile (Swidsinski et al., 2014; Castro et al., 2019). Certain *Gardnerella* species or genomic subgroups may have a reduced capacity to adhere to epithelial cells and form a biofilm (Castro et al., 2015; Janulaitiene et al., 2018), probably due to differences in proteins that contribute to and participate in these activities (Harwich et al., 2010; Marín et al., 2018).

Comparative transcriptomic analysis of the *G. leopoldii* AMD strain and several other *Gardnerella* spp. strains demonstrated that the transcription level of the *vly* gene was significantly lower in biofilms than in planktonic cells (Castro et al., 2017). As expected, the transcription of genes responsible for biofilm cell metabolic activity in biofilm cells also was reduced. The authors suggested that VLY was likely not required for the persistence of mono-species biofilm, which represents a long-lasting mode of vaginal colonization. The interactions between *Gardnerella* spp. and other BV-associated bacteria were analyzed using dual-species biofilm assemblies (Castro et al., 2019). Expression of the *vly* gene was significantly upregulated in biofilms when *Gardnerella* was associated with *Enterococcus faecalis* or *Actinomyces neuii*. By contrast, *vly* expression was only slightly upregulated when *Gardnerella* was associated with the BV-related bacteria *Prevotella bivia*. *Atopobium vaginae*, which

TABLE 1 | Summary of vaginolysin and inerolysin characteristics.

Characteristic	Vaginolysin	Inerolysin
CDC-producing bacteria	*Gardnerella* species	*Lactobacillus iners*
Presence of the CDC coding gene	Majority of isolates	Prevalence among isolates is not well known
Mode of action	Form β-barrel pores in the membrane	
Structural features	Display a four-domain structural organization; possess the key sequence motifs characteristic of CDCs	
Receptor	Human CD59 or membrane cholesterol	Membrane cholesterol
Pore-forming mechanism	Bind to the cholesterol-rich membranes, oligomerize into large oligomeric complexes (rings and arciform structures), undergo conformational changes leading to membrane perforation and pore formation	
Membrane cholesterol	Indispensable for the pore-forming activity	
Membrane cholesterol concentration required for CDC activity	>40 mol%	>20 mol%
pH activity range	5.0–7.5	4.5–6.0
Effect (1): lytic CDC concentrations	Direct cell damage: formation of water-filled pores in the target cellular cholesterol-containing membranes	
Effect (2): sublytic CDC concentrations	Non-lytic pores modulate signaling cascades: induce p38 MAPK activation and cytokine expression	
Effect (3): independent of pore formation	Not known	Not known
Pore size on synthetic cholesterol-rich membranes	Dominate large pores (ring structures)	Dominate smaller-size pores (aciform structures)
Presence in healthy vagina	Yes	Not known
Role in healthy vagina	VLY is not active	Not known
Presence in BV	Yes	Yes
Plausible role in BV	VLY-mediated cytotoxicity results in vaginal epithelial cell desquamation	INY-mediated disruption of the host cell integrity to obtain nutrients necessary for growth, outcompete other bacteria, and survive in the vaginal environment

has most frequently been detected within the *Gardnerella* biofilm matrix, does not affect the *vly* transcription level compared to the mono-species biofilm composed solely of *Gardnerella*. *Mobiluncus mulieris* also did not affect the *vly* transcription level, whereas *Brevibacterium ravenspurgense* repressed *vly* expression. Castro et al. (2019) reported that urogenital bacterial species that were seldom detected in BV had a higher effect on the expression of genes related to virulence than commonly BV-associated *A. vaginae* and *M. mulieris*. One strain of *Gardnerella* spp. isolated from BV-positive women was used in a dual-species biofilm approach. This does not exclude that other species or genomic variants of *Gardnerella* may experience diverse effects. In summary, the *vly* transcript level is lower in *Gardnerella* spp. biofilm than in the planktonic mode of growth. However, certain bacterial species found in the unique dual-species biofilm morphotypes affect *Gardnerella* spp. cells and increase *vly* expression (Castro et al., 2019). The authors proposed that vaginal epithelial cell desquamation characteristic of BV was connected with the increased transcription level of *vly* and VLY-mediated cytotoxicity. By contrast, *L. crispatus*, which is distinctive to a healthy vagina, reduced the cytotoxicity of several *Gardnerella* spp. isolates toward HeLa vaginal epithelial cells by repressing *vly* expression (Castro et al., 2018).

Application of the meta-transcriptomic approach revealed that INY was upregulated six-fold and VLY was upregulated 256-fold in BV compared to a healthy state (Macklaim et al., 2013). Mitchell and Marrazzo (2014) reported that it was not clear whether these changes occurred due to strain differences or because the microbial environment reflected the gene expression levels. These studies require further investigation and analysis to detect differences in the expression profiles of cytolysins isolated from a large number of vaginal samples.

DISCUSSION AND PERSPECTIVES

CDCs have evolved a sophisticated mechanism to punch a hole in host membranes and invade the cells. Cytolysin-mediated disruption of membrane integrity of the cell barrier leads to various consequences to the host, from the direct cell death to impaired cellular functions. Research challenges still remain, such as determining the structure-function relationships *in vivo*, although many issues regarding the structure of CDCs and their pore-forming activity have been elucidated. Some CDC activities are accomplished by pore-independent pathways. All these activities promote growth and invasion of bacterial pathogens; therefore, CDCs are accepted as important virulence factors contributing to the pathology of infectious diseases. Bacterial species that are not regarded as traditional human pathogens also produce cytolysins, including *Gardnerella* spp. and *L. iners*. Studies to elucidate the role of *Gardnerella* as a pathogen have been performed for more than 60 years. In the recent past, this taxon was underrated, but has been recently revived by the definition of vaginal *Gardnerella* polymicrobial biofilm as a characteristic of BV, and identifying species and genomic groups with potentially diverse roles in health and disease. Future work will continue to delineate the importance of *L. iners*.

This review provides an overview of structural and activity characteristics of the cytolysins VLY and INY (**Table 1**). The environmental pH, accessibility of membrane cholesterol, and cytolysin concentration may affect the activity and function of these CDCs on eukaryotic cells. However, many issues remained unsolved. The membrane composition that is more prone to cytolysin binding remains to be determined, which may condition the specificity to the target during infection and in a commensal state. The impact of hCD59 on some characteristics of VLY activity needs to be fully elucidated because the majority of studies on synthetic membranes did not include this receptor. Knowledge of the genetic regulation of *vly* and *iny* expression is lacking. It is unclear whether non-hCD59-responsive INY is sensitive to other binding targets besides cholesterol. *Gardnerella* spp. and *L. iners* cytolysins are considered as virulence factors by analogy with other CDCs, although their particular roles *in vivo* are unknown. This report also provides insights into the predicted and hypothetical functions of VLY and INY *in vivo* (**Table 1**). Prospectively, the effects of cytolysins *in vitro* should be studied by the inclusion of different *Gardnerella* species and *L. iners* strains, and strains lacking *vly* and *iny* genes. Models that more closely resemble *in vivo* conditions are needed to unravel the relevance of cytolysins to the pathogenesis of BV. The polymicrobial nature of BV is technically challenging for studying the effects of cytolysins, as it is an experimentally evident influence of neighboring vaginal bacteria on the virulence genes. It is crucial to study the role of cytolysins in the context of host microbiota, its fluctuations, and disturbance that condition the virulence potential of bacterial species.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Growth Forms of *Gardnerella* spp. and *Lactobacillus* spp. on Vaginal Cells

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Bacterial vaginosis (BV) is a common vaginal condition in women of reproductive age. During BV development, BV-associated bacteria may form a polymicrobial biofilm, which predispose women to recurrent BV. The aim of the study was to investigate the growth forms of *Gardnerella* spp. and *Lactobacillus* spp. and to determine the association between the bacterial growth forms and clinical characteristics [urinary tract infection (UTI) symptoms, human immunodeficiency virus (HIV) infection and abnormal vaginal discharge] in women attending a tertiary hospital in Pretoria, South Africa. A first-void urine specimen was collected from 196 women and BV was diagnosed using the Nugent scoring and the Ison-Hay criteria (vaginal smear microscopy). Fluorescence *in situ* hybridisation (FISH) was performed to classify the growth forms ["dispersed" or "biofilm"]. Bacterial cells were categorized as "dispersed" if cells were scattered separately and as "biofilm" if bacterial aggregates on the vaginal epithelial cells were observed. BV was detected in 52 women (52/196; 27%) and in these women, *Gardnerella* spp. were predominantly present in biofilms (46/52; 88% for Nugent scoring; and 45/50; 90% for Ison-Hay criteria), whereas *Lactobacillus* spp. were predominantly present in a dispersed form (38/52; 73% for Nugent scoring; and 37/50; 74% for Ison-Hay criteria). The odds of having BV increased when *Gardnerella* biofilms were present ($p < 0.001$), whereas the opposite was observed for *Lactobacillus* biofilms ($p = 0.001$). Neither *Gardnerella* spp. or *Lactobacillus* spp. (both dispersed or biofilms) had an association with the presence of UTI symptoms, HIV coinfection or abnormal vaginal discharge. In conclusion, this study demonstrated and confirmed that *Gardnerella* biofilms are associated with BV and that *Lactobacillus* spp. may form biofilms to protect against BV.

Keywords: bacterial vaginosis, biofilm, *Gardnerella* spp., *Lactobacillus* spp., fluorescence *in situ* hybridization

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INTRODUCTION

Bacterial vaginosis (BV) is the most common vaginal disease in women of reproductive age (Kenyon et al., 2013). BV may have a devastating impact on women's reproductive health due to its association with preterm birth (Leitich et al., 2003; Shimaoka et al., 2019), miscarriages (Hay et al., 1994) and tubal infertility (Wilson et al., 2002; van Oostrum et al., 2013). Furthermore, BV has been

suggested to increase the risk of acquisition of sexually transmitted infections (STIs) (Allsworth and Peipert, 2011) and human immunodeficiency virus type 1 (HIV-1) (Atashili et al., 2008; van de Wijgert et al., 2008).

BV is a polymicrobial dysbiotic condition, which is characterized by a decrease in the normally resident lactic-acid producing vaginal bacteria (usually *Lactobacillus* spp.) and an overgrowth of vaginal anaerobic bacteria (Hillier et al., 1993; Fredricks et al., 2005; Ravel et al., 2013). Although the exact pathogenesis of BV remains unknown, two main hypotheses are currently in debate to explain the BV etiology—that BV is triggered by the initial establishment of the "key bacteria" such as virulent strains of *Gardnerella* spp., *Prevotella bivia*, *Atopobium vaginae* and *Megasphaera* type 1; or it is caused by the sexual introduction of the polymicrobial community that predominantly consists of the above-mentioned "key bacteria" (Srinivasan and Fredricks, 2008; Muzny and Schwebke, 2013; Muzny et al., 2018, 2019). *Gardnerella* spp. have long been suggested as an etiological agent of BV since its discovery (described as *Haemophilus vaginalis*) by Gardner and Dukes (1955), but its role in BV etiology has remained controversial over the years because of its presence in the microbiota of "healthy" women (Zhou et al., 2004; Ravel et al., 2011; Shipitsyna et al., 2013). This controversy could partially be explained by the evidence that four different genotypes/clades (or 13 genomic species) may exist within the genus *Gardnerella* (Santiago et al., 2011; Ahmed et al., 2012; Vaneechoutte et al., 2019), and that there are differences in virulence potential between avirulent and virulent strains of *Gardnerella* spp. (Harwich et al., 2010; Janulaitiene et al., 2018).

During the initial establishment of BV, the BV-associated bacteria such as virulent strains of *Gardnerella* spp. may adopt the biofilm mode of growth (Swidsinski et al., 2005, 2014). The formation of a polymicrobial biofilm not only allows BV-associated bacteria to survive and to persist against the antibiotic therapy, but also provides a shelter to tolerate hydrogen peroxide (H_2O_2) and lactic acid produced by *Lactobacillus* spp. (Patterson et al., 2007; Swidsinski et al., 2008; Alves et al., 2014). Among many BV-associated bacteria that can form biofilms, virulent strains of *Gardnerella* spp. and *A. vaginae* are the predominant bacterial species actively involved in the formation of a polymicrobial BV biofilm (Swidsinski et al., 2005, 2014; Hardy et al., 2016). *Gardnerella* spp. are hypothesized as the "early colonizer species" that establishes a baseline for the formation of a BV biofilm, because of its strong initial adhesion ability and high biofilm formation capacity (Patterson et al., 2010; Machado et al., 2013a,b; Castro et al., 2015). Anaerobes such as *P. bivia*, *A. vaginae* and *Megasphaera* type 1 may then co-exist as a "second colonizer species" in a biofilm formed by *Gardnerella* spp. (Swidsinski et al., 2005; Hardy et al., 2016; Muzny et al., 2018, 2019; Castro et al., 2019).

As biofilms allow *Gardnerella* spp. and *A. vaginae* to persist after metronidazole treatment in recurrent BV (Bradshaw et al., 2006; Swidsinski et al., 2008), it could be important to study the growth form of BV-associated bacteria (especially BV biofilms), which is useful in the development of the treatment strategies. One approach to visualize the bacterial growth form and the bacterial composition in biofilms is by using fluorescence *in situ* hybridization (FISH) (Hardy et al., 2016). Many previous studies have successfully used FISH to demonstrate the importance of a polymicrobial biofilm in BV; however, none of these studies have directly compared each growth form of BV-associated bacteria with Ison-Hay criteria and clinical characteristics such as HIV status and urinary tract infection (UTI) symptoms (Swidsinski et al., 2005, 2014; Hardy et al., 2016). Therefore, the present study aimed to investigate the growth forms of BV-associated bacteria (*Gardnerella* spp. and *A. vaginae*) and *Lactobacillus* spp. on the vaginal epithelial cells in first-void urine specimens of women attending a tertiary hospital in Pretoria, South Africa, as well as to determine the association between bacterial growth forms and clinical characteristics (HIV status, presence of UTI symptoms and presence of abnormal vaginal discharge) in BV-positive women.

MATERIALS AND METHODS

Study Setting and Participants

Non-pregnant women attending a gynecology clinic and pregnant women attending a maternal and fetal unit (MAFU) at a tertiary hospital in Pretoria, South Africa were recruited from May 2014 to October 2014. The gynecology clinic provides care for outpatients who have gynecological conditions that cannot be treated at local clinics or have abnormal Pap smear test results, while the MAFU provides specialized care for pregnant women with high risk pregnancies. All adult women (18–75 years-old – see **Table 1**) were eligible to participate in the study if they gave written informed consent. Women were not enrolled in the study if any antibiotics were consumed at least two weeks prior to sample collection and if they menstruated at the time of sample collection or had abnormal vaginal bleeding. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional research ethics committee (Faculty of Health Sciences Research Ethics Committee) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (Ethics reference no.: 117/2014). Basic demographic and clinical data were obtained using a questionnaire. The UTI symptoms were defined as any of the following: frequent urination, hematuria, burning sensation, dysuria and nocturia.

Classification of Vaginal Smears According to Nugent Score and Ison-Hay Criteria

After the informed consent was obtained, a vaginal swab was collected by a physician and a smear was prepared on a frosted microscope slide (Merck KGaA, Darmstadt, Germany) immediately after the swab collection. Upon arrival at the laboratory, the vaginal smears were heat-fixed and Gram stained. Following Gram staining, vaginal smears were read and scored using the Nugent scoring system (Nugent et al., 1991) and Ison-Hay criteria (Ison and Hay, 2002). For the Nugent scoring system, a score of 0–10 is given based on the presence or absence and counted number of three bacterial morphotypes: (i)

TABLE 1 | Demographic, clinical and microbiological characteristics of the women included in the study.

Clinical characteristics	Non-pregnant women ($n = 129$)	Pregnant women ($n = 67$)	p-value[a]
Age			
Median (range, years)	39 (18–75)	29 (18–42)	**<0.001**
Ethnic groups			
African (black)	114 (88%)	56 (84%)	0.73[b]
Caucasian (white)	12 (9.3%)	7 (10%)	
Indian	3 (2.3%)	2 (3%)	
Inter-racial/multi-racial	0 (0%)	2 (3%)	
Clinical symptoms			
Abnormal vaginal discharge			
Present	22 (17%)	12 (18%)	0.88
Absent	107 (83%)	55 (82%)	
UTI symptoms[c]			
Present	28 (22%)	18 (27%)	0.42
Absent	101 (78%)	49 (73%)	
HIV[d] status			
Positive	67 (52%)	10 (15%)	**<0.001[b]**
Negative	57 (44%)	57 (85%)	
Unknown	5 (3.9%)	0 (0%)	
Vaginal microbiota (Nugent scoring)			
Nugent score 0–3 (normal vaginal microbiota)	69 (53.5%)	50 (72%)	**0.016**
Nugent score 4–6 (intermediate vaginal microbiota)	20 (15.5%)	5 (7%)	
Nugent score 7–10 (BV[e])	40 (31%)	12 (20%)	
Vaginal microbiota (Ison-Hay criteria)			
Class I (normal vaginal microbiota)	62 (48.1%)	48 (72%)	**0.013[g]**
Class II (intermediate vaginal microbiota)	24 (18.6%)	8 (12%)	
Class III (BV)	39 (30.2%)	11 (16%)	
Class IV (Gram-positive cocci only)	1 (0.8%)	0 (0%)	
Class 0 (Epithelial cells with no bacteria)[f]	3 (2.3%)	0 (0%)	

[a] χ^2 *test for independence. Significant p-values (<0.05) are indicated in bold.*
[b] *For African vs. Caucasian and for HIV-positive vs. HIV-negative.*
[c] *Urinary tract infection symptoms: frequent urination, hematuria, burning sensation, dysuria, nocturia.*
[d] *HIV: Human immunodeficiency virus.*
[e] *BV: bacterial vaginosis.*
[f] *Equivalent to a Nugent score of 4 (intermediate vaginal microbiota).*
[g] *Excluding classes IV and 0.*

Gram-positive rods (vaginal lactobacilli); (ii) Gram-negative to Gram-variable short rods (*Gardnerella* spp. /*Bacteroides* spp.); and (iii) Gram-negative curved rods (*Mobiluncus* spp.) (Nugent et al., 1991). A score of 0 to 3 is considered to be "normal" microbiota, a score of 4 to 6 is considered to be "intermediate" microbiota and a score of 7 to 10 is considered to be "bacterial vaginosis" (Nugent et al., 1991). The Ison-Hay criteria categorize the vaginal smears into five classes of microbiota, namely: (i) class I (normal microbiota, lactobacilli only); (ii) class II (intermediate microbiota, reduced lactobacilli with mixed bacterial morphotypes); class III (bacterial vaginosis, few or absent lactobacilli with mixed bacterial morphotypes); class IV (epithelial cells with only Gram-positive cocci) and class 0 (epithelial cells with no bacteria) (Ison and Hay, 2002). All slides were re-read at a later stage and were examined by a second examiner for quality control purposes.

Processing of Urine Specimens

A first-void urine specimen for the FISH examination of the bacterial growth forms on the vaginal epithelial cells was collected from each enrolled woman after informed consent was obtained. A first-void urine specimen was selected for practical purposes because it is easier to collect, and the fixed sample can be stored for long periods of time until the FISH assays are performed. Upon arrival at the laboratory, all collected first-void urine specimens were processed prior to performing the FISH assay as described in Swidsinski et al. (2010) with some modifications. In brief, the cells in 1.5 mL of first-void urine were collected by centrifugation (Spectrafuge™ 24D Digital Micro-centrifuge, Labnet International Inc., Edison, NJ, USA) at 6,000 × *g* for 6 min and were fixed in 1 mL of 4% (v/v) formaldehyde (Merck KGaA, Darmstadt, Germany) in 1x phosphate-buffered saline (PBS) buffer (pH 7.2; Gibco®, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 1 h. The pellet was washed with 1x PBS buffer (pH 7.2; Gibco®, Thermo Fisher Scientific Inc., Waltham, MA, USA) three times and was resuspended in a 1:1 mixture of 1x PBS and ice-cold 100% ethanol (Merck KGaA, Darmstadt, Germany). The fixed cell suspension was stored at 4°C until further analysis.

The Probe Specificity Test and the FISH Assay

The probe specificity tests were performed on the following probes: (i) GardV (Swidsinski et al., 2005); (ii) Ato291 (Harmsen et al., 2000); (iii) Lab158 (Harmsen et al., 1999); (iv) Enc38a (Frahm et al., 1998); and (v) Eub338 probes (Amann et al., 1990). GardV, Ato291 and Lab158 probes are oligonucleotide probes that were previously used to visualize *Gardnerella* spp., *A. vaginae* and *Lactobacillus* spp. in a study by Swidsinski et al. (2005) (see **Table 2** for information regarding the probes used in the FISH assay). As the Lab158 probe targets both *Lactobacillus* spp. and *Enterococcus* spp. (Harmsen et al., 1999), the Lab158-positive samples with a negative signal to the Enc38a probe specific to *Enterococcus* spp. were considered positive for *Lactobacillus* spp. The Eub338 probe is a broad-range oligonucleotide probe that targets eubacteria (Amann et al., 1990). The specificity of each probe was tested using reference strains (*G. vaginalis* ATCC® 14018™ and 14019™; *A. vaginae* ATCC® BAA-55™; *L. crispatus* ATCC® 33820™). For culturing of reference strains, a single pure colony of *A. vaginae* ATCC® BAA-55™ was inoculated in tryptone soya broth (TSB) (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA), *G. vaginalis* ATCC® 14018™ and 14019™ in brain heart infusion (BHI) broth (Lab M Limited, Heywood, UK) and *L. crispatus* ATCC® 33820™ in de Man, Rogosa and Sharpe

TABLE 2 | Oligonucleotide sequences of probes that were used in the FISH assay.

Probe name	Target	Nucleotide sequence (5′ to 3′)	Fluorochrome label	References
Assay 1				
Ato291	*Atopobium* cluster	GGTCGGTCTCTCAACCC	Cy3[a]	Harmsen et al., 2000
Eub338	Eubacteria	GCTGCCTCCCGTAGGAGT	6-FAM[b]	Amann et al., 1990
GardV	Probe derived from Bif662 with 0 mismatches to *G. vaginalis*	CCACCGTTACACCGCGAA	Cy5[b]	Swidsinski et al., 2005
Assay 2				
Enc38a	*Enterococcus* spp.	CTCTACCTCCATCATTCT	Cy5	Frahm et al., 1998
Eub338	Eubacteria	GCTGCCTCCCGTAGGAGT	6-FAM	Amann et al., 1990
Lab158	*Lactobacillus* spp. and *Enterococcus* spp.	GGTATTAGCA(C/T)CTGTTTCCA	Cy3	Harmsen et al., 1999

[a] *Cy3 refers to carbocyanine attached at the 3′ end of oligonucleotides.*
[b] *Cy5 and 6-FAM are attached at the 5′ end of oligonucleotides.*

(MRS) broth (Oxoid, Thermo Fisher Scientific Inc., Waltham, MA, USA). The TSB inoculated with *A. vaginae* ATCC® BAA-55™ and the MRS broth inoculated with *L. crispatus* ATCC® 33820™ were incubated at 37°C for 48 h in an anaerobic jar (bioMérieux, Marcy l'Etoile, France) using an anaerobic gas generator (GENbox anaer, bioMérieux, Marcy l'Etoile, France). The BHI broth inoculated with either *G. vaginalis* ATCC® 14018™ or *G. vaginalis* ATCC® 14019™ was incubated in a carbon dioxide (CO_2) incubator (HR212UV, Shanghai Lishen Scientific Equipment Co. Ltd., Shanghai, China) with 5% CO_2 at 37°C for 48 h. The FISH protocol by Amann (1995) with some modifications was followed for hybridization reactions of the oligonucleotide probes. A volume of 2 mL cultured broth was centrifuged (Spectrafuge™ 24D Digital Micro-centrifuge, Labnet International Inc., Edison, NJ, USA) at 6,000 × *g* for 6 min to obtain a pellet. The supernatant was discarded, and the cell pellet was resuspended in 100 μL of PBS buffer (pH 7.2; Gibco®, Thermo Fisher Scientific Inc., Waltham, MA, USA). A volume of 100 μL 4% (v/v) formaldehyde (Merck KGaA, Darmstadt, Germany) in a PBS buffer (pH 7.2; Gibco®, Thermo Fisher Scientific Inc., Waltham, MA, USA) was added to fixate the cells and the mixture was incubated at room temperature (25 ± 5°C) for 1 h. After incubation, the mixture was washed twice with 1x PBS buffer (pH 7.2; Gibco®, Thermo Fisher Scientific Inc., Waltham, MA, USA) to remove the formaldehyde completely. The washed mixture was vortexed (Vortex Mixer VX-200, Labnet International Inc., Edison, NJ, USA) and diluted 1:10 in a PBS buffer (pH 7.2; Gibco®, Thermo Fisher Scientific Inc., Waltham, MA, USA). An aliquot of 10 μL of the dilution was added to each well of the polytetrafluoroethylene (PTFE)-coated microscope slide (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the slide was air-dried for 20 min at 50°C (Techne HB-1D, Bibby Scientific Limited, Stone, UK). A volume of 20 μL of 1% (w/v) (10 $mg{\cdot}mL^{-1}$) lysozyme solution (Sigma-Aldrich Co., St. Louis, MO, USA) was added to each well of the slide prior to hybridization and the slide was incubated for 30 min at 56°C (Techne HB-1D, Bibby Scientific Limited, Stone, UK). After incubation, 8 μL of hybridization buffer [containing 5 M NaCl solution, 1 M Tris-HCl, 10% sodium dodecyl sulfate (SDS) and sterile water] and 1 μL of working solution of each probe (30 $ng{\cdot}\mu L^{-1}$ for Cy3 and Cy5 labeled probes; 50 $ng{\cdot}\mu L^{-1}$ for 6-FAM labeled probes) were added to the wells. Hybridization of oligonucleotide probes (GardV, Ato291, Lab158, Enc38a, and Eub338) was performed in a hybridization oven (Techne HB-1D, Bibby Scientific Limited, Stone, UK) at 50°C for 16 h. After hybridization, the slides were rinsed briefly with distilled water and the 4′, 6′-diamidino-2-phenylindole (DAPI) stain (PureBlu™ DAPI Nuclear Staining Dye, Bio-Rad Laboratories Inc., Hercules, CA, USA) was added to counter-stain the DNA of bacteria, fungi and host cells present. The slides were incubated in the dark at room temperature (25 ± 5°C) for 15 min. Finally, a few drops of anti-fading mounting agent (ProLong® Diamond Antifade Mountant, Thermo Fisher Scientific Inc., Waltham, MA, USA) were applied and a cover slip was placed on each slide.

Two triplex FISH assays (assay 1: GardV-Cy5, Ato291-Cy3, and Eub338-FAM probes; assay 2: Lab158-Cy3, Enc38a-Cy5, and Eub338-FAM probes) were performed without formamide at 50°C for 16 h according to the standard protocol by Amann (1995) with some modifications. An aliquot of 10 μL of the fixed cell suspension was added to each well of the polytetrafluoroethylene (PTFE)-coated microscope slide (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the slide was air-dried for 20 min at 56°C (Techne HB-1D, Bibby Scientific Limited, Stone, UK). The cell smears were dehydrated for 5 min each in an increasing ethanol series [50, 80, and 96% ethanol (v/v)] (Merck KGaA, Darmstadt, Germany). A volume of 20 μL of lysozyme solution (10 $mg{\cdot}mL^{-1}$) (Merck KGaA, Darmstadt, Germany) was added to each well of the slide prior to hybridization and the slide was incubated for 30 min at 56°C (Techne HB-1D, Bibby Scientific Limited, Stone, UK) to allow better penetration of the oligonucleotide probes. After incubation, hybridization reaction was performed at 50°C (Techne HB-1D, Bibby Scientific Limited, Stone, UK) for 16 h after adding the hybridization buffer and working solution of each probe to the wells as described above. The microscope slides were rinsed briefly after the incubation in the washing buffer [containing 5 M NaCl solution, 1 M Tris-HCl, 10% sodium dodecyl sulfate (SDS), and sterile water] and were incubated at

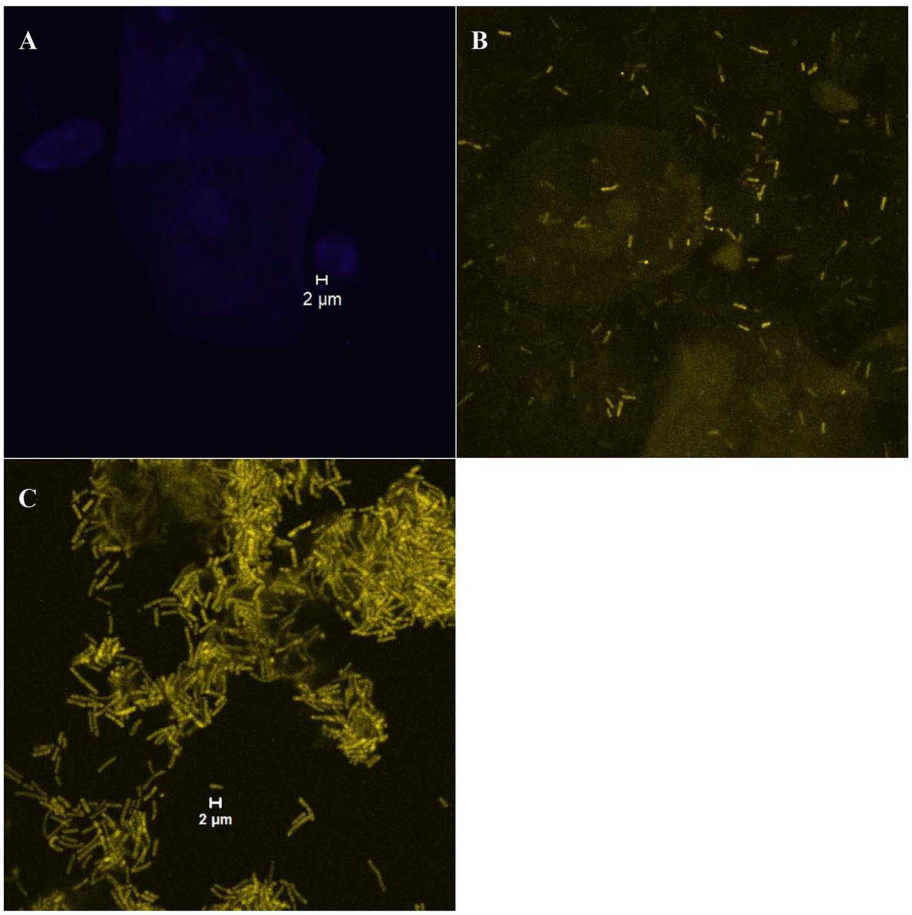

FIGURE 1 | Examples of different bacterial growth forms; **(A)** "absent" category with no bacteria present on vaginal cells (visualized with the laser diode 405 that detects the DAPI stain); **(B)** "dispersed" category [an example of scattered cells of *Lactobacillus* spp. (rods in yellow color) visualized with the helium-neon laser 2 that detects the Lab158-Cy3 probe]; **(C)** "biofilm" category ([an example of the *Lactobacillus* biofilm (rods in yellow color) visualized with the helium-neon laser 2]).

50°C (Techne HB-1D, Bibby Scientific Limited, Stone, UK) for 10 min. The slides were rinsed briefly with distilled water and the 4′, 6′-diamidino-2-phenylindole (DAPI) stain (PureBlu™ DAPI Nuclear Staining Dye, Bio-Rad Laboratories Inc., Hercules, CA, USA) was added to counter-stain the DNA of bacteria, fungi and host cells present. The slides were incubated in the dark at room temperature (25 ± 5°C) for 15 min. Finally, a few drops of anti-fading mounting agent (ProLong® Diamond Antifade Mountant, Thermo Fisher Scientific Inc., Waltham, MA, USA) were applied and a cover slip was placed on each slide.

Fluorescence Microscopic Visualization and Bacterial Growth Phenotype

The Zeiss LSM 510 META Laser Scanning Confocal Microscope (Carl Zeiss Microscopy GmbH, Jena, Germany; objective: Plan-Neofluar 100x/1.3 oil immersion lens) and its accompanying software (Version 3.2 service pack 2, Carl Zeiss Microscopy GmbH, Jena, Germany) were used to detect the fluorescence signal of the hybridized oligonucleotide probes. The fluorescent cells were observed using the Plan-Neofluar objective (100x, oil immersion lens; Carl Zeiss Microscopy GmbH, Jena, Germany). An argon laser (detecting excitation at 458, 477, 488, and 514 nm) was used to detect the EUB338 probe, while a helium-neon laser 1 (detecting excitation at 543 nm) and helium-neon laser 2 (detecting excitation at 633 nm) were used for the Cy3-labeled (Ato291 or Lab158) and Cy5-labeled probes (GardV or Enc38a), respectively. Lastly, a laser diode 405 (detecting excitation at 405 nm) was used to observe the cells stained with the DAPI stain. Ten microscopic fields were inspected per each sample to determine the presence or absence of each growth form. The fluorescence signal for species-specific probes (GardV, Ato291, Lab158, Enc38a) was considered positive if a fluorescence signal for the universal bacterial probe (Eub338) was detected simultaneously at the same position.

The growth forms of *Gardnerella* spp. and *Lactobacillus* on vaginal cells were classified into two categories, i.e., "dispersed" or "biofilm". The samples were evaluated as the "dispersed" category if bacterial cells were separately scattered (**Figure 1B**) and as the "biofilm" category if characteristic bacterial aggregates attached on the vaginal epithelial cells were observed (**Figure 1C**). The

TABLE 3 | Probe specificity test results of the oligonucleotide probes using reference strains.

Bacterial species	Strains	Ato291	GardV	Lab158	Enc38a	Eub338
Atopobium vaginae	ATCC® BAA-55™	+[a]	-[b]	-	-	+
Gardnerella vaginalis	ATCC® 14018™	-	+	-	-	+
Gardnerella vaginalis	ATCC® 14019™	-	+	-	-	+
Lactobacillus crispatus	ATCC® 33820™	+	-	+	-	+

[a](+): presence of hybridization.
[b](–): absence of hybridization.

samples with no bacteria present on vaginal cells were classified into the "absent" category (**Figure 1A**).

Statistical Analysis

Statistical analysis was performed using the Stata 14.0 software (StataCorp LP, College Station, TX, USA). Descriptive statistics were provided as number (proportion) or median (range); proportions were compared between groups using the χ^2 test. The agreement between Nugent scoring and the Ison-Hay criteria was measured using the Kappa (κ) value. Forward stepwise logistic regression models based on the likelihood ratio method were fitted with BV status (BV-positive and BV-negative) as outcome, two bacterial growth forms ("dispersed" or "biofilm") as exposure (dependent variable) and the "absent" category as a reference category. Lastly, the association between two categories of bacterial growth forms and three clinical characteristics (HIV status, presence/absence of UTI symptoms and presence/absence of abnormal vaginal discharges) was determined, in which only BV-positive women were included in the analysis. Fisher's exact test was used for comparing proportions between bacterial growth forms and clinical characteristics as these categories had small sample size. The odds ratios (ORs) were reported with the 95% confidence intervals (CI). All statistical values were considered significant for $p < 0.05$ (95% CI).

RESULTS

Study Participants/Population

One hundred and twenty-nine non-pregnant women from the gynecology clinic and 67 pregnant women from the MAFU were included in the study. Women recruited at the gynecology outpatient clinic were generally older (39 years vs. 29 years; $p < 0.001$), more often HIV-infected (52% vs. 15%; $p < 0.001$) and more often diagnosed with BV (31% vs. 20%; $p = 0.016$ for Nugent Score; 30% vs. 16%; $p = 0.013$ for Ison-Hay criteria) (**Table 1**). The Nugent scoring system revealed that 52 women (27%) were BV-positive, while 25 women (13%) had intermediate vaginal microbiota and 119 women (61%) had normal vaginal microbiota. Nine smears that had normal vaginal microbiota by Nugent scoring (9/119; 7.6%) were assigned to class II by the Ison-Hay criteria; one smear that had intermediate vaginal microbiota (1/21; 5%) were assigned to class III and three smears that were BV-positive by Nugent scoring (3/52; 6%) were assigned to class II. Among the smears that had intermediate vaginal microbiota by Nugent scoring, three smears were assigned to class 0 (3/25; 12%) and one smear was assigned to class IV (1/25; 4%). The κ value of agreement between Nugent scoring and Ison-Hay criteria was 0.88 (95% CI: 0.77 – 0.98).

Probe Specificity Results

The GardV and Lab158 probes were specific for each target (i.e., had a positive signal for *G. vaginalis* ATCC® 14018™ and 14019™ and *L. crispatus* ATCC® 33820™, respectively and did not cross-hybridize with other reference strains) (**Table 3**). The Ato291 probe had a positive signal for *A. vaginae* ATCC® BAA-55™; however, the Ato291 probe cross-hybridized with *L. crispatus* ATCC® 33820™. Consequently, the FISH results obtained from the Ato291 probe hybridization were excluded from the analysis.

Growth Forms of *Gardnerella* spp. and *Lactobacillus* spp. on Vaginal Epithelial Cells

Gardnerella spp. were mostly present in biofilms (76/196; 39%), whereas *Lactobacillus* spp. were mostly present in a dispersed form (115/196; 59%). Among the samples that contained *Gardnerella* spp. only (i.e., samples without *Lactobacillus* spp.; 16/196; 8.2%), 15 had biofilms (15/16; 94%) and one had a dispersed form (1/16; 6%). Among the samples that contained *Lactobacillus* spp. only (i.e., samples without *Gardnerella* spp.; 98/196; 50%), 60 had a dispersed form (60/98; 61%) and 38 formed biofilms (38/98; 39%). Dispersed *Gardnerella* spp. (9/196; 4.6%) were observed with equal amounts of dispersed form (4/9; 44%) and biofilm (4/9; 44%) of *Lactobacillus* spp., respectively. *Gardnerella* biofilms were mostly observed when *Lactobacillus* spp. were dispersed (51/76; 67%) but some samples with *Gardnerella* biofilms were also observed with *Lactobacillus* biofilms (10/76; 13%). Thirteen samples (13/196; 6.6%) did not contain either *Gardnerella* spp. or *Lactobacillus* spp.

Gardnerella biofilms were detected in 88% (46/52) of samples from BV-positive women and no dispersed *Gardnerella* spp. was observed in samples from BV-positive women (**Table 4**). There was a significant difference ($p < 0.001$) between growth forms of *Gardnerella* spp. and between Nugent score categories. The majority of dispersed *Lactobacillus* spp. (63/115; 55%) and the *Lactobacillus* biofilms (43/52; 83%) were observed in samples from women with normal vaginal microbiota (**Table 4**). There was also a significant difference ($p < 0.001$) between growth forms of *Lactobacillus* spp. and between Nugent score categories. Similar observations were obtained for the Ison-Hay criteria (**Table 4**).

TABLE 4 | Association of growth forms of *Gardnerella* spp. and *Lactobacillus* spp. in relation to BV status.

Bacterial growth form		Nugent scoring			OR[a] (95% CI)	*p*-value[d]		Ison-Hay criteria[b]			OR[c] (95% CI)	*p*-value[d]
	Total *N*	Nugent 0–3 (%) (*n* = 119)	Nugent 4–6 (%) (*n* = 25)	Nugent 7–10 (%) (*n* = 52)			Total *N*	Class I (%) (*n* = 110)	Class II (%) (*n* = 32)	Class III (%) (*n* = 50)		
***Gardnerella* spp.**												
Absent	111	95 (86[e])	10 (9)	6 (5)	Ref[f]	**<0.001**[g]	108	91 (84[h])	12 (11)	5 (5)	Ref[f]	**<0.001**[g]
Dispersed forms	9	8 (89)	1 (11)	0 (0)	-[i]	-	9	8 (89)	1 (11)	0 (0)	-	-
Biofilms	76	16 (21)	14 (18)	46 (61)	27 (11 – 69)	**<0.001**	75	11 (15)	19 (25)	45 (60)	31 (11 – 85)	**<0.001**
***Lactobacillus* spp.**												
Absent	29	13 (45[e])	6 (21)	10 (35)	Ref[f]	**0.002**[g]	27	11 (41[h])	7 (26)	9 (33)	Ref[f]	**0.003**[g]
Dispersed forms	115	63 (55)	14 (12)	38 (33)	-	-	113	59 (52)	17 (15)	37 (33)	-	-
Biofilms	52	43 (83)	5 (10)	4 (8)	0.17 (0.057 – 0.49)	**0.001**	52	40 (77)	8 (15)	4 (8)	0.17 (0.058 – 0.50)	**0.001**

[a]*Odds ratio (95% confidence intervals); Exposure: bacterial growth forms; Outcome: BV-positive (Nugent score 7-10) vs. BV-negative (Nugent score 1-3 and 4-6); Entering significance level of 0.05; Removing significance level of 0.1; Only significant OR was displayed.*

[b]*Class IV and 0 omitted for comparison with Nugent scoring.*

[c]*Odds ratio (95% confidence intervals); Class IV and 0 omitted for comparison with Nugent scoring; Exposure: bacterial growth forms; Outcome: BV-positive (Class III) vs. BV-negative (Classes I and II); Entering significance level of 0.05; Removing significance level of 0.1; Only significant OR was displayed.*

[d]*Significant p-values (<0.05) are indicated in bold.*

[e]*Row percentage between Nugent score categories.*

[f]*Used as a reference category for OR.*

[g]χ^2 *test.*

[h]*Row percentage between classes I, II, and III.*

[i]*Not available or not applicable.*

Association of Biofilms With Bacterial Vaginosis and Clinical Characteristics

The odds of having BV (Nugent score 7–10 or class III) increased when *Gardnerella* biofilms were present (OR 27; CI 11 – 69; $p <$ 0.001 for Nugent scoring; and OR 31; CI 11– 85; $p < 0.001$ for Ison-Hay criteria) (**Table 4**). In contrast, the odds of having BV decreased when *Lactobacillus* spp. were present in biofilms (OR 0.17; CI 0.057 – 0.49; $p = 0.001$ for Nugent scoring; and OR 0.17; CI 0.058 – 0.50; $p = 0.001$ for Ison-Hay criteria). There was no significant relationship between the tested clinical characteristics and the bacterial growth forms (**Tables 5**, **6**).

DISCUSSION

The present study set out to investigate the growth forms of *Gardnerella* spp. and *Lactobacillus* spp. on vaginal cells in first-void urine specimens of enrolled South African women with normal vaginal microbiota, intermediate vaginal microbiota and BV using FISH. The findings in this study demonstrate that *Gardnerella* spp. are predominantly present in biofilms on vaginal cells of BV-positive women, while *Lactobacillus* spp. are present in a dispersed growth form. The study also confirms that biofilms formed by *Gardnerella* spp. increase the odds of having BV and that *Lactobacillus* biofilms have the opposite effect.

The presence of *Gardnerella* biofilms in the majority of BV-positive women and the association of *Gardnerella* biofilms with BV observed in this study is a common observation confirmed by previous studies (Swidsinski et al., 2005, 2010; Hardy et al., 2015). It can be hypothesized that *Gardnerella* spp. may prefer to choose the biofilm form of growth during BV as it provides protection against antimicrobial substances like H_2O_2 and lactic acid (Patterson et al., 2007), as well as antibiotics/antiseptics (Swidsinski et al., 2008, 2015). Indeed, virulent strains of *Gardnerella* spp. are known strong biofilm formers with good adherence ability, which outcompete other BV-associated anaerobes (Patterson et al., 2010; Machado et al., 2013a; Alves et al., 2014; Castro et al., 2015). For this reason, *Gardnerella* spp. have recently been suggested as the main "early colonizer species" that displace lactobacilli and form a biofilm to create a favorable environment for other BV-associated bacteria such as *Prevotella bivia*, *A. vaginae* and *Megasphaera* type 1 (Machado et al., 2013a; Swidsinski et al., 2014; Hardy et al., 2015; Muzny et al., 2019).

Interestingly, the present study revealed that *Lactobacillus* biofilms can decrease the odds of having BV in women. The *Lactobacillus* biofilms were observed more frequently in women with normal vaginal microbiota than in BV-positive women (43/52; 83% vs. 4/52; 8% for Nugent scoring and 40/52; 77% vs. 4/52; 8% for Ison-Hay criteria) and had an inverse association with BV ($p = 0.001$ for Nugent scoring; and $p = 0.001$ for Ison-Hay criteria) in this study. These observations could possibly support previous findings that some vaginal lactobacilli may form a biofilm to disrupt and prevent the colonization of pathogenic bacteria (Saunders et al., 2007; Leccese Terraf et al., 2014; Ventolini, 2015). It could also be proposed that lactobacilli may form biofilms to prevent the

TABLE 5 | Association of growth forms of *Gardnerella* spp. and *Lactobacillus* species in relation to clinical characteristics in BV-positive women (Nugent scoring).

Bacterial growth form	HIV status				UTI symptoms				Abnormal vaginal discharge			
	HIV-negative (%) (*n* = 19)	HIV-positive (%) (*n* = 33)	OR[a] (95% CI)	*p*-value	Absent (%) (*n* = 39)	Present (%) (*n* = 13)	OR[b] (95% CI)	*p*-value	Absent (%) (*n* = 41)	Present (%) (*n* = 11)	OR[c] (95% CI)	*p*-value
***Gardnerella* spp.**												
Absent	2 (11[d])	4 (12)	Ref[e]	1.0[f]	4 (10[d])	2 (15)	Ref[e]	0.63[f]	6 (15[d])	0 (0)	Ref[e]	0.32[f]
Dispersed	0 (0)	0 (0)	-[g]	-	0 (0)	0 (0)	-	-	0 (0)	0 (0)	-	-
Biofilm	17 (89)	29 (88)	-	-	35 (90)	11 (85)	-	-	35 (76)	11 (100)	-	-
***Lactobacillus* species**												
Absent	1 (5[d])	9 (27)	Ref[e]	0.058[f]	8 (21[d])	2 (15)	Ref[e]	0.08[f]	7 (17[d])	3 (27)	Ref[e]	0.53[f]
Dispersed	15 (79)	23 (70)	-	-	30 (77)	8 (62)	-	-	30 (73)	8 (73)	-	-
Biofilm	3 (16)	1 (3)	-	-	1 (3)	3 (23)	-	-	4 (9.8)	0 (0)	-	-

[a]*Odds ratio (95% confidence interval) adjusted by pregnancy status; Exposure: bacterial growth forms; Outcome: HIV-negative vs. HIV-positive; Entering significance level of 0.05; Removing significance level of 0.1; Only significant OR was displayed.*

[b]*Odds ratio (95% confidence interval) adjusted by pregnancy status; Exposure: bacterial growth forms; Outcome: UTI symptoms absent vs. UTI symptoms present; Entering significance level of 0.05; Removing significance level of 0.1; Only significant OR was displayed.*

[c]*Odds ratio (95% confidence interval) adjusted by pregnancy status; Exposure: bacterial growth forms; Outcome: abnormal vaginal discharge absent vs. abnormal vaginal discharge present; Entering significance level of 0.05; Removing significance level of 0.1; Only significant OR was displayed.*

[d]*Column percentage between bacterial growth forms.*

[e]*Used as a reference category for OR.*

[f]*Fisher's exact test.*

[g]*Not available or not applicable.*

TABLE 6 | Association of growth forms of *Gardnerella* spp. and *Lactobacillus* species in relation to clinical characteristics in BV-positive women (Ison-Hay criteria[a]).

Bacterial growth form	HIV status				UTI symptoms				Abnormal discharge			
	HIV-negative (%) (*n* = 19)	HIV-positive (%) (*n* = 31)	OR[b] (95% CI)	*p*-value	Absent (%) (*n* = 37)	Present (%) (*n* = 13)	OR[c] (95% CI)	*p*-value	Absent (%) (*n* = 40)	Present (%) (*n* = 10)	OR[d] (95% CI)	*p*-value
***Gardnerella* spp.**												
Absent	2 (11[e])	3 (10)	Ref[f]	1.0[g]	3 (8[e])	2 (15)	Ref[f]	0.60[g]	5 (13[e])	0 (0)	Ref[f]	0.57[g]
Dispersed	0 (0)	0 (0)	-[h]	-	0 (0)	0 (0)	-	-	0 (0)	0 (0)	-	-
Biofilm	17 (89)	28 (90)	-	-	34 (92)	11 (85)	-	-	35 (88)	10 (100)	-	-
***Lactobacillus* species**												
Absent	1 (5[e])	8 (26)	Ref[f]	0.065[g]	7 (19[e])	2 (15)	Ref[f]	0.11[g]	7 (18[e])	2 (20)	Ref[f]	0.86[g]
Dispersed	15 (79)	22 (71)	-	-	29 (78)	8 (62)	-	-	29 (73)	8 (80)	-	-
Biofilm	3 (16)	1 (3)	-	-	1 (3)	3 (23)	-	-	4 (10)	0 (0)	-	-

[a]*Class IV and 0 omitted for comparison with Nugent scoring.*

[b]*Odds ratio (95% confidence interval) adjusted by pregnancy status; Exposure: bacterial growth forms; outcome: HIV-negative vs. HIV-positive; Entering significance level of 0.05; Removing significance level of 0.1; only significant OR was displayed.*

[c]*Odds ratio (95% confidence interval) adjusted by pregnancy status; Exposure: bacterial growth forms; outcome: UTI symptoms absent vs. UTI symptoms present; Entering significance level of 0.05; Removing significance level of 0.1; only significant OR was displayed.*

[d]*Odds ratio (95% confidence interval) adjusted by pregnancy status; Exposure: bacterial growth forms; outcome: abnormal vaginal discharge absent vs. abnormal vaginal discharge present; Entering significance level of 0.05; Removing significance level of 0.1; only significant OR was displayed.*

[e]*Column percentage between bacterial growth forms.*

[f]*Used as reference category for OR.*

[g]*Fisher's exact test.*

[h]*Not available or not applicable.*

formation of a polymicrobial BV biofilm. However, lactobacilli were absent in some women with normal vaginal microbiota (13/119; 10.9% for Nugent scoring and 11/110; 10% for Ison-Hay criteria), as previously reported (Zhou et al., 2004; Ravel et al., 2011). According to Ravel et al. (2011) and Zhou et al. (2004), some women may still maintain a "normal" vaginal environment without lactobacilli and that these women may contain BV-associated vaginal bacteria like *A. vaginae*, *Leptotrichia* spp. and *Megasphaera* spp., which are capable of producing lactic acid.

Lastly, the present study has attempted to determine the association between different bacterial growth forms and three clinical characteristics (HIV status, UTI symptoms and abnormal vaginal discharge) in BV-positive women. However, there was no significant association observed between the bacterial growth forms and tested clinical characteristics. This could be due to a small sample size of BV-positive women.

The present study acknowledges that there are limitations. The first limitation was that the FISH results of *A. vaginae* could not be included in the analysis due to the suboptimal specificity of the Ato291 probe. Cross-hybridization of the Ato291 probe was also observed in a study by Hardy et al. (2015), indicating that use of the peptide nucleic acid (PNA) probe instead of oligonucleotide probe could be useful in future. Another limitation is the usage of the urine specimens for detecting growth forms on the vaginal cells, which might not have adequate amounts of vaginal epithelial cells for the FISH assay as compared to vaginal swabs. Therefore, future studies should utilize the vaginal swab eluates for the FISH assays to improve results. Furthermore, as this study did not determine the clades/subgroups of *Gardnerella* spp., it could be beneficial to perform the clade-specific PCR assays along with the FISH assays to determine associations between the growth forms with different strains of *Gardnerella* spp.

In conclusion, this study demonstrated and confirmed that *Gardnerella* spp. form biofilms on vaginal epithelial cells of BV-positive women, whereas *Lactobacillus* spp. display a dispersed growth form on vaginal cells. This study also revealed that *Lactobacillus* spp. may form biofilms to have a protective role against BV. This study encourages further studies covering a wide array of BV-associated bacteria including *A. vaginae* to uncover the mystery of biofilm formation mechanisms in BV.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Faculty of Health Science Research Ethics Committee, University of Pretoria (Ethics ref. no. 117/2014). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HJ was involved in the concept design, experimental work, data and statistical analysis as well as the writing and editing of the manuscript. ME, HL, MR, and MK were involved in the concept design. ME, RP, HL, MR, and MK critically reviewed the manuscript. RP and JB were involved in the statistical analysis of the acquired data.

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Gardnerella vaginalis Enhances *Atopobium vaginae* Viability in an *in vitro* Model

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Bacterial vaginosis (BV) is the most common vaginal infection among women of reproductive age. A hallmark of BV is the presence of a highly structured polymicrobial biofilm on the vaginal epithelium, presumably initiated by facultative anaerobes of the genus *Gardnerella*, which then becomes a scaffold for other species to adhere to. One of the species often found incorporated in *Gardnerella* mediated biofilms is *Atopobium vaginae*. Interestingly, *A. vaginae* is very rarely found without the presence of *Gardnerella*. However, not much is known regarding the interactions between *A. vaginae* and *Gardnerella* species. This study assessed biological interactions between *Gardnerella vaginalis* and *A. vaginae*. In our *in vitro* model, by using specific *Gardnerella* and *A. vaginae* Peptide Nucleic Acid (PNA)-Fluorescence *In Situ* Hybridization (FISH) probes, we confirmed that *A. vaginae* was able to incorporate a pre-formed *G. vaginalis* biofilm, accounting for up to 20% of the total number of biofilm cells. However, our findings showed that almost 92% of *A. vaginae* cells lost viability after 48 h of mono-species planktonic growth, but were able to maintain viability when co-cultured with *Gardnerella* or after pre-conditioning with cell-free supernatant of *Gardnerella* cultures. While the *in vitro* conditions are very different from the *in vivo* microenvironment, this study contributes to a better understanding of why *A. vaginae* vaginal colonization rarely occurs in the absence of *Gardnerella*. Overall, this highlights the importance of microbial interactions between BV-associated bacteria and demands more studies focused on the polymicrobial bacterial communities found in BV.

Keywords: bacterial vaginosis, polymicrobial biofilms, *Gardnerella*, *Atopobium vaginae*, PNA-FISH

INTRODUCTION

Bacterial vaginosis (BV) is the most prevalent bacterial vaginal infection in women of reproductive age (Jung et al., 2017; van de Wijgert and Jespers, 2017; Rosca et al., 2019). BV is characterized by a change in the microbial composition of the vaginal ecosystem where the prevailing *Lactobacillus* spp., associated with an optimal vaginal microbiota, are outnumbered by other microorganisms, including species of the genus *Gardnerella* and *Atopobium vaginae* (Ferris et al., 2004; Verhelst et al., 2004; dos Santos Santiago et al., 2012; Jung et al., 2017; Muzny et al., 2019). Noteworthy, the involvement of *A. vaginae* in BV rarely occurs in the absence of *Gardnerella* (Bradshaw et al., 2006; Hardy et al., 2015, 2016).

It should be also noted that *Gardnerella vaginalis* was the only recognized species in its genus for four decades (Castro et al., 2020), but very recently a whole-genome analysis of 81 *Gardnerella* isolates carried out by Vaneechoutte and coworkers showed the existence of 13 species within the genus *Gardnerella* (Vaneechoutte et al., 2019). Of these 13 species, three new were officially described (*G. leopoldii*, *G. piotii*, and *G. swidsinskii*) and *G. vaginalis* was amended. Nine genomospecies were defined but not described because the authors did not have the strains (needed for the official description). Following this renewed taxonomy of the genus *Gardnerella*, in this article, the term *Gardnerella* spp. will be used to discuss previous publications, because we cannot rule out the fact that other *Gardnerella* species were involved.

An important discovery of the last decade was the observation that a highly structured polymicrobial biofilm on the vaginal epithelium, presumably initiated by *Gardnerella* spp., becomes a scaffold for other species to adhere to (Swidsinski et al., 2005, 2013). While many bacterial species have been found associated with BV, the contribution of those species to the biofilm formation process is not well documented, remaining unclear its role in the development of BV infection. An interesting example is the case of *A. vaginae*. Evidence of a possible dependent relationship between *Gardnerella* spp. and *A. vaginae* has been demonstrated on BV-associated biofilms (Swidsinski et al., 2005; Hardy et al., 2015, 2016). Nevertheless, as biological interactions in BV-associated biofilms are still poorly understood, we aimed to analyze the interactions between *G. vaginalis* and *A. vaginae*, using a previously described *in vitro* dual-species biofilm model (Castro et al., 2019). We then evaluated cell viability when these bacterial species were grown for 48 h in either mono- or co-cultures through fluorescence *in situ* hybridization (FISH) method.

METHODS

Reclassification of *Gardnerella* Species

Our collection of fourteen *Gardnerella* spp. isolates first identified by partial sequencing of the16S rRNA coding gene as *G. vaginalis* (Castro et al., 2015) and according to the clade classification system (Ahmed et al., 2012; Castro et al., 2018), were herein reclassified by MALDI-TOF protein profiling as described by Vaneechoutte et al. (2019). Briefly, eight peptide spectra were generated from all strains after ethanol/acetic acid extraction using the Microflex Biotyper™ spectrometer (Bruker Daltonics, Germany). Raw data spectra were imported in BioNumerics Software (Applied Maths, Belgium), used to make one summary spectrum per strain. The summary spectra were then used to classify the strains as *G. vaginalis*, *G. leopoldii*, *G. piotii*, or *G. swidsinskii* according to the peptide biomarkers described by Vaneechoutte and coworkers.

Strains and Culture Conditions

G. vaginalis strain ATCC 14018^T and *A. vaginae* strain ATCC BAA-55^T were used as controls in all the experimental assays. Then, for the bacterial viability experiments, five additional strains of *G. vaginalis,* namely UM121 and UM137 (Castro et al., 2015) and UGent09.01, UGent09.07, and UGent25.49 (Vaneechoutte et al., 2019), and five additional strains of *A. vaginae,* namely BVS065, BVS067, BVS069, FB106b, VMF0907Col23 (Henriques et al., 2012), were used. All strains were grown in supplemented BHI (sBHI) [Brain-heart infusion (Liofilchem, Rosetodegli, Abruzzi, Italy) containing 2% (wt/vol) gelatin (Liofilchem), 0.5% (wt/vol) yeast extract (Liofilchem), and 0.1% (wt/vol) soluble starch (Panreac, Barcelona, Spain)] for 24 h (for biofilm experiments) or 48 h (for bacterial viability experiments) at 37°C under anaerobic conditions [controlled atmosphere composed of 10% carbon dioxide, 10% helium and 80% nitrogen generated by a cylinder (Air Liquid, Algés, Portugal) coupled to an anaerobic incubator (Plas-Labs, Lansing, MI)].

Biofilm Formation and Biomass Quantification

Dual-species biofilms were initiated by inoculating a 10^7 CFU/mL bacterial suspension of *G. vaginalis* strain ATCC 14018^T into 24-well tissue culture plates (Orange Scientific, Braine L'Alleud, Belgium) and by incubating the plate for 24 h, at 37°C and under anaerobic conditions. After 24 h, planktonic cells were removed, and fresh medium was added to each well. Then, 10^7 CFU/mL of *A. vaginae* strain ATCC BAA-55^T was inoculated in the pre-formed *G. vaginalis* biofilms and incubated for another 24 h. Mono-species biofilms were grown as controls. To quantify the biomass of mono- and dual-species biofilms, we used the crystal violet (CV) method, which is the most frequently employed approach (Peeters et al., 2008; Azeredo et al., 2017). 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 phosphate-buffered saline, and bound CV was released with 33% (vol/vol) acetic acid (Thermo Fisher Scientific, Lenexa, KS). To estimate total biomass, the optical density (OD) of the resulting solution was measured at 595 nm. Biofilm assays were repeated three times on separate days, with four technical replicates assessed each time.

Quantification of Bacterial Populations in Dual-Species Biofilms by PNA-FISH

The bacterial population within the biofilms formed was discriminated according to FISH method (Machado et al., 2013), by using peptide nucleic acid (PNA) probes specific for *G. vaginalis* (Gard162) (Machado et al., 2013) and for *A. vaginae* (AtoITM1) (Hardy et al., 2015). Before counting the percentage of cells detected by PNA-FISH, any non-adherent cells were removed by two gentle washes with PBS and, thereafter, biofilms were scraped vigorously from the well. For mono- and dual-cultures, 30 μL of each bacterial suspension was spread on epoxy-coated microscope glass slides (Thermo Fisher Scientific) and the slides air-dried. 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 then by 50% (vol/vol) ethanol (Thermo Fisher Scientific) for

10 min. After the fixation step, the samples were covered with 10 μL of each PNA probe and incubated in a hybridization oven (Nahita, drying oven, model 631/2) in humid conditions, at 60°C for 90 min. Afterward, the slides were immersed in a washing solution containing 5 mM Tris base (Thermo Fisher Scientific), 15 mM NaCl (Liofilchem) and 0.1% (vol/vol) Triton X-100 (Fisher Bioreagents, Pennsylvania, USA) for 30 min at 60°C. After this washing step, the slides were air-dried in the dark and at room temperature. 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) and the PNA AtoITM1 probe (BP 470-490, FT500, LP 516 sensitive to the Alexa Fluor 488 molecule attached to the AtoITM1 probe). Twenty fields were randomly acquired in each sample. The number of bacteria was counted using *ImageJ Software* (Rasband, 1997). Biofilm assays were repeated three times on separate days.

Confocal Laser Scanning Microscopy Analysis of Biofilm Bacterial Distribution

To analyze the bacterial distribution of dual-species biofilms, the intact biofilm structure was evaluated by confocal laser scanning microscopy (CLSM) using the PNA Gard162 and PNA AtoITM1

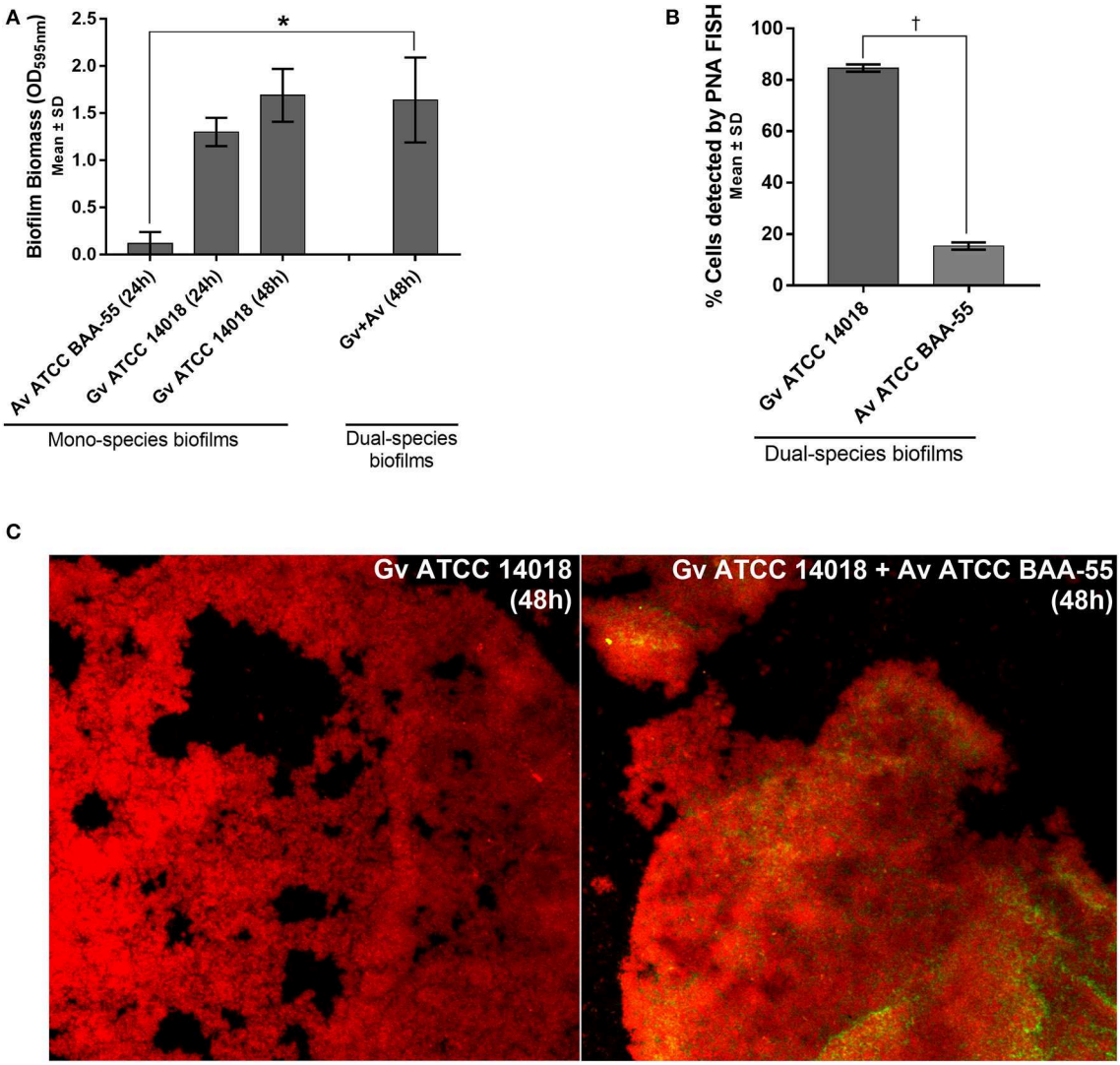

FIGURE 1 | Interactions between *G. vaginalis* strain ATCC 14018 and *A. vaginae* strain ATCC BAA-55 cultured under biofilms conditions. **(A)** Total biomass of mono- and dual-species BV-associated biofilms was determined by staining with crystal violet. Each data point represents the mean ± s.d. of three independent assays, with four technical replicates assessed each time. **(B)** Percentage of cells detected by PNA-FISH for 48 h biofilms. Each data point represents the mean ± s.d. of three independent assays. For each assay, 20 fields were randomly acquired in each sample and the number of bacteria per image was counted using *ImageJ Software*. **(C)** Example of data set on the organization of the dual BV-associated biofilms by confocal laser scanning microscopy (CLSM). *Gardnerella vaginalis* (Gv) and *Atopobium vaginae* (Av) cells were differentiated by hybridization with PNA-probes Gard162 (red color) and AtoITM1 (green color), respectively. *Values are significantly different between the dual-species consortium and the mono-species culture (independent samples *t*-test, $P < 0.05$). † Values are significantly different between the bacterial populations of *G. vaginalis* and *A. vaginae* in dual-species biofilms (paired samples *t*-test, $P < 0.05$).

probes as described above. For this experiment, biofilms were formed as described above but on an eight-well chamber slide (Thermo Fisher Scientific™ Nunc™ Lab-Tek™, Rochester, NY). The CLSM images were acquired with an Olympus™ Fluo View FV1000 confocal laser scanning microscope (Olympus), using a ×10 objective and with a 640 × 640 resolution (pixels). All assays were repeated three times with two technical replicates.

Coaggregation Assays

To determine the extent of the coaggregation between *G. vaginalis* strain ATCC 14018^T and *A. vaginae* strain ATCC BAA-55^T, we used an experimental model suggested by Reid et al. (1990). Of note, coaggregation assays were carried out using planktonic cultures and aimed to assess the possible mechanism behind the development of bacterial biofilms (Rickard et al., 2003). However, it has been reported that there is not always a direct relation between coaggregation and biofilm formation (Karched et al., 2015). In brief, 500 µL of *G. vaginalis* (10^7 CFU/mL) was combined with 500 µL of *A. vaginae* (10^7 CFU/mL) in 24-well plates (Thermo Fisher Scientific) and incubated for 4 h, at 37°C, under anaerobic conditions. The aggregates were visualized using an inverted light microscope Leica DMI 3000B (Leica Microsystems, Wetzlar,

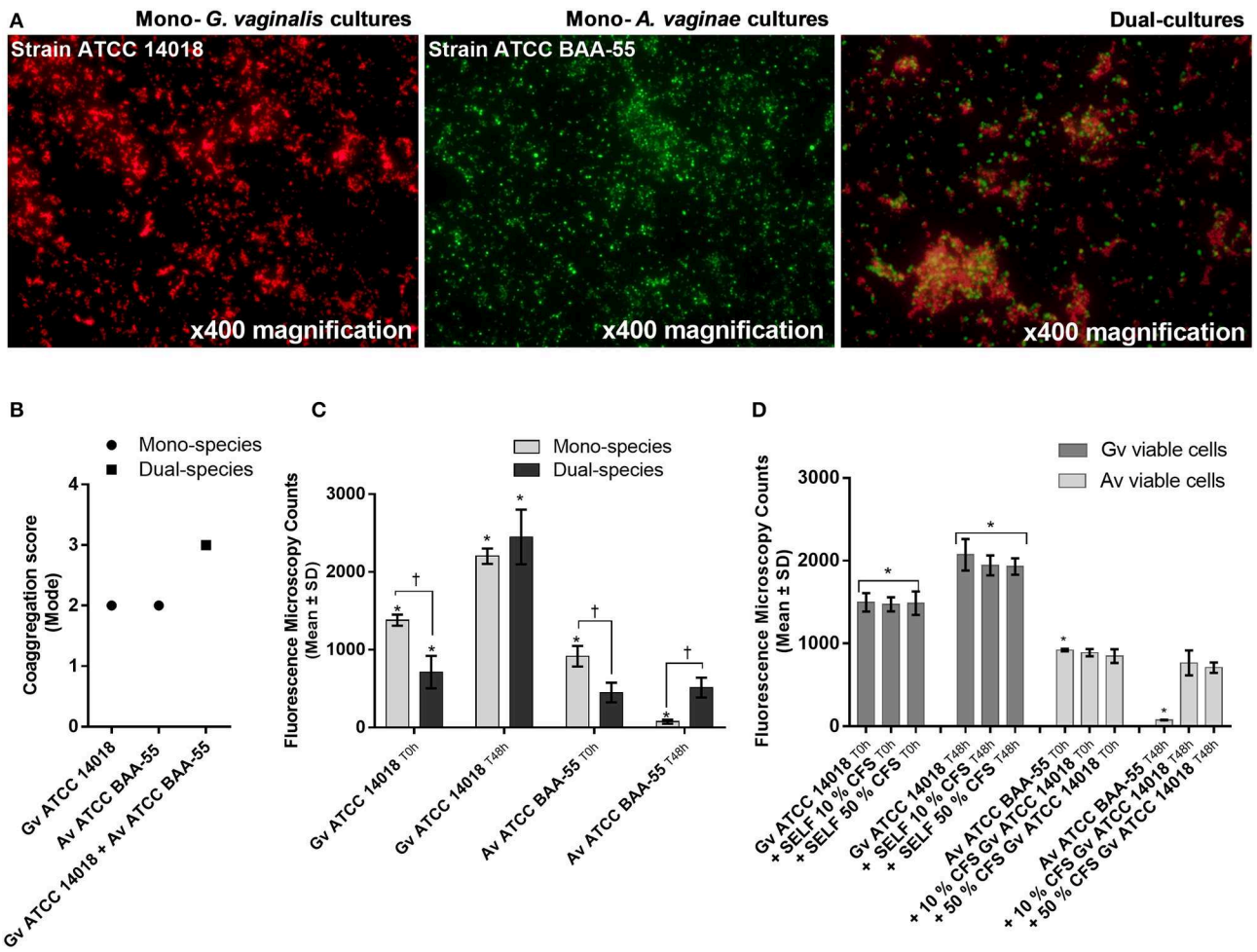

FIGURE 2 | Bacterial interactions in mono- and co-cultures of *G. vaginalis* strain ATCC 14018 and *A. vaginae* strain ATCC BAA-55 cultured under planktonic conditions. **(A)** An example of data set on the organization of microbial aggregates of mono- or dual-bacterial species. **(B)** Coaggregation score of mono- vs dual-bacterial species. Auto-aggregation was also assessed for each bacterial species. Coaggregation score was evaluated as follows: 0, no aggregation; 1, small aggregates comprising small visible clusters of bacteria; 2, aggregates comprising larger numbers of bacteria, settling to the center of the well; 3, macroscopically visible clumps comprising larger groups of bacteria which settle to the center of the well; 4, maximum score allocated to describe a large, macroscopically visible clump in the center of the well. Each data point represents the mode. **(C)** Fluorescence microscopy counts of *G. vaginalis* and *A. vaginae* in mono- and dual-species planktonic cultures. *G. vaginalis* and *A. vaginae* cells were differentiated by hybridization with PNA-probes Gard162 and AtoITM1, respectively. **(D)** Effect of 10% (vol/vol) and 50% (vol/vol) cell-free supernatant (CFS) of *G. vaginalis* on *A. vaginae* viability. The influence of CFS of *G. vaginalis* on *G. vaginalis* growth was also analyzed as a control ("self-CFS"). Each data point represents the average ± s.d. of three experiments. For each assay, 20 fields were randomly acquired in each sample and the number of bacteria per image was counted using *ImageJ Software*. *Values are significantly different between T0h and T48h for each growth condition (Kruskal-Wallis test, $P < 0.05$). † Values are significantly different between mono- or dual-species cultures for each time point (Kruskal-Wallis test, $P < 0.05$).

Germany) and the score was evaluated as following: 0, no aggregation; 1, small aggregates comprising small visible clusters of bacteria; 2, aggregates comprising larger numbers of bacteria, settling to the center of the well; 3, macroscopically visible clumps comprising larger groups of bacteria which settle at the center of the well; 4, maximum score allocated to describe large clumps, macroscopically visible at the center of the well. Auto-aggregation was assessed for each isolate. All assays were performed in duplicate and repeated three different times.

Bacterial Viability on Mono- and Co-cultures Under Planktonic Conditions

G. vaginalis and *A. vaginae* cell viability was assessed before and after 48 h of planktonic growth in mono- and in co-cultures (contact-dependent interactions), by PNA-FISH, as previously described. In brief, for contact-dependent interactions assays, monocultures were grown as described above (section Strains and Culture Conditions). For dual-cultures planktonic growth, 2 mL of *G. vaginalis* suspension was mixed with 2 mL of *A. vaginae* suspension. As such, each species is present at half of the concentration in the respective dual-culture. Furthermore, the influence of 10 and 50% (vol/vol) of cell-free supernatant (CFS) of *G. vaginalis* on *A. vaginae* cells viability was also evaluated (contact-independent interactions) based on the protocol described by Khan et al. (2019). Briefly, CFS was generated by centrifuging the 48 h inoculum of *G. vaginalis* for 30 min at 3,000 × g. The supernatant was filter-sterilized using 0.22 μm filters and was used on the same day. Filter-sterilized CFS was streaked on Columbia base agar supplemented with 10% defibrinated horse blood (Thermo Fisher Scientific) to confirm sterility. To test the effect of CFS on *A. vaginae*, we added 0.4 or 2 mL of CFS in individual tubes with a *A. vaginae* suspension (final volume of 4 mL) and then the tubes were incubated for 48 h. *A. vaginae* was also grown in control tubes with media containing no CFS. Of note, the effect of CFS of *G. vaginalis* on *G. vaginalis* growth was also analyzed as a control ("self-CFS"). All assays were performed in duplicate and repeated three times.

Statistics

All numerical data were subjected to statistical analysis using One-way ANOVA test or non-parametric Kruskal-Wallis test, when data that did not follow a normal distribution according to Kolmogorov–Smirnov's test. Statistical software package SPSS 17.0 (SPSS Inc. Chicago, IL) was used. Data are presented as mean ± standard deviation (s.d.), unless stated otherwise.

RESULTS

Biofilm Assays

We observed that in our *in vitro* conditions, *A. vaginae* was not able to form mono-species biofilms (**Figure 1A**). Interestingly, although *A. vaginae* did not significantly enhance the dual-species biomass when compared with 48 h *G. vaginalis* mono-species biofilms, this species was able to incorporate the biofilm, accounting for up to ∼20% of the total number of cells, as determined by PNA-FISH (**Figure 1B**). CLSM analysis using specific PNA probes revealed that *A. vaginae* was found well distributed across the biofilm, in small clusters of cells (**Figure 1C**).

TABLE 1 | Reclassification of the *Gardnerella* isolates according to MALDI-TOF protein profiling.

Strain	Accession number[a]	Clade[b]	Species identification[c]
Gardnerella sp. UM016	KP996686.1	1	*Gardnerella vaginalis*
Gardnerella sp. UM034	KP996684.1	4	*Gardnerella leopoldii*
Gardnerella sp. UM035	KP996685.1	2	*Gardnerella piotii*
Gardnerella sp. UM060	KP996673.1	1	*Gardnerella vaginalis*
Gardnerella sp. UM061	KP996674.1	1	*Gardnerella vaginalis*
Gardnerella sp. UM067	KP996675.1	2	*Gardnerella* sp.[d]
Gardnerella sp. UM085	KP996679.1	1	*Gardnerella vaginalis*
Gardnerella sp. UM094	KP996680.1	4	*Gardnerella swidsinskii*
Gardnerella sp. UM121	KP996681.1	1	*Gardnerella vaginalis*
Gardnerella sp. UM131	KP996676.1	2	*Gardnerella* sp.[d]
Gardnerella sp. UM137	KP996682.1	1	*Gardnerella vaginalis*
Gardnerella sp. UM224	KP996678.1	4	*Gardnerella leopoldii*
Gardnerella sp. UM241	KP996683.1	1	*Gardnerella* sp.[d]
Gardnerella sp. UM246	KP996677.1	1	*Gardnerella* sp.[d]

[a] *The partial 16S ribosomal RNA gene sequences of vaginal isolates are downloadable from NCBI. The strains were phenotypically characterized by Castro et al. (2015). UM, University of Minho, Portugal.*

[b] *The results regarding the genotyping of Gardnerella isolates based on the clades described by Ahmed et al. (2012) were described in Castro et al. (2018).*

[c] *The reclassification of the Gardnerella species was performed by comparing our generated MALDI-TOF spectra with the species-specific peaks defined by Vaneechoutte et al. (2019).*

[d] *MALDI-TOF spectra not matching with any of the described Gardnerella species-specific spectra (i.e., G. vaginalis, G. piotii, G. leopoldii, and G. swidsinskii). Hence, these strains were considered as Gardnerella species (but not G. vaginalis, G. piotii, G. leopoldii, and G. swidsinskii).*

Coaggregation Assays

Coaggregation-mediated interactions between *G. vaginalis* and *A. vaginae* were also analyzed since co-aggregation is believed to facilitate the integration of new bacterial species into polymicrobial communities (Rickard et al., 2003). As such, we evaluated the ability of each mono- and mixed-species cultures to coaggregate. As shown in **Figure 2A**, macroscopic clusters formed in the dual-species planktonic cultures contained both species. Furthermore, the presence of both species enhanced the co-aggregation ability (**Figure 2B**).

Planktonic Assays

Interestingly, we observed that in planktonic monocultures, ∼92% of *A. vaginae* cells had lost their viability after 48 h of growth. However, when growing in mixed cultures, *A. vaginae* was able to maintain the same level of viability, according to the PNA-FISH counts (**Figure 2C**). Furthermore, by using sBHI medium conditioned by prior growth of *G. vaginalis* to culture *A. vaginae,* we demonstrated that the observed effect was not dependent on physical contact with *G. vaginalis*, suggesting that

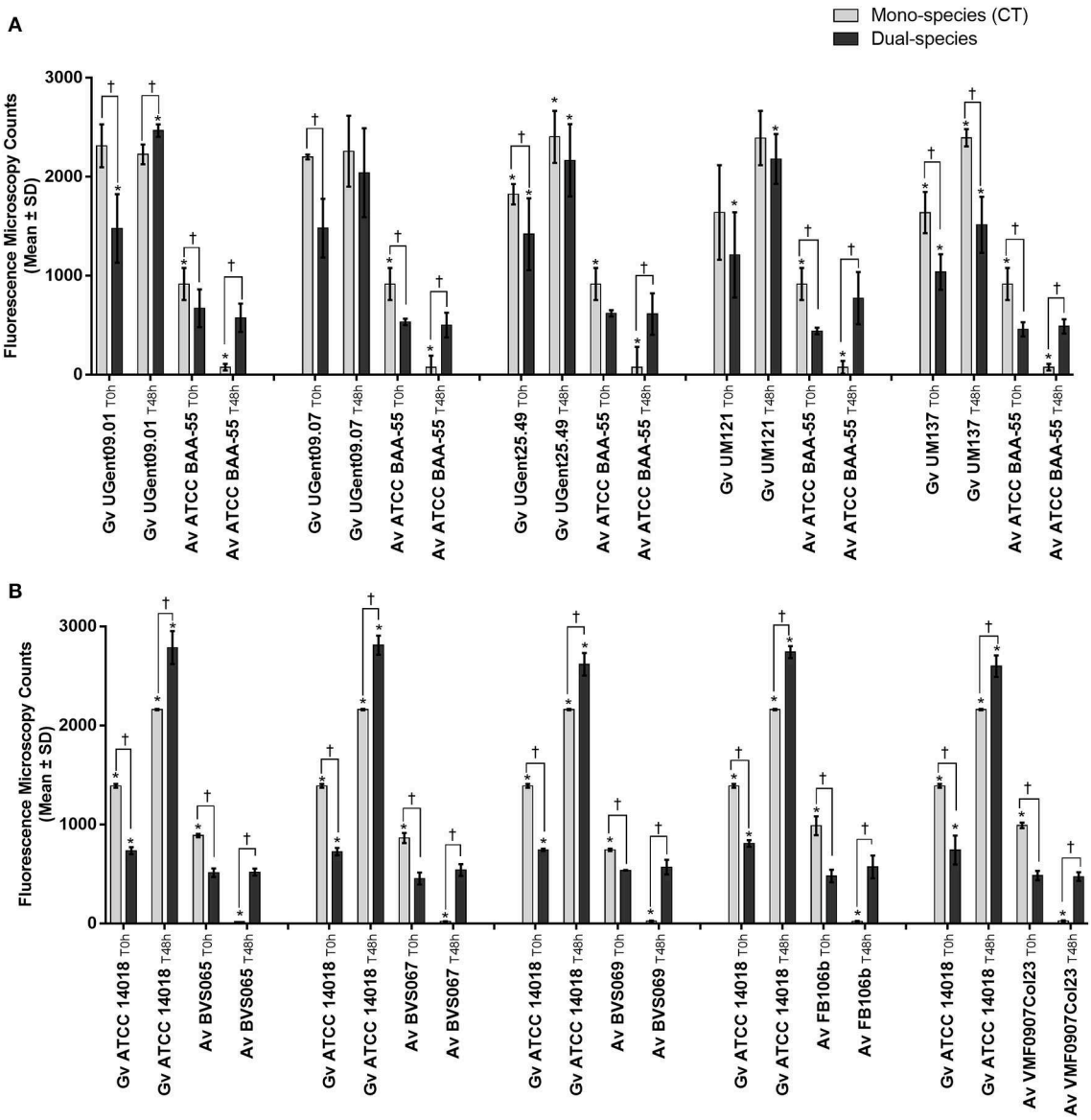

FIGURE 3 | Fluorescence microscopy counts of *G. vaginalis* and *A. vaginae* in mono- and dual-species planktonic cultures. **(A)** Experiments conducted with five other isolates of *G. vaginalis*. **(B)** Experiments conducted with five other isolates of *A. vaginae*. *G. vaginalis* and *A. vaginae* cells were differentiated by hybridization with PNA-probes Gard162 and AtoITM1, respectively. For each assay, 20 fields were randomly acquired in each sample and the number of bacteria per image was counted using *ImageJ Software*. Each data point represents the average ± s.d. of three experiments. *Values are significantly different between T0h and T48h for each growth condition (Kruskal-Wallis test, $P < 0.05$). † Values are significantly different between mono- or dual-species for each time point (Kruskal-Wallis test, $P < 0.05$).

A. vaginae maintains cell viability due to some extracellular molecules produced by *G. vaginalis* (**Figure 2D**).

In order to verify to what extent this was strain-specific, we evaluated *A. vaginae* cells viability in co-culture with five other *G. vaginalis* strains. Due to the recent reclassification of species in the genus *Gardnerella* (Vaneechoutte et al., 2019), we characterized our collection of strains by MALDI-TOF, and selected, for this study, isolates of *G. vaginalis* (**Table 1**). As shown in **Figure 3A**, all tested isolates of *G. vaginalis* resulted in a comparable increase in *A. vaginae* cells viability. Identical observations were also observed after repeating the experiment with five other *A. vaginae* strains, as shown in **Figure 3B**. This confirmed that *A. vaginae* was only viable in the presence of *G. vaginalis* and that this observation is independent of the strains used.

DISCUSSION

Despite the evidences suggesting that *Gardnerella* spp. might be the initial colonizer, establishing early biofilm structures to which *A. vaginae* can attach (Swidsinski et al., 2005; Castro et al., 2019; Muzny et al., 2019), there is a lack of studies

addressing why *A. vaginae* is almost always accompanied by *Gardnerella* spp. in the vaginal microbiota (Verhelst et al., 2004; Bradshaw et al., 2006; Menard et al., 2010; Hardy et al., 2015, 2016). In this regard, some important attempts have been made to analyze the co-occurrence of these bacterial species in BV-associated biofilms by using the FISH methodology (Frickmann et al., 2017). Importantly, by using a broad range of probes to assess the composition and spatial organization of bacteria in BV-associated biofilms, an *ex vivo* study carried out by Swidsinski and colleagues on vaginal biopsies specimens, demonstrated that adherent biofilms were mainly composed by *Gardnerella* spp. (∼60%) and *A. vaginae* (∼40%) (Swidsinski et al., 2005). Later, Hardy and colleagues conducted a study on vaginal samples which demonstrated that *A. vaginae* was always part of *Gardnerella* biofilms, but *Gardnerella* biofilms could be found without *A. vaginae* (Hardy et al., 2015). In a subsequent study, Hardy and coworkers demonstrated that adherent *Gardnerella* spp. and *A. vaginae* were visualized, respectively, in 82 and 54% of samples with BV-associated biofilms (Hardy et al., 2016). This was, therefore, the basis for suggesting that *Gardnerella* spp. and *A. vaginae* could establish a relationship in a BV-associated biofilm (Hardy et al., 2016). Furthermore, other studies that addressed the interactions between these two bacterial species, showed that the co-occurrence of *Gardnerella* spp. and *A. vaginae* provides to both bacterial species increased antibiotic resistance (Bradshaw et al., 2006; Swidsinski et al., 2008), and increased expression of genes related to *Gardnerella* virulence (Castro et al., 2019). Such bacterial interactions in the female lower genital tract might have important clinical implications, namely in preterm birth (Menard et al., 2010; Bretelle et al., 2015; Redelinghuys et al., 2017; Mendling et al., 2019). Interestingly, Redelinghuys and colleagues hypothesized that high vaginal concentrations of *Gardnerella* and *A. vaginae* might create a permissive environment for anaerobic Gram-negative bacteria (Redelinghuys et al., 2017). This hypothesis is supported by earlier findings, which showed that women containing high concentrations of *G. vaginalis* and anaerobic Gram-negative bacteria might have higher levels of proinflammatory cytokines and, according to the authors, it could be a reason to the increased risk for spontaneous preterm delivery (Genc et al., 2004; Genc and Onderdonk, 2011).

Given the fact that *A. vaginae* seems to be almost always accompanied by *Gardnerella* in BV biofilms, we hypothesized that *A. vaginae* could be taking advantage of *G. vaginalis* to survive in the vaginal ecosystem. Evidence that supports our hypothesis is the fact that *A. vaginae* does not seem to be able to form mono-species biofilms *in vitro*, as shown by Patterson et al. (2010) and as confirmed herein, at least in the conditions used in both studies. However, it is important to note that the method used in our study to quantify biofilm biomass has some limitations. Despite its widespread use, CV has been associated with lack of reproducibility (Peeters et al., 2008) and absence of a standardized protocol, resulting in a broad variety of staining protocols that make comparison of results between studies difficult (Stepanovic et al., 2007). Additionally, the nonspecific nature of CV does not allow species differentiation in polymicrobial communities (Azeredo et al., 2017).

Interestingly, Faro suggested that a possible explanation for the co-occurrence of *G. vaginalis* and *A. vaginae* could be related to low oxygen levels that prevail in the vaginal environment (Faro, 2006). As such, *Gardnerella* might consume the oxygen, creating a more favorable environment for *A. vaginae*, a strict anaerobe (Faro, 2006). Our results shed new light on these bacterial-interspecies interactions as we demonstrated that the enhanced viability of *A. vaginae* cells was not related to the consumption of oxygen by *G. vaginalis* because (*i*) our experimental design involved strictly anaerobic conditions and (*ii*) the physical presence of *G. vaginalis* was not required. It is noteworthy that even contact-independent interactions provided benefits for *A. vaginae*. A similar effect was described before for *Peptostreptococcus anaerobius,* which could only grow in co-culture with *Prevotella bivia* or in medium conditioned by prior growth of *P. bivia* (Pybus and Onderdonk, 1998).

In conclusion, the results from this *in vitro* study demonstrated that *A. vaginae* benefits from *G. vaginalis* to survive, providing a strong indication of the importance of the biological interactions between both taxa. This strengthens the idea that microbial interactions between BV-associated bacteria can be essential in BV pathogenesis. Therefore, future research should address the complex interplay between *G. vaginalis*, *A. vaginae,* and other BV-associated species. Understanding the molecular basis and biological effect of these inter-bacterial processes may provide novel information fundamental to define new targets and strategies for BV control.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

AUTHOR CONTRIBUTIONS

JC and AR performed the experiments. PC and MV performed the reclassification of *Gardnerella* species. NC and MV designed the study. JC and NC drafted the manuscript. All authors critically reviewed and approved the final manuscript.

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Asymptomatic Bacterial Vaginosis Is Associated With Depletion of Mature Superficial Cells Shed From the Vaginal Epithelium

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Previous studies have described bacterial vaginosis (BV) as associated with increased cell-shedding from the cervicovaginal epithelium. Cell-shedding in excess of cell-proliferation is thought to decrease epithelial barrier function and increase susceptibility to infection. This study evaluated the number of shed cells in mid-vaginal smears from women with a diagnosis of symptomatic BV (sBV, $n = 17$), asymptomatic BV (aBV, $n = 71$), or no BV ($n = 104$) by Amsel criteria. The sBV smears contained significantly more shed cells (median 158/100X field) than no BV smears (median 91/100X field), $p = 7.2e{-}9$. However, we observed that aBV smears contained significantly fewer shed cells (median 35/100X field) than no BV smears, $p = 22.0e{-}16$. The sizes of cell-aggregates (cells shed in sometimes multilayered sections with intact cell-cell attachments) followed the same pattern. Cell-aggregates in sBV smears were significantly larger (median ~220,000 μm^2) than those in no BV smears (median ~50,000 μm^2), $p = 1.8e{-}6$, but cell-aggregates in aBV smears were significantly smaller (median ~7,000 μm^2) than those in no BV smears, $p = 0.0028$. We also compared the superficial cell index (SCI), a measure of cervicovaginal epithelial cell maturity, in no BV and aBV smears with relatively low numbers of shed cells (≤50/100X field). The SCI of no BV smears was significantly higher (median 0.86) than that of aBV smears (median 0.35), $p = 4.3e{-}98$, suggesting a depletion of mature cells with exposure and shedding of underlying immature cells in aBV with low number of shed cells. These results indicate that aBV may contribute disproportionately to the increased susceptibility to reproductive tract infections associated with BV. Our findings remained true when considering only those smears in which the microbiota comprised a diverse set of strict and facultative anaerobic bacteria [Community State Type IV ($n = 162$)], thus excluding those dominated by *Lactobacillus* spp. This is consistent with our developing hypothesis that high-shedding sBV and low-shedding aBV could be temporally separated phases of the same condition, rather than two separate forms of BV. These findings might inform future work on clinical management of symptomatic and asymptomatic bacterial vaginosis.

Keywords: women's health, gynecology, BV, vaginal microbiome, vaginal microbiota

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INTRODUCTION

The cervicovaginal microbiota is known to contribute to a woman's protection against and susceptibility to reproductive tract infections (RTIs). Women whose cervicovaginal microbiota is composed predominantly of *Lactobacillus* spp. are at decreased risk of many sexually transmitted infections, including HIV (Taha et al., 1998; Gosmann et al., 2017), HPV (Mitra et al., 2016), HSV-2 (Cherpes et al., 2003), trichomoniasis (Brotman et al., 2012), gonorrhea and chlamydia (Wiesenfeld et al., 2003) compared to women with microbiota comprising strict and facultative anaerobic bacteria. The latter is what broadly characterizes bacterial vaginosis (BV), a condition that is defined differently in clinical and research settings (Mckinnon et al., 2019). Clinically, Amsel-BV (Amsel et al., 1983) is characterized by the presence of certain clinical signs with patients reporting symptoms (symptomatic BV [sBV]) or no symptoms (asymptomatic BV [aBV]) on direct questioning (Fleury, 1983; Eschenbach et al., 1988). In research studies, Nugent-BV is characterized, through microscopic examination of a vaginal smear, by a predominance of Gram-negative *Gardnerella* and *Bacteroides* spp. morphotypes rather than Gram-positive *Lactobacillus* spp. morphotypes (Nugent et al., 1991). Vaginal microbiota assessment using culture-independent approaches based on the quantification and characterization of bacterial 16S rRNA gene sequences defines molecular-BV when a microbiota both lacks high relative abundance of *Lactobacillus* spp. and is composed predominantly of a wide array of strict and facultative anaerobic bacteria [Community State Type (CST) IV] (Gajer et al., 2012). Neither Nugent-BV nor molecular-BV can distinguish between sBV and aBV in the absence of a clinical examination and patient questioning. Although the concordance among the methods is not complete, it is generally true that Amsel-BV, Nugent-BV, and molecular-BV are progressively more inclusive: in a given population, more women are positive for Nugent-BV than for Amsel-BV, and more are positive for molecular-BV than for Nugent-BV (Mckinnon et al., 2019).

The cervicovaginal epithelium is the site at which multiple defenses against STIs and RTIs are deployed (Hickey et al., 2011) and *Lactobacillus* spp. contribute to: 1/ physical defenses, including mucus-trapping of pathogens (Nunn et al., 2015), suppression of inflammatory de-keratinization of epithelial cells (Zárate et al., 2009) and control of cell proliferation often necessary for infection (Edwards et al., 2019); 2/ immunological defenses, including suppression of pro-inflammatory signaling through toll-like receptors (Mirmonsef et al., 2011) and suppression of pro-inflammatory cytokine expression (Jespers et al., 2017); and 3/ biochemical defenses, including inducing expression of protective peptides (Yarbrough et al., 2015) and production of lactic acid (Tachedjian et al., 2017).

The homeostatic balance between the proliferation/maturation and shedding/loss of cells on the vaginal epithelium is likely critical in maintaining these defenses. Balanced proliferation/maturation and shedding/loss means the luminal surface consists of dead detaching cells and the adherence of pathogens to these sloughing cells has been hypothesized as a mechanism to provide some protection to the underlying living cells, which are vulnerable to productive infection (Anderson et al., 2014). Accelerated shedding/loss of cells from the vaginal epithelium, therefore, could be expected to decrease this protective function and thus increase susceptibility to STIs and RTIs. The composition of the cervicovaginal microbiota may affect the proliferation/maturation and shedding/loss balance: approximately twice as many shed epithelial cells were found in a study of vaginal smears from women with Nugent-BV, vs. women with predominantly *Lactobacillus* spp. microbiota (Amegashie et al., 2017). Additionally, the composition of the cervicovaginal microbiota has been shown to control the expression of the host microRNA miR-193b which reduces cell proliferation (Edwards et al., 2019). Cell proliferation was lower in women with a BV-associated microbiota (Edwards et al., 2019), as were host proteins associated with epithelial maturation (Zevin et al., 2016). Further supporting this finding are observations of fewer mature and more immature epithelial cells in cervicovaginal samples from women lacking lactobacilli (Fowler, 2012). At the same time, increased cell-shedding could eliminate non-*Lactobacillus* spp. bacteria attaching to the epithelium, such as *G. vaginalis* or *Atopobium vaginae,* which can form resilient biofilms (Hardy et al., 2016). Only symptomatic Amsel-BV is typically treated (Workowski and Bolan, 2015); however, inflammatory changes on the cervicovaginal epithelium are found in women with Nugent-BV (Thurman et al., 2015; Jespers et al., 2017) and molecular-BV (Anahtar et al., 2015), and so it is not surprising that the increased susceptibility to infections associated with BV also accrues to women with Nugent-BV (Cherpes et al., 2003; Wiesenfeld et al., 2003) and molecular-BV (Brotman et al., 2012, 2014; Gosmann et al., 2017).

The objective of the current study was to determine whether the number and maturity of shed epithelial cells observed in sBV and aBV differ, utilizing a collection of cervicovaginal smears for which microbiota composition and clinical data were available.

MATERIALS AND METHODS

Clinical Samples

The current study utilized samples and data from the UMB-HMP study (Ravel et al., 2013). A total of 135 non-pregnant, reproductive-aged women took part in a 10-weeks observational longitudinal study during which participants self-collected daily vaginal samples, under a protocol approved by the Institutional Review Boards of the University of Alabama at Birmingham and of the University of Maryland School of Medicine. Written informed consent was obtained from all participants.

The study included examination by a clinician at enrollment, week 5 and 10, or at interim times if vaginal symptoms were reported. None of the samples used in this study were positive for STI or yeast infection. The clinician's record for each examination included a diagnostic of BV performed according to the Amsel criteria (Amsel et al., 1983). A diagnosis of symptomatic BV (sBV) was established when the participant reported symptoms on direct questioning and fulfilled at least three of the four Amsel's criteria; a diagnosis of asymptomatic BV (aBV) was established when the participant did not report

symptoms but fulfilled at least three of the Amsel's criteria. Each day, participants self-collected a vaginal swab, smeared it on a microscope slide and placed the slide in a protective sleeve. Participants dropped off the slides to the clinic every week. The slides were Gram-stained and scored for Nugent-BV (Nugent et al., 1991) by three independent readers (Ravel et al., 2013). Another daily swab stored in Amies transport medium and immediately frozen at −20°C was used for extraction and purification of genomic DNA using a QIAsymphony robotic platform and QIAGEN CellFree 500 kits (QIAGEN, Valencia CA); Metataxonomic analysis (Marchesi and Ravel, 2015): the V3-V4 hypervariable regions of bacterial 16S rRNA genes were amplified and sequenced on an Illumina MiSeq Instrument to obtain the bacterial composition and abundance of each sample as described previously (Fadrosh et al., 2014). Sequencing was performed at the Institute for Genome Sciences' Genomic Resource Center (GRC) at the University of Maryland School of Medicine (marylandgenomics.com). CST (Gajer et al., 2012) were assigned using VALENCIA, a novel nearest centroid classification algorithm based on the classification of over 13,000 vaginal microbiota dataset. This approach to CST assignment is more robust than standard within-study hierarchical clustering and allows for between-studies comparisons (Ravel, 2018).

Microscopy

Vaginal smears collected on the day of clinical examination (study entry, week 5 and 10 for each participants) were evaluated as described below and the findings evaluated with respect to associated clinical and microbiota data.

A total of 192 smears from 126 women met the criteria above and were available to be examined. Following the method previously described to evaluate cervicovaginal epithelial cell-shedding (Amegashie et al., 2017), slides were visualized using a Zeiss Plan-ACHROMAT 10x objective on a Zeiss Primo Star microscope (Carl Zeiss Microscopy LLC, Thornwood NY) for a total magnification of 100x, and images of three "representative" fields were captured from each slide using a Zeiss Axiocam ICc3 camera and software. "Representative" fields were located within the main body of the smear, not near the start, end, or margins; "representative" fields were

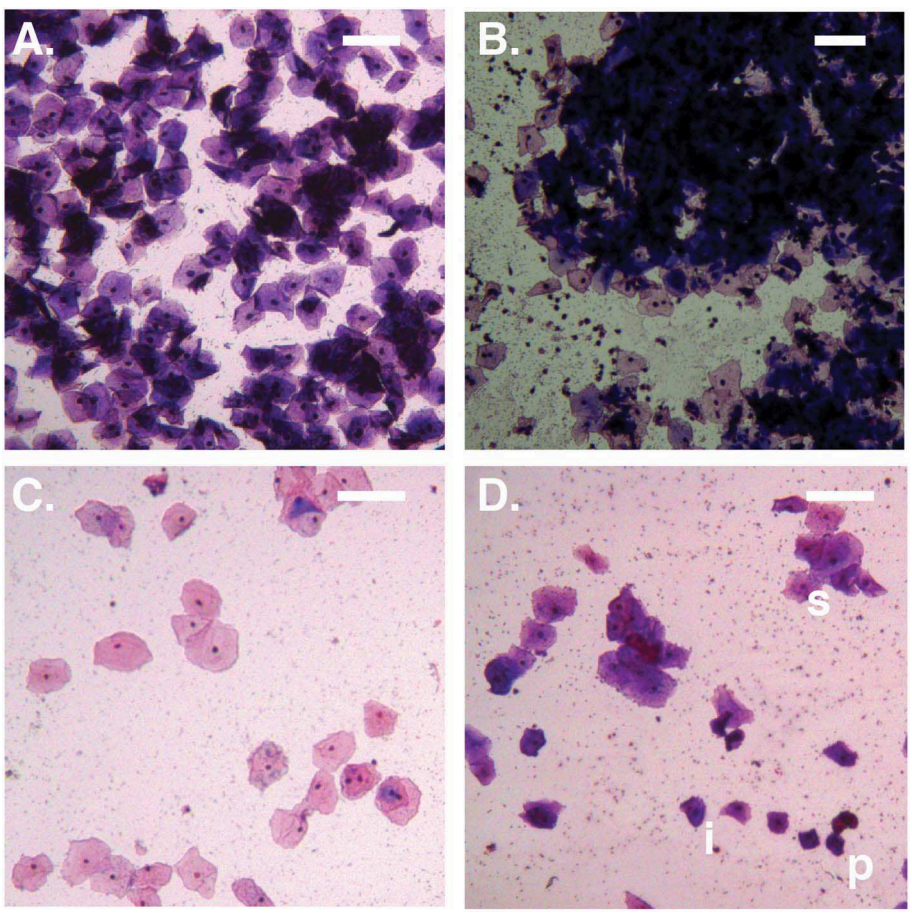

FIGURE 1 | Bright-field micrographs (100X total magnification). **(A)** No Amsel-BV sample with cell count >100/100X field, showing predominantly superficial cells shed as singletons. **(B)** Symptomatic Amsel-BV sample with cell count >100/100X field, showing predominantly superficial cells shed in large aggregates. **(C)** No Amsel-BV sample with cell count <50/100X field, showing predominantly superficial cells. **(D)** Asymptomatic Amsel-BV sample with cell count <50/100X, showing superficial cells (example marked s) shed in combination with intermediate (example marked i) and parabasal (example marked p) cells. Scale bars represent 100 μm.

chosen to exclude aggregations of epithelial cells too dense to distinguish cells. All complete epithelial cells in the three images were counted (i.e., cells falling partly outside the image were not counted) and the mean count calculated. Images of three "representative" cell-aggregations were captured, also from locations within the main body of the smear, away from the start, end, or margins. Cell-aggregation size was measured using the "measure > outline" tool of the imaging software (unit μm^2) (AxioVision v 4.8.2.0); the mean cell-aggregation size was calculated. When the mean epithelial cell count was $\leq$50, the three captured images were further analyzed, counting the number of superficial cells (i.e., cells with a condensed nucleus, large cytoplasmic space, and polygonal shape with thin, angular margins; Anderson et al., 2014). From this, the superficial cell index (SCI = number of superficial cells/number of all epithelial cells) was calculated (range 0.0–1.0; Stupnicki and Teter, 1970). A SCI of 1.0 corresponds to high numbers of mature cells and low or no immature cells, while a low SCI corresponds to low numbers of mature epithelial cells and high numbers of immature epithelial cells. Example of different types of smears are shown on **Figure 1**. All measurements are listed in **Supplementary Table 1**.

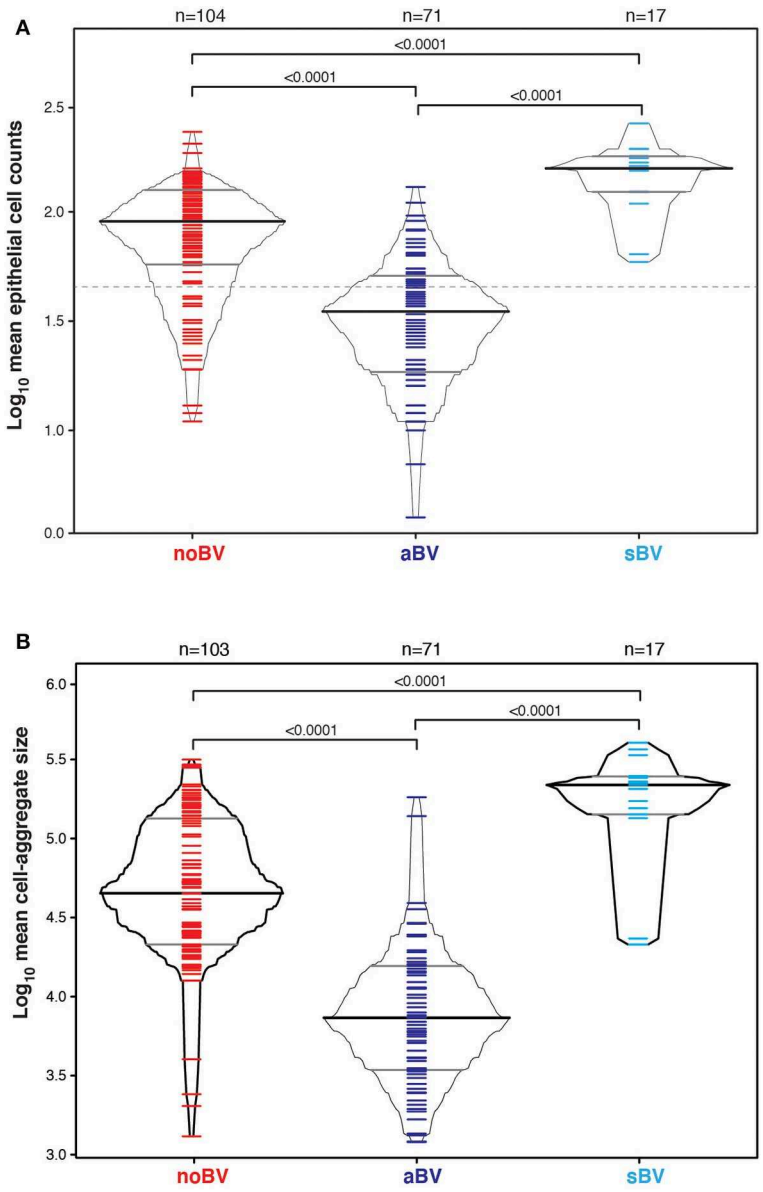

FIGURE 2 | (A) Box-percentile plot of log_{10} mean epithelial cell counts in different Amsel diagnostic groups. Dash line indicates 50 mean epithelial cells. **(B)** Box-percentile plot of log_{10} mean cell-aggregate sizes in different diagnostic groups. The median, 25 and 75th percentiles are marked with line segments across each box. Numbers at the top axis indicate the number of samples in each group. Ticks within percentile-boxes show individual sample values.

Statistical Analysis

Cell counts, cell-aggregate sizes, and SCI values are reported as median and interquartile range. Comparisons of cell counts were performed by fitting a Bayesian Laplace subject-wise random effects model to the $\log_{10}$ transformed data. Comparisons of cell-aggregate sizes were made in the same way. SCI values were computed within a Bayesian binomial mixed effects model with subject-wise random intercept. Adjustment for multiple testing was performed using false discovery rate (FDR). For all comparisons, exact p-values are reported. Within woman pairwise comparison of $\log_{10}$ cell counts and aggregation sizes was made using a Bayesian Poisson within subject two group comparison model. All scripts used in this study are available on GitHub at https://github.com/ravel-lab/BV_CELL_SHEDDING.

RESULTS

Comparison of Total Epithelial Cell Counts Between Asymptomatic, Symptomatic and No BV Smears

Cell counts were first considered based on diagnostic group only, without reference to microbiota CST information. The median cell count of samples with no diagnosis of Amsel-BV (noBV, $n = 104$) was 91/100X field (interquartile range 58–126). This was significantly lower than the median cell count for samples with a diagnosis of symptomatic Amsel-BV (sBV, $n = 17$), which had median 158/100X field (IQR 124–179), $p = 7.2e{-}09$. Additionally, the median cell count of noBV samples was significantly higher than that of samples with a diagnosis of asymptomatic Amsel-BV (aBV, $n = 71$), which had a median 35/100X field (IQR 19–50), $p = 2.4e{-}16$ (**Figure 2A**). In addition, median cell counts of sBV and aBV samples were significantly different, $p < 10e{-}12$.

Comparison of Cell-Aggregates Size Between Asymptomatic, Symptomatic and No BV Smears

Variability of cell-aggregates on the slides was apparent. Some slides had evenly dispersed cells with aggregates of only a few cells each (**Figure 3A**), while other slides had multiple aggregations so extensive and dense that they could be distinguished without magnification (**Figure 3B**). Cell-aggregate size measurements indicated that in noBV samples the median cell-aggregate size (50,000 μm^2, IQR 20,000–140,000 μm^2) was significantly smaller than that for sBV samples, which had a median cell-aggregate size of 220,000 μm^2 (IQR ~140,000–240,000 μm^2), $p = 1.6e{-}14$. Additionally, the median cell-aggregate size of noBV samples was significantly larger than that of aBV samples, which had a median cell-aggregate size of ~7,000 μm^2 (IQR 3,000–14,000 μm^2), $p < 2.22e{-}16$ (**Figure 2B**). Lastly, the median cell-aggregate sizes of sBV and aBV samples were significantly different, $p = 2.2e{-}55$.

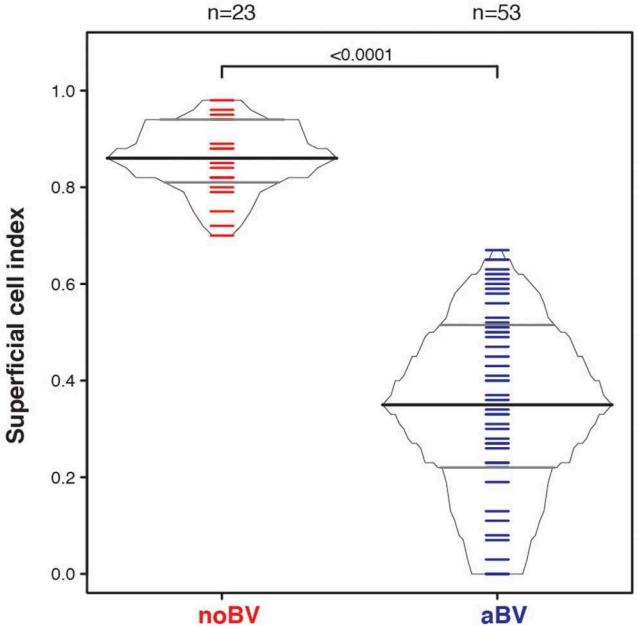

FIGURE 4 | Box-percentile plot of superficial cell index in no Amsel-BV and asymptomatic Amsel-BV samples with a mean epithelial cell counts of <50.

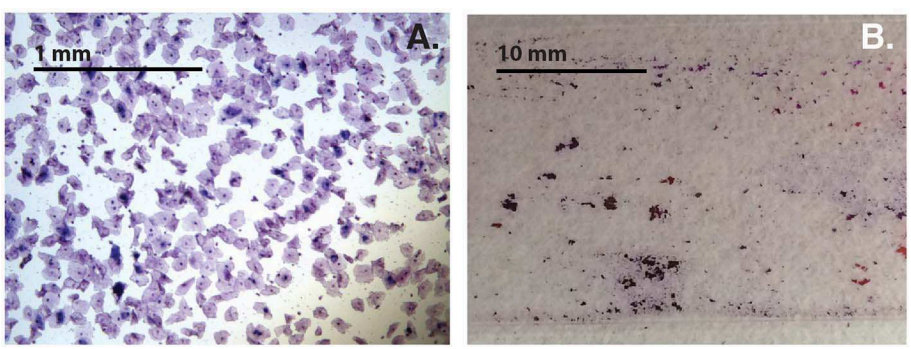

FIGURE 3 | (A) Bright-field micrograph (100X total magnification) showing sample with single cells and small cell-aggregates. **(B)** Photograph without magnification showing samples with cell-aggregates large enough to see with the naked eye.

Comparison of the Superficial Cell Index Between Asymptomatic, Symptomatic and No BV Smears

The superficial cell index (SCI) was calculated for samples with a mean cell count of ≤50/100X field, corresponding to a total of 78 samples from 54 women. Mean cell count of ≤50/100X field was chosen since it included almost all aBV samples and was well-represented among noBV samples. The median SCI of noBV samples ($n = 23$) was 0.86 (IQR 0.81–0.94), significantly higher than that of aBV samples ($n = 53$), which had a median SCI of 0.35 (IQR 0.22–0.51), $p = 4.3e{-}98$ (**Figure 4**). No sBV samples had mean cell count ≤50/100X field.

Comparison of Total Epithelial Cell Counts, Cell-Aggregate Sizes and SCI Stratified by Amsel Diagnostic Groups and CSTs

Interestingly, differences between noBV, sBV, and aBV remained when samples were considered based on Amsel diagnostic groups and CSTs. Among samples assigned to CST IV (microbiota comprising a wide range of facultative and strict anaerobic

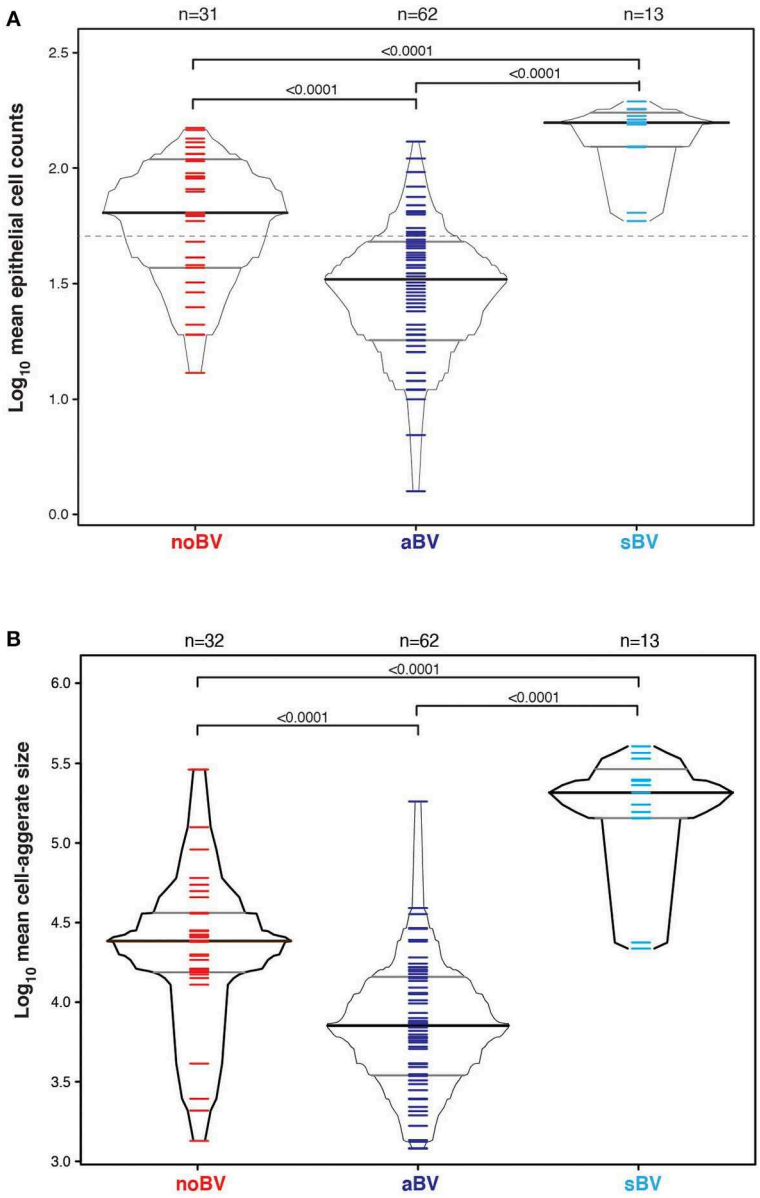

FIGURE 5 | (A) Box-percentile plot of $\log_{10}$ mean epithelial cell counts in different diagnostic groups for only CST IV samples. Dash line indicates 50 mean epithelial cells. **(B)** Box-percentile plot of $\log_{10}$ mean cell-aggregate sizes in different diagnostic groups for only CST IV samples. The median, 25 and 75th percentiles are marked with line segments across each box. Numbers at the top axis indicate the number of samples in each group. Ticks within percentile-boxes show individual sample values.

bacteria and a lack of *Lactobacillus* spp.), the median cell count of noBV samples (64/100X field, IQR 38–112), $n = 31$, Amsel-BV negative, but molecular-BV positive was significantly lower than that of sBV samples (157/100X field, IQR 157–171, $n = 13$), $p = 9.5e{-}7$, and significantly higher than aBV samples (33/100X field, IQR 18–48), $n = 62$), $p = 0.00011$ (**Figure 5A**). In addition, median cell counts of sBV and aBV samples assigned to CST IV were significantly different, $p = 1.3e{-}24$. Similarly, the median cell-aggregate size of CST IV noBV samples (20,000 μm^2, IQR 15,000–25,000 μm^2) was significantly smaller than that of CST IV sBV samples (25,000 μm^2, IQR 15,000–36,000 μm^2), $p = 5.8e{-}16$, and significantly larger than that of aBV samples (7,000 μm^2, IQR 3,600–13,000 μm^2), $p = 1.06e{-}9$ (**Figure 5B**). Further, the median cell-aggregate sizes of sBV and aBV samples assigned to CST IV were significantly different, $p = 3.15e{-}45$. Further, Lastly, among the 61 samples with mean cell count of $\leq$50/100X field and that were CST IV, the median SCI of noBV samples (0.94 IQR 0.84–0.95), $n = 11$) was significantly higher than that of aBV samples (0.34, IQR 0.22–0.51, $n = 48$), $p = 5.3e{-}73$ (**Figure 6**). There were too few samples with a microbiota dominated by *Lactobacillus* spp. that were categorized as sBV ($n = 4$, all CST III) or aBV ($n = 4$, one CST I and three CST III) to permit any analysis for CSTs other than CST IV.

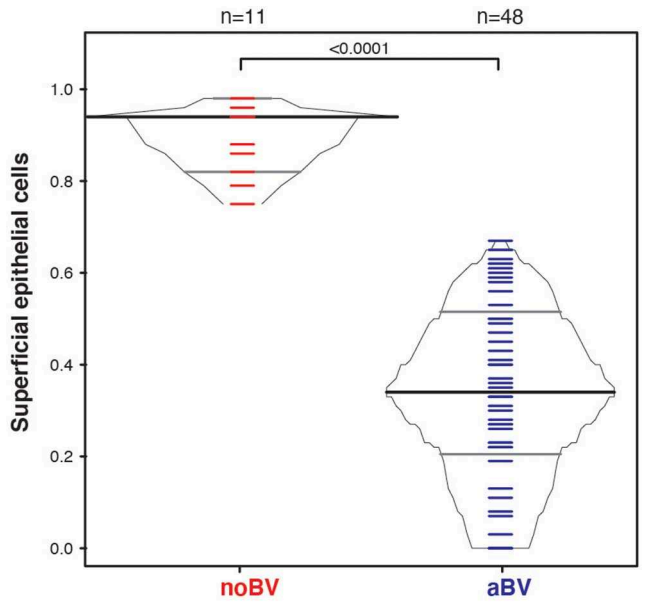

FIGURE 6 | Box-percentile plot of superficial cell index in no Amsel-BV and asymptomatic Amsel-BV samples for only CST IV samples with a mean epithelial cell counts of <50.

DISCUSSION

The equilibrium among the continuous processes of cell proliferation, maturation and shedding on the vaginal epithelium is probably essential to maintaining an effective barrier against pathogenic and non-pathogenic microbes alike. Here, we report

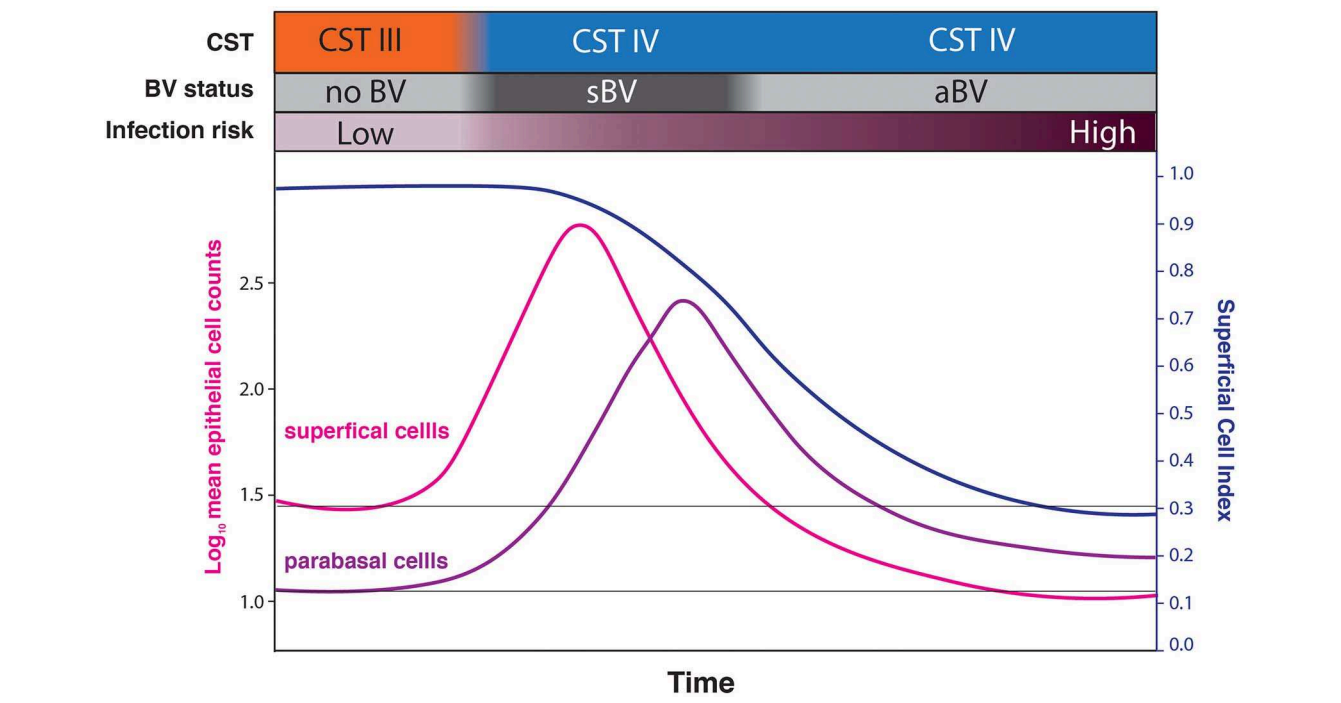

FIGURE 7 | Conceptual model hypothesizing that sBV may be rapidly followed by aBV. Under this hypothesis, the increased cell-shedding events associated with sBV exhaust the superficial and intermediate epithelial cell layers resulting in reduced cell-shedding, and exposure and loss of immature epithelial cells (low SCI) associated with aBV. This progression is hypothesized to be associated with increased risk of infections.

an association of symptomatic Amsel-BV (sBV) with evidence of increased cell-shedding, and of asymptomatic Amsel-BV (aBV) with both decreased cell-shedding and increased presence of immature epithelial cells.

These findings raise two intriguing possibilities. Firstly, rather than sBV and aBV being two forms of the condition, we hypothesize that they are sometimes two phases of a single form. Under this model (**Figure 7**), sBV may be rapidly followed by aBV. Though the dataset presented here is effectively cross-sectional and cannot directly address this possibility (there were only 7 women who provided both sBV and aBV samples, collected several weeks apart). Under this hypothesis, the increased cell-shedding events associated with sBV exhaust the superficial and intermediate epithelial cell layers resulting in reduced cell-shedding, and exposure and loss of immature epithelial cells associated with aBV. This hypothesis is supported by the finding that there are no significant differences in microbiota structure between aBV and sBV [high frequency of CST IV (93.94 and 76.47%, respectively, $p = 0.205$) and CST III (4.55 and 23.53%, respectively, $p = 0.096$)] (**Supplementary Figure 1**), although absolute abundances of bacteria have not been evaluated and could be different between sBV and aBV. We are currently undertaking larger, longitudinal studies to address these possibilities.

Secondly and more importantly, our findings suggest that aBV might be associated with more severe disruption of the vaginal epithelium than sBV, and hence lead to a greater increase in susceptibility to infections. By analogy, the spermicide nonoxynol-9 (Hillier et al., 2005) is known to cause accelerated cell-shedding (Niruthisard et al., 1991), thinning (Vincent et al., 2011), disruption (Hoffman et al., 2004) and pro-inflammatory changes (Smith-Mccune et al., 2015) on the vaginal epithelium, and was found to increase women's susceptibility to HIV (Wilkinson et al., 2002), as well as susceptibility to HSV-2 (Cone et al., 2006) and HPV (Roberts et al., 2007) in a mouse model. Since aBV is associated with similar physiological effects on the epithelium, we hypothesize it would be associated with an increased risk of infections.

If validated, the hypothesis that aBV does have a particularly disruptive effect on the cervicovaginal epithelium would make it necessary to reevaluate the relative importance of sBV and aBV. While vaginal symptoms have been linked in part to a patient's self-report (Klebanoff et al., 2004) thus symptoms perception, six women in this study presented with sBV and aBV at separate clinical visits over the course of the study (**Supplementary Table 1**), indicating that women who have had aBV also recognize sBV. However, we hypothesize that based on our findings, there may be relevant biological differences between aBV and sBV. Current CDC guidelines recommend antibiotic treatment only for sBV, not aBV (Workowski and Bolan, 2015). Future work may lead clinicians to reconsider the merits of treating aBV and amending counseling messages for women with aBV to manage their increased susceptibility to infection, for example by avoiding unprotected sexual intercourse. However, expanded screening based on high-resolution molecular assays of both the microbiota and the state of the epithelium would need to be implemented at routine gynecological visits, and more importantly, the development of specific therapies to address the epithelial disruption associated with aBV would be needed.

In our study, the median cell count of noBV samples assigned to CST IV (70/100X field, $n = 32$) was significantly lower than that of other noBV samples (93/100X field, $n = 69$), $p = 0.0384$ (**Supplementary Figure 2**). This is likely because molecular-BV broadly defines BV and is not limited to Amsel-sBV and Amsel-aBV (as evidenced by our data set, in which 31/104 noBV samples were CST IV). Interestingly, it has been noted that the BV-associated increased risk of HIV acquisition is higher in studies using molecular-BV (defined as all forms of CST IV) as the exposure variable, compared to studies using Amsel-BV or Nugent-BV (Mckinnon et al., 2019). A recent meta-analysis showed that Amsel-BV was associated with a 2-fold increase in risk for HIV acquisition (OR: 1.93, 95% CI: 1.45–2.57; Atashili et al., 2008). A subsequent study suggested that molecular-BV was associated with a 4-fold hazard ratios compared to *L. crispatus*-dominated samples (HR:4.41, 95% CI: 1.17–16.61, $p = 0.028$; Gosmann et al., 2017). Molecular-BV is associated with proinflammatory cytokines (Gosmann et al., 2017) and activated HIV-target cells (Anahtar et al., 2015) in the cervicovaginal epithelium and thus with increased risk of HIV acquisition. These findings support a higher risk associated with asymptomatic molecular-BV, and suggest mechanisms involving impaired physiological state of the epithelium and increased pro-inflammatory markers. The above epidemiologic studies of BV and risk for HIV suggest that molecular-BV might trend toward higher point estimates for HIV risk than Amsel-BV because molecular-BV includes more women with at-risk aBV states. To our knowledge, no studies have directly assessed the risk of HIV or other STI acquisition in women with molecular-BV vs. sBV; further studies are needed to test our hypothesis.

DATA AVAILABILITY STATEMENT

Raw counts and metadata used in this study are available in **Supplementary Table 1**.

ETHICS STATEMENT

Samples used in this study were archived vaginal smears obtained in accordance with protocols approved by the University of Maryland Baltimore Institutional Review Board.

AUTHOR CONTRIBUTIONS

DO'H, RB, and JR designed the study. DO'H executed the laboratory part of the study. PG performed the statistical analyses. DO'H, PG, RB, and JR wrote/edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00106/full#supplementary-material

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Conflict of Interest: JR is co-founder of LUCA Biologics, a biotechnology company focusing on translating microbiome research into live biotherapeutics drugs for women's health.

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.

frontiers in Cellular and Infection Microbiology

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Comparative Metagenome-Assembled Genome Analysis of "*Candidatus* Lachnocurva vaginae", Formerly Known as Bacterial Vaginosis-Associated Bacterium−1 (BVAB1)

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Bacterial vaginosis-associated bacterium 1 (BVAB1) is an as-yet uncultured bacterial species found in the human vagina that belongs to the family *Lachnospiraceae* within the order *Clostridiales*. As its name suggests, this bacterium is often associated with bacterial vaginosis (BV), a common vaginal disorder that has been shown to increase a woman's risk for HIV, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* infections as well as preterm birth. BVAB1 has been further associated with the persistence of BV following metronidazole treatment, increased vaginal inflammation, and adverse obstetrics outcomes. There is no available complete genome sequence of BVAB1, which has made it difficult to mechanistically understand its role in disease. We present here a circularized metagenome-assembled genome (cMAG) of BVAB1 as well as a comparative analysis including an additional six metagenome-assembled genomes (MAGs) of this species. These sequences were derived from cervicovaginal samples of seven separate women. The cMAG was obtained from a metagenome sequenced with long-read technology on a PacBio Sequel II instrument while the others were derived from metagenomes sequenced on the Illumina HiSeq platform. The cMAG is 1.649 Mb in size and encodes 1,578 genes. We propose to rename BVAB1 to "*Candidatus* Lachnocurva vaginae" based on phylogenetic analyses, and provide genomic and metabolomic evidence that this candidate species may metabolize D-lactate, produce trimethylamine (one of the chemicals responsible for BV-associated odor), and be motile. The cMAG and the six MAGs are valuable resources that will further contribute to our understanding of the heterogeneous etiology of bacterial vaginosis.

Keywords: women's health, gynecology, microbial genomics, odor, vaginal microbiome

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INTRODUCTION

Bacterial vaginosis (BV) is a common vaginal infection affecting approximately 30% of US reproductive-aged women, with both African- and Mexican-Americans disproportionately afflicted (Allsworth and Peipert, 2007; Koumans et al., 2007). Though treatable with antibiotics, BV has a high rate of recurrence (Bradshaw et al., 2006; Schwebke and Desmond, 2007). The microbiological diagnosis of BV is established using Nugent scoring of Gram-stained vaginal smears and is defined by a low abundance of *Lactobacillus* spp. morphotypes and a wide array of strict and facultative Gram-negative anaerobes (Nugent et al., 1991). The clinical diagnosis of BV is made when 3 of the 4 Amsel's criteria are met: vaginal pH > 4.5, homogenous vaginal discharge, a positive whiff test, and the presence of clue cells upon wet mount examination (Amsel et al., 1983). Aside from the burdensome symptoms of vaginal discharge and fishy odor, BV is also associated with increased risk to adverse health outcomes including preterm birth (Leitich et al., 2003), increased risk of sexually transmitted infections acquisition and transmission, including HIV (Taha et al., 1998; Cherpes et al., 2003; Ness et al., 2005; Atashili et al., 2008; Cohen et al., 2012) and pelvic inflammatory disease (Ness et al., 2004).

A critical step along the path to understanding the ecology and pathogenic potential of a bacterial species is the characterization of its genome. Yet many of the bacteria associated with BV have thus far been uncultivable, further complicating genome sequencing efforts. BV-associated bacterium 1 (BVAB1) is one such organism for which there is limited genetic information available. BVAB1 was first identified by Fredricks et al. (2005) using 16S rRNA gene amplicons Sanger sequencing of samples associated with BV, and has eluded cultivation efforts since. Based on the sequence of its 16S rRNA gene, BVAB1 belongs to the family *Lachnospiraceae* (Muzny et al., 2014) and has often been misidentified as belonging to the genus *Shuttleworthia* (Lamont et al., 2011; Petrova et al., 2013). Interestingly, Gram-negative curved rods designated *Mobiluncus* morphotypes on Gram stain in Nugent scoring have been shown to likely be BVAB1 (Srinivasan et al., 2013). Further, vaginal communities in which BVAB1 16S rRNA gene sequence is detected have been associated with vaginal inflammation and persistent BV in African women (Lennard et al., 2018). BVAB1 remains uncultured and aside from detection of this species via partial 16S rRNA gene amplicon sequencing, little is known about its metabolism, pathogenic potential, or ecology in the vaginal environment, especially during BV. Further understanding of the genetic and physiological properties of BVAB1 will help to dissect the complex etiology of BV. Previously, a 94 contig BVAB1 metagenome-assembled genome from short-read sequencing was produced (Fettweis et al., 2019).

In this study, we characterize the first circularized metagenome assembled genome (cMAG) of BVAB1 constructed from a metagenome sequenced using the PacBio Sequel II long read platform. In addition, we compare this cMAG to an additional six metagenome-assembled genomes (MAGs). All genomes originate from different women with symptomatic or asymptomatic BV. Based on phylogenetic analysis of full-length 16S rRNA gene sequences obtained from the genomic assemblies, we propose to rename this bacterium "*Candidatus* Lachnocurva vaginae".

MATERIALS AND METHODS

Sample Collection

Vaginal samples used in this study were identified as containing a high relative abundance (> 60%) of BVAB1 using 16S rRNA gene amplicon sequencing of the V3–V4 regions as previously reported (Holm et al., 2019). Cervicovaginal lavages from six participants that were collected as part of the NIH Longitudinal Study for Vaginal Flora (LSVF) (Klebanoff et al., 2004) by washing the vaginal walls with 3 mL sterile, deionized water, aspiration from the vaginal vault via pipette and stored at −80°C, were included in this study. Gram stain smears were prepared for Nugent scoring as previously described (Nugent et al., 1991). DNA was extracted from 200 μL of lavage fluid using the MagAttract Microbial DNA Kit (QIAGEN Inc., Germantown MD) automated on a Hamilton Star robotic platform according the manufacturer recommendations. DNA was eluted in a final volume of 110 μL nuclease-free water.

An additional swab sample collected as part of the UMB-HMP study was used in this study (Ravel et al., 2013). The swab was self-collected by a participant using a Copan Eswab re-suspended into 1 mL Amies transport medium (ESwab, Copan Diagnostics Inc.), frozen at −20°C for no more than a week, and then transferred to −80°C until analyzed. High molecular weight DNA was extracted from this sample using the MasterPure DNA purification kit (Lucigen) with two phenol/chloroform cleanups prior to DNA precipitation. DNA extraction was quantified on a TapeStation 2200 instrument run with a Genomic DNA tape (Agilent).

Metagenomic Library Construction and Sequencing on the PacBio Sequel II Platform

The extracted DNA from the swab collected as part of the UMB-HMP study (Ravel et al., 2013) was found to be of sufficient concentration (5.49 ng/μL in 200 μL) for long-read sequencing using the Pacific Biosciences Sequel II platform (Pacific Biosciences). The sequencing library was prepared with SMRTBell Template Prep Kit 1.0 and was size-selected on a BluePippen (Sage Science) with a cutoff of 5 kb. The library was barcoded and sequenced as part of a multiplexed run with four other unrelated samples. Sequencing was performed on a PacBio Sequel II instrument with an 8M cell loaded at 60 pM at the Genomic Resource Center of the University Maryland School of Medicine.

Long Read Quality Filtering, Host-Read Removal, and cMAG Construction

Raw reads were demultiplexed with *lima* (version 1.9.0) using default parameters except for minimum barcode score set at 26 and a minimum read length of 50 bp after clipping of the barcode was enforced. Both tools are part of the SMRTLink 6.0.1 software package with updated CCS version 3.4.1. Human reads were detected using pbalign v0.4.1 (Pacific Biosciences) and the

human genome build 38 (GRCh38.p12). Remaining reads were corrected and assembled via Canu v1.7 and the "-pacbio-raw" protocol (Koren et al., 2017). The largest resulting contig was ca. 1.6 Mb in length, much larger than the second longest contig (ca. 600 kb). Four copies of the 16S rRNA gene were detected on this contig and were identical to the existing BVAB1 16S rRNA gene reference AY724739 Fredricks et al., 2005 (NEJM). The contig had 6.5 kb of overlapping ends and was determined to be circular by Canu. This contig was then manually circularized using Geneious version 2019.2.1 (Galens et al., 2011), searched for the *dnaA* gene, and rotated so that *dnaA* was the first gene (Kearse et al., 2012). The circularized metagenome-assembled genome (cMAG) was annotated using the IGS Prokaryotic Annotation Pipeline (Galens et al., 2011). Translated gene sequences were assessed against KEGG using the BlastKOALA algorithm for insights into "*Candidatus* Lachnocurva vaginae" metabolism (Kanehisa et al., 2016). Bacteriophages were detected using PHASTER (Zhou et al., 2011; Arndt et al., 2016). Completeness and contamination of the cMAG was analyzed using CheckM version 1.0.18 (Parks et al., 2015) and the taxonomy_wf flag specifying Order *Clostridiales* and rerun specifying Family *Lachnospiraceae*. Metabolic reconstruction was examined with the cMAG using the metabolic modeling function in KBase (Arkin et al., 2018). Complete media for gapfilling and a Gram-positive template were used.

Metagenomic Library Construction and Sequencing on the Illumina HiSeq 4000 Platform

Metagenomic libraries for the six samples from the LSVF study were prepared using the KAPA HyperPlus Kit (Kapa Biosystems) with KAPA Single-Indexed Adapter Kit Set B. A fixed volume (35 μL) of genomic DNA was used as input, and libraries were prepared following the manufacturer's protocol with modifications based on their amount of input DNA as in **Supplemental Data Sheet 1**. For samples with 0.5 or 0.2 ng input DNA, the fragmentation enzyme was diluted 1:2 or 1:5 with water. All samples were fragmented at 37°C for 5 min. Adapter concentrations varied according to the input DNA as listed in **Supplemental Data Sheet 1**, and the adapter ligation was carried out overnight at 4°C for all samples. The post-ligation cleanup was performed with 0.8x Ampure XP beads (Beckman Coulter, Indianapolis IN) and 20 μL of sample was used in library amplification. Amplification library cycles varied by input DNA as listed in **Supplemental Data Sheet 1**. Post-amplification cleanup was performed with 1x Ampure XP beads; libraries with remaining adapter dimer peaks were cleaned a second time. The final elution was in 25 μL of nuclease-free water. Libraries were run on a TapeStation instrument with a D1000 tape (Agilent) to assess quality and concentration. Libraries were sequenced (8 libraries/lane) on an Illumina HiSeq 4000 instrument using the 150 bp paired-end protocol.

Short Read Quality Filtering, Host-Read Removal, and Metagenomic-Assembled Genome Reconstruction

Metagenomic reads were quality filtered using Trimmomatic v0.36 (Bolger et al., 2014) to remove sequencing adapters allowing for 2 mismatches, a palindromic clip threshold of 30, and a simple clip threshold of 10. Bases with quality scores < 3 were removed from the beginning and end of reads combined with a 4 bp sliding window which trimmed a read if the average quality score within that window fell below 15. Reads < 75 bp in length were removed. Human reads were detected by mapping to the human genome build 38 (GRCh38.p12) with Bowtie 2 v2.3.4.1 and default settings (Langmead and Salzberg, 2012), and removed using samtools v1.9 (Li et al., 2009) and bedtools v2.27.1 (Quinlan and Hall, 2010; Quinlan, 2014) (see **Supplemental Data Sheet 2** for specific scripts). Metagenomic assemblies were produced using SPAdes genome assembler v3.13.0 (Bankevich et al., 2012) with the careful setting. Resulting

TABLE 1 | Participants demographics and cervicovaginal lavage microbial compositions for samples used in metagenomic reconstruction of the "*Ca.* Lachnocurva vaginae" circularized metagenome-assembled genome using long-read (*) and shotgun sequencing (*).

Sample ID	Age	Race	Microbiota Relative Abundances of Vaginal Samples (16S rRNA Gene –V3V4)	BV Status	Nugent Score	Vaginal pH	Clinician-Observed Discharge	Positive Whiff Test
UAB071*	22	Black		Asym	8	5	-	Yes
Y3207	37	Black		No	6	6.5	No	No
Y2266	34	Black		Asym	10	5.3	Mild visible—Gray/White	Yes
Y2337	36	Black		Asym	8	5.5	Mild visible—Gray/White	Yes
Y3255	24	Black		Asym	9	5.5	No	Yes
Y2624	27	Black		Asym	7	5.5	Mild visible—"Mucousy"	Yes
Y2694	34	White		Sym	7	5.5	Mild visible—Gray/White	Yes

■, *Ca. Lachnocurva vaginae*; ■, *Gardnerella vaginalis*; ■, *Megasphaera*; ■, *Sneathia sanguinegens*; ■, *Prevotella*; ■, *Atopobium vaginae*; ■, *Prevotella amnii;* ■, *Mageeibacillus indolicus*; ■, *Other*.

Other (gray, includes all taxa except the 8 most abundant taxa). Asym, asymptomatic BV; Sym, symptomatic BV.

contigs were aligned to the cMAG using NUCmer with the –mum setting and allowing for 5,000 bp breaklength, filtered to remove aligned contigs < 100 bp, and minimum contig coverage of 25%. MAGs were annotated with the Live Annotate & Predict tool from Geneious version 2019.2.1 (Kearse et al., 2012) using the "*Ca.* Lachnocurva vaginae" cMAG as annotation

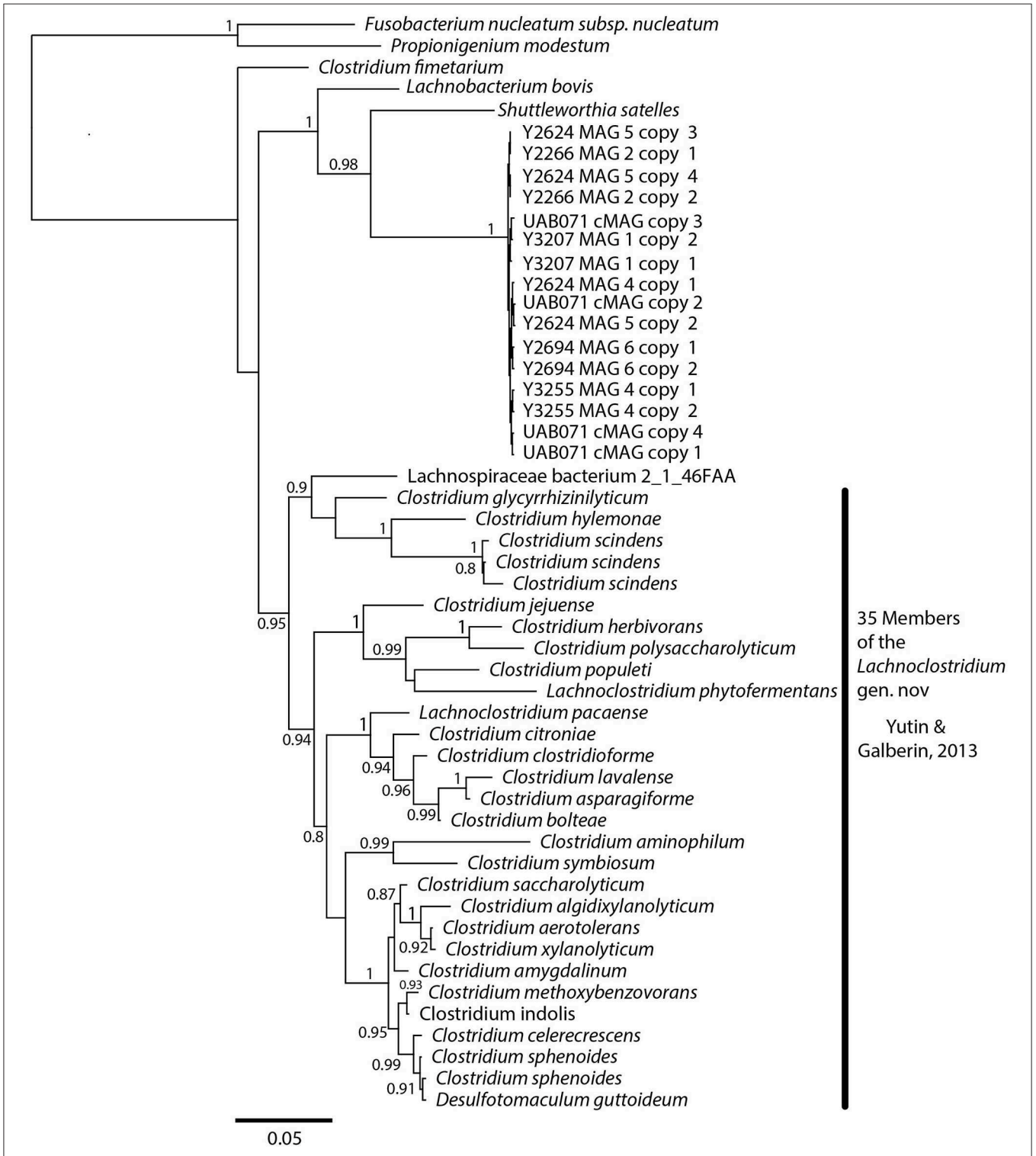

FIGURE 1 | "*Ca.* Lachnocurva vaginae" is related to *Shuttleworthia satelles*; the full-length 16S rRNA gene sequences are 89–90% identical.

source (sequence similarity of ≥ 95% required). Genes with no similarity to the cMAG were annotated with Prokka v1.13 (Seemann, 2014). Phages were detected using PHASTER (Zhou et al., 2011). A circle plot was constructed with the BLAST Ring Image Generator v0.95 (Alikhan et al., 2011), and the annotated cMAG.

Metabolomic Analysis

Metabolomics analysis was conducted as previously described (Nelson et al., 2018) by Metabolon Inc. using 200 μL of each LSVF lavage sample used in this study or 200 μL of a frozen dry swab eluted in 1 ml of PBS for the sample from the UMB-HMP study (Ravel et al., 2013). The abundances of 561 compounds were quantified using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS). Quantities were corrected for instrument block variability and reported as normalized area-under-the-curve estimates. Figures were generated using ggplot2 v3.2.0 (Wickham, 2009).

Comparative Genome and Phylogenetic Analyses

Average nucleotide identities (ANI) were calculated using FastANI v1.1 with fragment lengths of 1,000 bp, which is about the mean length of coding sequences (Jain et al., 2018). Maximum-likelihood phylogenetic trees of full-length 16S rRNA gene sequences alignments were generated with MUSCLE v3.8.425 using default parameters (Edgar, 2004) and FastTree v2.1.11 using the Generalized Time Reversible model and default parameters (Price et al., 2009, 2010) in Geneious v11.0.3 (Kearse et al., 2012). The tree was rooted with *Fusobacterium nucleatum* (AJ133496) and *Propionigenium modestum* (X54275) (Domingo et al., 2009), and members of the genus *Lachnoclostridium* were included as neighbors (Yutin and Galperin, 2013) as well as 16S rRNA genes from *Shuttleworthia satelles* (NR_028827), and *Lachnospiraceae* bacterium 2_1_46FAA (NR_025127). In addition, the cMAG was submitted for taxonomic placement using the TrueBacID tool from EZBioCloud (Yoon et al., 2017). cMAG and MAG pseudomolecules were aligned and visualized using AliTV v1.0.6 (Ankenbrand et al., 2017). Genes were ordered by gene synteny of the cMAG. To examine homology to other reference sequences, the cMAG was aligned to the NCBI protein reference database (downloaded 1/28/2019), as well as the genomes of *Shuttleworthia satelles* (NZ_ACIP00000000), *Lachnobacterium bovis* (GCF_900107245.1), and *Lachnospiraceae* bacterium 2_1_46FAA (ADLB02000001.1) using BLAST v2.8.1+. The top 3 hits were chosen by lowest e-value, highest percent identity, and longest alignment length, in that order. Genomic and pathogenicity islands were explored using IslandViewer4 (Bertelli et al., 2017).

RESULTS AND DISCUSSION

Participant Information

All six participants in the LSVF study were of reproductive age and had high Nugent scores (6–10) (**Table 1**). Four were diagnosed with asymptomatic Amsel-BV, one with symptomatic Amsel-BV, and one was negative for Amsel-BV (Mckinnon et al., 2019). The woman who participated in the UMB-HMP study (Ravel et al., 2013) had a Nugent score of 8 and was diagnosed with asymptomatic Nugent-BV. The vaginal microbiota of these 7 samples had >60% BVAB1 as defined by 16S rRNA gene V3-V4 amplicon sequencing (Ravel et al., 2013).

Phylogenetic Analysis

A phylogenetic analysis using the full-length 16S rRNA genes extracted from all MAGs revealed BVAB1 belongs in the *Clostridales* family *Lachnospiraceae*, and that *S. satelles* is the closest known relative (**Figure 1**), though nucleotide identity is only 89.2%. Similar results were obtained from EZBioCloud's TrueBacID (**Supplemental Data Sheet 3**). We therefore propose a new candidate species for BVAB1: "*Candidatus* Lachnocurva vaginae", which represents the phylogenetic placement, the curved morphology of the cells, and the source of this cMAG. Considering the entire cMAG, the ANI between "*Ca.* Lachnocurva vaginae" and the *S. satelles* draft genome (NZ_ACIP00000000) was 81.85%. Relative to each other, "*Ca.* Lachnocurva vaginae" 16S rRNA genes were >99.7% identical and the cMAG and MAG average nucleotide identities were also high (98.6–99.2%, **Table 2**).

Overall Genomic Features

The cMAG of "*Ca.* Lachnocurva vaginae" is 1,649,642 bp in size with 31.8% GC content, encodes 1,578 genes and was

TABLE 2 | Average Nucleotide Identity (ANI) between the "*Ca.* Lachnocurva vaginae" circularized metagenome-assembled genome, each metagenome-assembled genome, and the *Shuttleworthia satelles* draft genome NZ_ACIP00000000.

		"*Ca.* Lachnocurva vaginae" MAG ID						
		Y2694_MAG_6	**Y2624_MAG_5**	**Y3255_MAG_4**	**Y2337_MAG_3**	**Y2266_MAG_2**	**Y3207_MAG_1**	**UAB071**
"*Ca.* Lachnocurva vaginae" MAG ID	Y2624_MAG_5	99.2	–	–	–	–	–	–
	Y3255_MAG_4	99.0	99.01	–	–	–	–	–
	Y2337_MAG_3	98.95	98.99	98.97	–	–	–	–
	Y2266_MAG_2	99.06	99.01	98.93	99.05	–	–	–
	Y3207_MAG_1	98.98	98.95	99.07	98.99	99.06	–	–
	UAB071	99.15	99.00	98.92	99.09	99.13	98.98	–
	Shuttleworthia satelles	80.80	81.90	81.51	81.63	81.30	81.86	81.85

estimated to be 99.1% complete and 0% contaminated at the order level and 90.9% complete and 0% contaminated at the family level. The relatively low completion estimate at the family level was due to 22 missing marker genes out of 312 (**Supplemental Table 2**). Mean coverage of the cMAG assembly by long reads was 124X (range: [26–285]). The "*Ca.* Lachnocurva vaginae" MAGs constructed using the short-read Illumina HiSeq 4000 metagenomic sequence data were 1.48–1.62 Mb in size (mean: 1.57 Mb) with a number of contigs ranging from 26 to 152 (mean N50: 99,943 bp) and 31.6% GC (**Table 3**). Genome annotation indicated the presence of four complete rRNA operon copies (**Figure 2**) and 42 tRNA genes in the cMAG. As expected, partial rRNA operons were identified in the Illumina-based MAGs (**Supplemental Image 1**).

Genomic Features of "*Ca.* Lachnocurva vaginae"

Metabolic modeling and reconstruction of the "*Ca.* Lachnocurva vaginae" cMAG contained 122 genes, 480

TABLE 3 | "*Ca.* Lachnocurva vaginae" MAG assembly characteristics.

"*Ca.* Lachnocurva vaginae" MAG ID	No. CDS	Total Length (Mb)	N50	%GC	No. Contig	No. tRNAs	No. 5S rRNA	No. 16S rRNA	No. 23S rRNA
UAB071	1,578	1.64	–	31.8	1	42	4	4	4
Y3207_MAG_1	1,463	1.60	42,408	31.6	152	41	1	1	1
Y2266_MAG_2	1,322	1.48	297,822	31.3	26	22	1	1	0
Y2337_MAG_3	1,492	1.62	60,576	31.6	143	42	0	0	0
Y3255_MAG_4	1,433	1.59	80,762	31.5	49	38	1	1	0
Y2624_MAG_5	1,459	1.61	168,048	31.8	31	43	2	2	2
Y2694_MAG_6	1,379	1.52	156,872	31.5	30	28	1	1	0

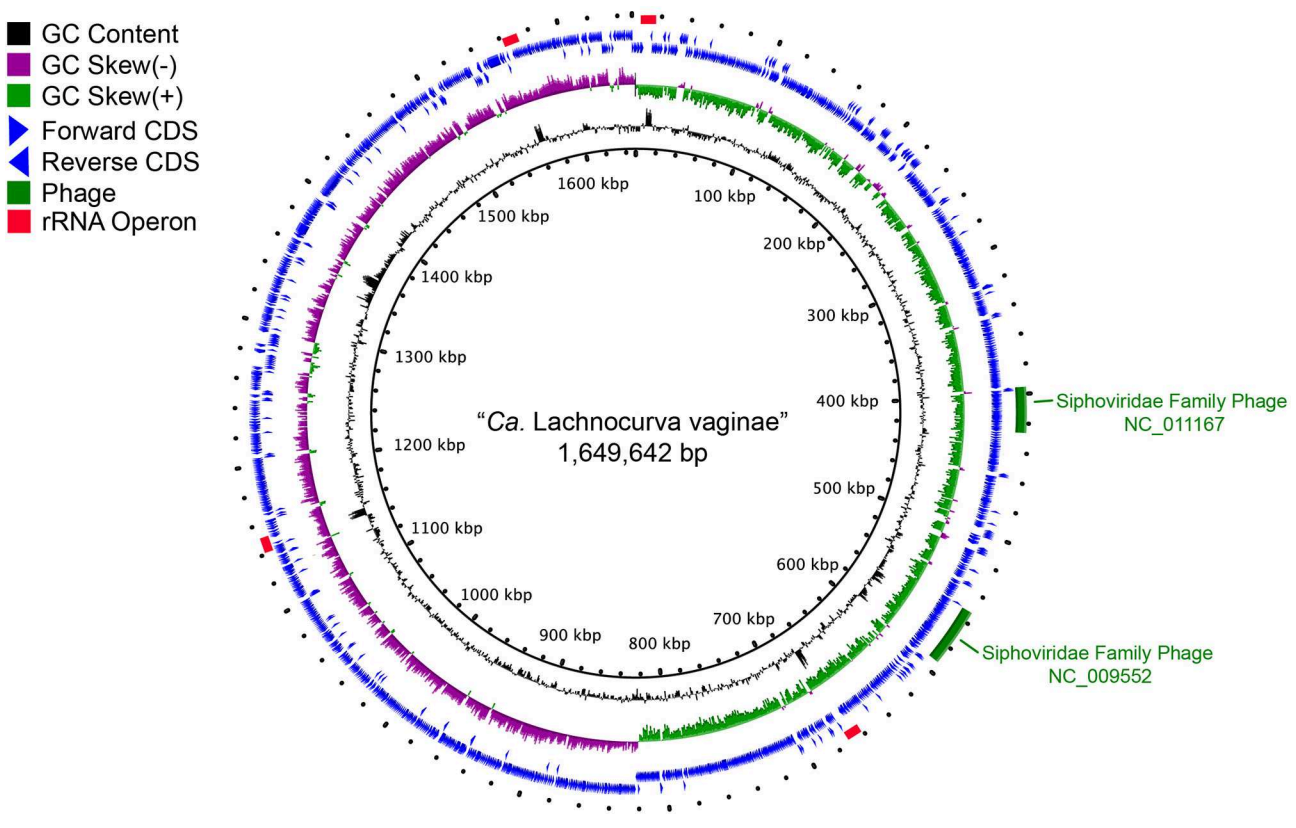

FIGURE 2 | Circle plot of the "*Ca.* Lachnocurva vaginae" circularized metagenome-assembled genome (cMAG). Circles are from inside to outside: Circle 1: cMAG position; Circle 2: GC content, 3: GC skew, 4: forward-direction coding sequences, 5: reverse-direction coding sequences, and 6: phage detected in cMAG (green bars) and rRNA operons (red bars).

reactions, 581 metabolites, and no mass imbalance was found. Of the 1,578 genes detected, 255 genes had best blast matches of 80% sequence identity covering > 80% of the query gene. Of these, 165 matches were within the order *Clostridiales*, and 90 were not (**Supplemental Image 2**). Genes encoding transporters for mannose, fructose, and L-ascorbate were identified in all MAGs (**Figure 3** and **Supplemental Table 1, LCVA_199-201, LCVA_1491-1494**), as were complete pathways for glycolysis, pyruvate oxidation, and the non-oxidative phase of the pentose phosphate pathway. Mannose was noticeably absent, or below the limit of detection, in the metabolome of all the samples, but present in other BV-like samples that did not contain a high relative abundance of the candidate species (**Figure 4**). We found "*Ca.* Lachnocurva vaginae" to have the genetic capability to uptake and metabolize mannose (**LCVA_1184**), suggesting mannose could also be a carbohydrate source for the candidate species (**Figure 3**).

The D-lactate dehydrogenase gene (**LCVA_41**), but not an L-lactate dehydrogenase gene was also observed in all MAGs. This result was unexpected, as the production of D-lactate in the vaginal environment is thought to be a key and somewhat unique feature of certain *Lactobacillus* spp. (Witkin et al., 2013; France et al., 2016). Metabolomic data for each sample were explored for the presence of lactate to provide evidence supporting this genomic finding. While lactate was observed in most samples, its abundance was substantially lower than that from representative samples that were dominated by *Lactobacillus crispatus*, a known D-lactate producer (**Figure 4**). Thus, either the enzyme exhibits lower activity in "*Ca.* Lachnocurva vaginae" or is instead used in the reverse reaction to consume *Lactobacillus* spp.-produced D-lactate to produce pyruvate. This is a strategy described for another vaginal anaerobic Gram-negative coccus, *Veillonella parvula*, which is able to grow on lactate as the sole source of carbon (Gronow et al., 2010), and would possibly contribute to an increase in vaginal pH, thereby creating a more favorable growth environment. However, for lactate to convert to pyruvate in anaerobic bacteria, an electron-bifurcating complex with an electron transfer flavoprotein alpha and beta subunit (EtfAB) would be necessary to make the reaction favorable (Weghoff et al., 2015). An EtfAB homolog was not observed in the "*Ca.* Lachnocurva vaginae" cMAG, suggesting that another mechanism may be involved. Instead, succinate was the most abundant short chain fatty acid in the communities from which "*Ca.* Lachnocurva vaginae" MAGs originated (**Figure 4**). Not surprisingly, succinate has been associated with bacterial vaginosis, a condition in which "*Ca.* Lachnocurva vaginae" is often found (Srinivasan et al., 2015). Interestingly, all "*Ca.* Lachnocurva vaginae" MAGs lacked genes for all steps of the tricarboxylic acid cycle, indicating it is unable to produce succinate via this pathway. It is possible that "*Ca.* Lachnocurva vaginae" produces metabolite(s) other species in the community can convert into succinate or produces it via a yet to be

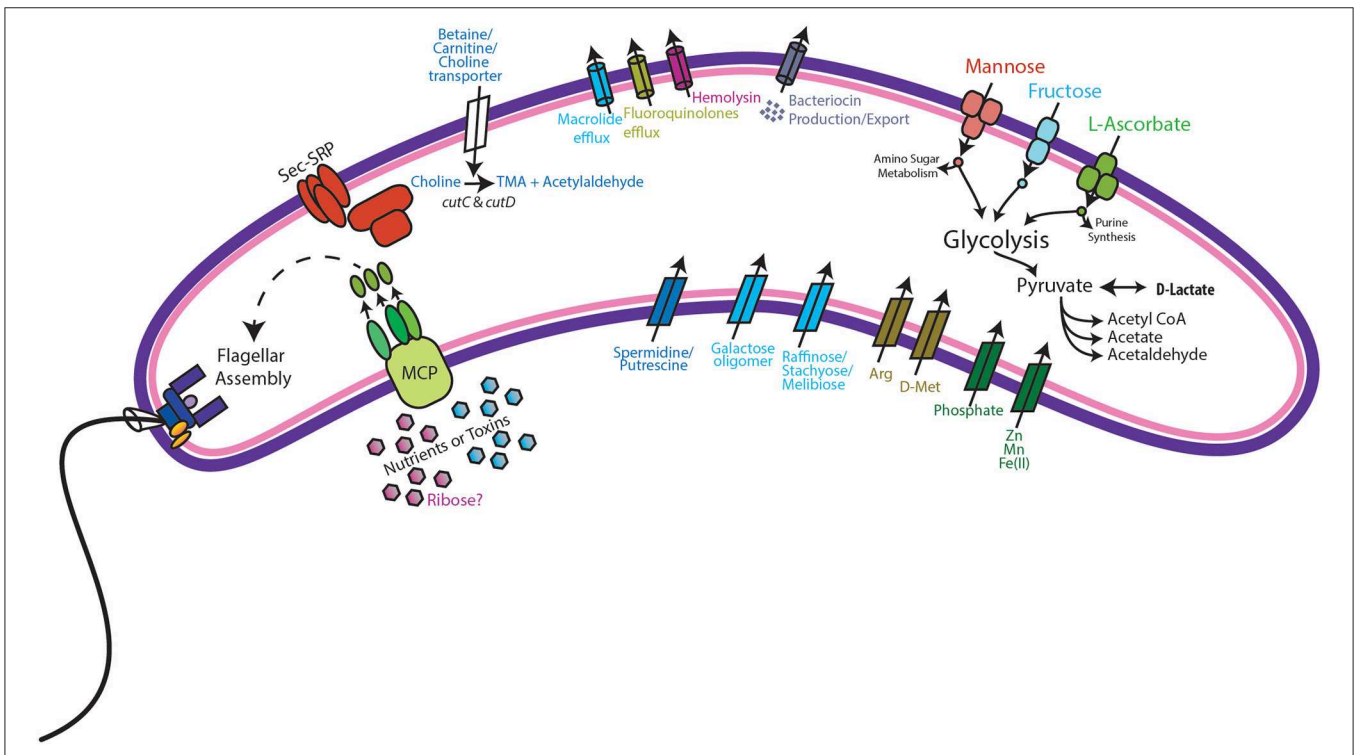

FIGURE 3 | Genomic features of "*Ca.* Lachnocurva vaginae". Genes coding for the following functions were observed: Methyl-accepting chemotaxis (MCP) and subsequent flagella assembly, Sec-SRP secretion systems, choline import, and metabolism to trimethylamine (TMA), bacteriocin production and export, mannose, fructose, and L-ascorbate transport and metabolism, and D-lactate dehydrogenase.

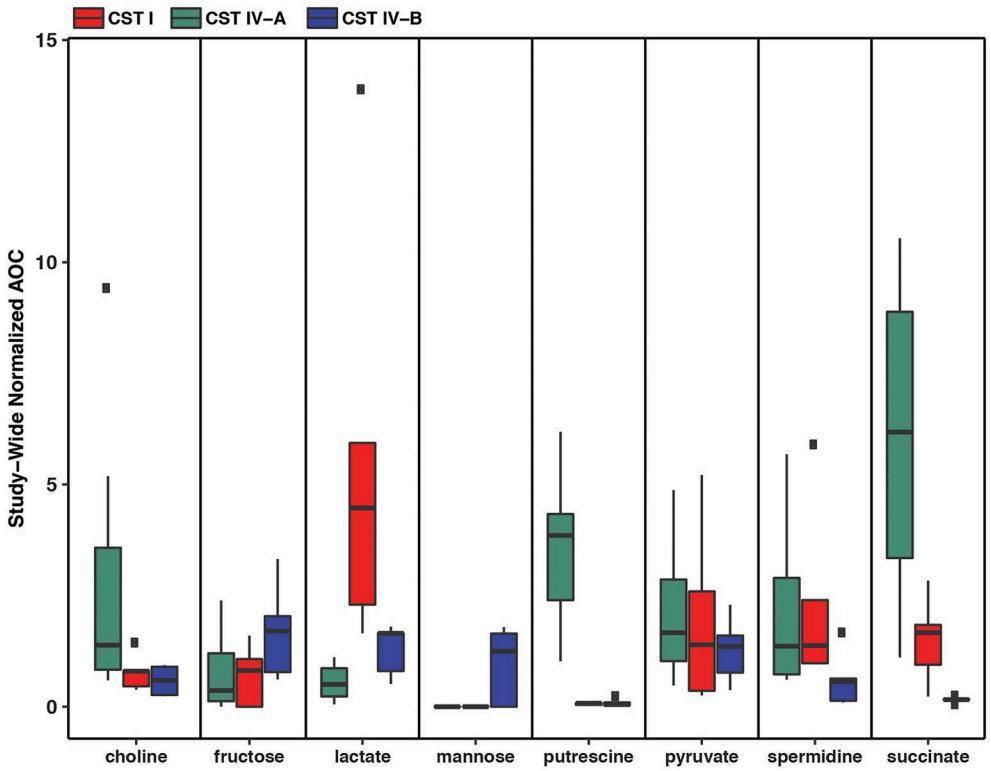

FIGURE 4 | Metabolomic signatures for biochemicals of interest in vaginal community state type (CST) IV-A (high abundance of "*Ca.* Lachnocurva vaginae"), CST I (dominated by *Lactobacillus crispatus*), and CST IV-B (high abundance of *Gardnerella vaginalis*).

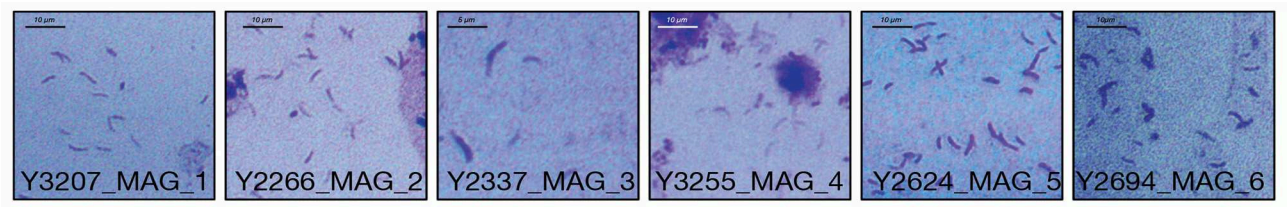

FIGURE 5 | Gram stains of LSVF samples containing >70% "*Ca.* Lachnocurva vaginae" and used in this study. Morphologically "*Ca.* Lachnocurva vaginae" is a curved rod. Images are labeled with the sample ID.

determined pathway. While "*Ca.* Lachnocurva vaginae" does not seem to be able to produce succinate, it has the genetic machinery to produce acetate from pyruvate (i.e., *ackA* and *pta* **LCVA_939** and **LCVA_1006**). We were, however, unable to detect acetate in the metabolome through the methods used, as it is too small of a molecule.

A full suite of genes required for flagella assembly was observed in all MAGs, as well as genes required for methyl-accepting chemotaxis (**LCVA_205, LCVA_992, LCVA_1053, LCVA_1122)** and for the downstream signal process that mediates flagellar response, *cheY* (**LCVA_362**, **LCVA_688)** (Bren and Eisenbach, 2000). Flagella have yet to be visually observed on "*Ca.* Lachnocurva vaginae" and were not observed in the source samples used for this study (**Figure 5**).

We found the cMAG and some MAGs to also include genes that encode protection against antibiotics including the *tetO* gene (**LCVA_1310**, also observed in MAG Y3255) and drug efflux pumps for macrolides (*macB* **LCVA_500**, also observed in MAGs Y2337 and Y2266) and fluoroquinolones (**LCVA_1202**, present in all MAGs). A gene for hemolysin, *tlyA,* was also observed in all MAGs (**LCVA_1031**), suggesting direct interaction between "*Ca.* Lachnocurva vaginae" and host tissues.

Additionally, choline transporters, as well as the *cutC* and *cutD* genes were detected in all MAGs (**LCVA_1194** and **LCVA_1193**, respectively). The *cut* genes metabolize choline and produce trimethylamine (TMA) (Martinez-Del Campo et al., 2015). Aside from transport of exogenous choline, another potential source of choline would be via phospholipase D

hydrolysis of phosphotidyl choline into choline as seen in gut bacteria (Chittim et al., 2019), however no genes encoding such activity were found in the cMAG of "*Ca.* Lachnocurva vaginae". Comparing the translated *cutC* amino acid sequence from "*Ca.* Lachnocurva vaginae" to those reported by Martinez-Del Campo et al. (2015), we observed three of the five active sites conserved in "*Ca.* Lachnocurva vaginae" (Cys489, Glu491, Gly821, data not shown) indicating that it is likely functional. TMA is one of the substances believed to be responsible for the fishy odor associated with BV (Brand and Galask, 1986; Wolrath et al., 2002), however it is rarely detected in metabolomics analyses as it is highly volatile, unless it is performed on freshly collected samples (Wolrath et al., 2002).

Two intact bacteriophages were detected in the cMAG (**Figure 2**). Partial matches to the same phages were observed in all MAGs (**Supplemental Image 1**). Best BLAST hits indicated that both bacteriophages belong to the Siphoviridae family of double-stranded viruses (NC_009552, NC_011167) which can exhibit both lytic and lysogenic phases. Bacteriophages of this family have previously been reported in the vaginal species *Lactobacillus jensenii* (Martin et al., 2010). Genomic islands were detected at multiple sites (**Supplemental Table 1** and **Figure 2**). Analysis of proteins encoded in the largest island (65.5 kb, coordinates 1,331,959–1,391,037, **LCVA_1280-1330**) showed similarity to proteins from *Shuttleworthia satelles*, and other taxa from the *Clostridiales* (**Supplemental Table 3** and **Supplemental Image 2**) and several transposons and integrases, as well as tetracycline resistance proteins (TetO, **LCVA_1310**), and ABC transporters. We ruled out the presence of genomic islands due to mis-assembly by analyzing the long-read coverage spanning the 5′ and 3′ ends of genomic islands. More than 100 long PacBio reads of a means size of 9 kb span the junctions of all detected genomic islands. Further, mean coverage of the genomic islands was similar to that of the mean cMAG coverage of 124X. Portions of these islands were observed in the other MAGs assembled in this study (**Supplemental Image 1** and **Supplemental Table 1**). The stringency of the mapping would not recover reads to regions of sequence diversity, thus these results may indicate this "*Ca.* Lachnocurva vaginae" likely contains multiple regions of genetic fluidity or diversity. However, the missing regions in MAGs may also be an artifact of metagenomic assembly.

CONCLUSION

We present here a circularized MAG of "*Ca.* Lachnocurva vaginae" and six MAGs of the candidate species, previously known as BVAB1, an important member of the human vaginal microbiota associated with bacterial vaginosis and other adverse outcomes. Short-read metagenomic assembles do not perform well and lead to sub-optimal assemblies with missing regions, whereas long read metagenomic assemblies are promising and can generate circularized metagenome assembled genomes, as shown in this study. Our inability to culture this bacterium has limited our understanding of its ecological role in the vaginal environment and its relation to women's health. We have shown that "*Ca.* Lachnocurva vaginae" has the genomic potential for motility and chemotaxis, and is likely capable of resisting several antibiotics via drug efflux systems. Our analysis indicates this candidate species may contribute to the fishy odor characteristic of bacterial vaginosis through the production of TMA from choline. This crucial genomic data could be used in metabolic modeling experiments to define a culture medium suitable for the cultivation of "*Ca.* Lachnocurva vaginae", a critical step to further understand its role in the vaginal microbiome.

DATA AVAILABILITY STATEMENT

Sequence data have been submitted to NCBI under BioProject PRJNA562728.

ETHICS STATEMENT

Samples used in this study were archived and de-identified cervicovaginal lavages and swabs. The samples were originally collected after obtaining informed consent by all participants, who also provided consent for storage of the samples and use in future research studies related to women's health. The original study was approved by the University of Maryland School of Medicine Institutional Review Board.

AUTHOR CONTRIBUTIONS

EM and CR performed the extraction and library preparations from the raw samples. JH, AM, LT, MF, and BM performed bioinformatics analyses to generate the MAGs. JH, RB, and JR designed the study. JH, MF, BM, and JR executed the study, contributed to the analyses. JH, RB, MF, BM, and JR wrote and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00117/full#supplementary-material

Supplemental Data Sheet 1 | Metagenomic library preparation modifications based on input amounts of DNA.

Supplemental Data Sheet 2 | Processing scripts for analysis.

Supplemental Data Sheet 3 | Taxonomic and phylogenetic placement of "Ca. Lachnocurva vaginae" (UAB071) from EZBioCloud TrueBacID system.

Supplemental Table 1 | List of genes annotated in the "Ca. Lachnocurva vaginae" cMAG. Excludes rRNA and tRNA.

Supplemental Table 2 | List of marker genes used in Family-level CheckM analysis and presence (1) or absence (0) in the "Ca. Lachnocurva vaginae" cMAG.

Supplemental Table 3 | Top 3 best BLAST matches to the genes of the "Ca. Lachnocurva vaginae" cMAG.

Supplemental Image 1 | Visualization of "Ca. Lachnocurva vaginae" whole genome alignments of cMAG and other MAGs.

Supplemental Image 2 | Circle plot of "Ca. Lachnocurva vaginae" cMAG with best BLAST hits indicated as either Clostridiales or not (Ring 5). If hit was within Clostridiales and >80% identical to cMAG, the specific taxon of close relatives was indicated in Ring 4. Included BLAST hits covered at least 80% of the query gene.

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Conflict of Interest: JR is co-founder of LUCA Biologics, a biotechnology company focusing on translating microbiome research into live biotherapeutics drugs for women's health.

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Pathobionts in the Vaginal Microbiota: Individual Participant Data Meta-Analysis of Three Sequencing Studies

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Sequencing studies have shown that optimal vaginal microbiota (VMB) are lactobacilli-dominated and that anaerobes associated with bacterial vaginosis (BV-anaerobes) are commonly present. However, they overlooked a less prevalent but more pathogenic group of vaginal bacteria: the pathobionts that cause maternal and neonatal infections and pelvic inflammatory disease. We conducted an individual participant data meta-analysis of three VMB sequencing studies that included diverse groups of women in Rwanda, South Africa, and the Netherlands (2,044 samples from 1,163 women in total). We identified 40 pathobiont taxa but only six were non-minority taxa (at least 1% relative abundance in at least one sample) in all studies: *Streptococcus* (54% of pathobionts reads), *Staphylococcus, Enterococcus*, *Escherichia/Shigella, Haemophilus*, and *Campylobacter*. When all pathobionts were combined into one bacterial group, the VMB of 17% of women contained a relative abundance of at least 1%. We found a significant negative correlation between relative abundances ($\rho = -0.9234$), but not estimated concentrations ($r = 0.0031$), of lactobacilli and BV-anaerobes; and a significant positive correlation between estimated concentrations of pathobionts and BV-anaerobes ($r = 0.1938$) but not between pathobionts and lactobacilli ($r = 0.0436$; although lactobacilli declined non-significantly with increasing pathobionts proportions). VMB sequencing data were also classified into mutually exclusive VMB types. The overall mean bacterial load of the ≥20% pathobionts VMB type (5.85 $\log_{10}$ cells/μl) was similar to those of the three lactobacilli-dominated VMB types (means 5.13–5.83 $\log_{10}$ cells/μl) but lower than those of the four anaerobic dysbiosis VMB types (means 6.11–6.87 $\log_{10}$ cells/μl). These results suggest that pathobionts co-occur with both lactobacilli and BV-anaerobes and do not expand as much as BV-anaerobes do in a dysbiotic situation. Pathobionts detection/levels were increased in samples with a Nugent score of 4–6 in both studies that conducted Nugent-scoring. Having pathobionts was positively associated with young age, non-Dutch origin, hormonal contraceptive use, smoking, antibiotic use in the 14 days prior to sampling,

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HIV status, and the presence of sexually transmitted pathogens, in at least one but not all studies; inconsistently associated with sexual risk-taking and unusual vaginal discharge reporting; and not associated with vaginal yeasts detection by microscopy. We recommend that future VMB studies quantify common vaginal pathobiont genera.

Keywords: vaginal microbiota, bacterial vaginosis, *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Escherichia*, pathobionts, ethnicity

INTRODUCTION

Understanding of the vaginal microbiota (VMB) has increased significantly since the turn of the century due to the increased availability of molecular laboratory techniques such as next-generation sequencing (van de Wijgert et al., 2014). Molecular studies have shown that most women have a VMB consisting of lactobacilli (most commonly *Lactobacillus crispatus* or *L. iners*), but that vaginal dysbiosis is highly prevalent worldwide (van de Wijgert and Jespers, 2017). The most common type of vaginal dysbiosis is anaerobic dysbiosis, which is characterized by a decrease of lactobacilli and an increase of fastidious anaerobes (van de Wijgert et al., 2014). Clinicians refer to symptomatic anaerobic dysbiosis as bacterial vaginosis (BV): patients typically have mild vaginal inflammation and a fishy-smelling vaginal discharge. It should be noted, however, that anaerobic dysbiosis is also frequently asymptomatic. The VMB of most women with anaerobic dysbiosis consists of a highly diverse mixture of fastidious anaerobes, usually including *Gardnerella vaginalis*. However, a substantial proportion of women with anaerobic dysbiosis are dominated by *G. vaginalis*, and this type of low diverse anaerobic dysbiosis is often overlooked. Recent studies have suggested that these women might be more difficult to treat, potentially due to the presence of a *G. vaginalis*-initiated vaginal mucosal biofilm (Verwijs et al., 2019a).

Another clinically relevant type of vaginal dysbiosis that has systematically been overlooked is the presence of bacterial pathobionts in the VMB (van de Wijgert and Jespers, 2017). Microbiologists define the term pathobiont as any potentially pathological organism which, under normal circumstances, lives as a non-harming symbiont. In the vaginal niche, this would include—among others—*Streptococcus agalactiae* (Group B streptococcus), *Staphylococcus aureus*, and species in the *Enterobacteriaceae* family. These bacteria have often been associated with maternal and neonatal infections (Cools et al., 2016; Black et al., 2018), as well as invasive infections in non-pregnant women such as pelvic inflammatory disease (Brunham et al., 2015). Some clinical researchers have hypothesized that a distinct type of vaginitis (desquamative inflammatory vaginitis), which is characterized by much more severe vaginal inflammation than BV and with desquamation of vaginal epithelial cells including parabasal cells (Sobel, 1994; Paavonen and Brunham, 2018), may be caused by pathobionts in the VMB (Donders et al., 2017). Two cases that appear to have been triggered by toxic shock syndrome toxin-1-producing *Staphylococcus aureus* strains have indeed been reported (Pereira et al., 2013). However, others believe that the condition is caused by estrogen deficiency or an immunologic disorder, and that vaginal dysbiosis develops secondarily (Sobel et al., 2011). A recent study found that most patients with vaginitis, parabasal cells, and lactobacilli-deficiency by microscopy did not have consistent VMB patterns by VMB sequencing (Oerlemans, 2019). We conclude that there is sufficient evidence to consider VMB pathobionts clinically relevant, but that the evidence-base related to both symptoms and complications is weak.

An important reason why the evidence-base is weak is because pathobionts are often not assessed properly. For example, neonatal invasive infection studies have focused on only one pathobiont (*S. agalactiae*) by culture (Kwatra et al., 2016), and VMB sequencing studies have systematically under-reported pathobionts. Authors of such studies typically use bioinformatical methods, such as hierarchical clustering, to summarize the sequencing data into a few VMB types. The first set of VMB types were published by Ravel et al. (2011) based on a study in asymptomatic American women: they referred to these as community state types I (*L. crispatus*-dominated), II (*L. gasseri*-dominated), III (*L. iners*-dominated), IV (diverse group), and V (*L. jensenii*-dominated (Ravel et al., 2011). The only pathobiont that was mentioned in this publication was *Streptococcus*, as one of the taxa included in the "diverse group." However, hierarchical clustering only takes relative abundances into account and not the pathogenic potential of individual bacteria. Pathobionts usually occur at lower levels than BV-anaerobes, but have a higher pathogenic potential: these lower levels may therefore be clinically relevant. Because pathobionts rarely dominate the VMB, samples that contain pathobionts are often classified based on the other bacteria that are also present in that sample. For example, a sample containing 70% *L. iners* and 30% *S. agalactiae* would be classified as community state type III (*L. iners*-dominated) in most studies.

We believe that this vaginal pathobionts knowledge gap is hampering clinical progress in the field. We therefore performed an individual participant data meta-analysis of three VMB sequencing studies that enrolled diverse groups of women in Rwanda, South Africa, and the Netherlands (2,044 samples from 1,163 women in total), with as main aim to describe the presence and levels of all pathobionts identified in the sequencing data, their correlations with lactobacilli and BV-anaerobes, and their associations with participant sociodemographic, behavioral, and clinical/laboratory characteristics.

MATERIALS AND METHODS

Studies Included in the Meta-Analysis

We performed an individual participant data meta-analysis of three VMB sequencing studies that were conducted in three different countries to account for regional and ethnic differences

in VMB composition: (1) a clinical trial of intermittent oral metronidazole or vaginal probiotic use in Kigali, Rwanda (referred to as the Rwanda VMB study); (2) the VMB sub-study of the South African HPV in Africa Research Partnership (HARP) study in Johannesburg, South Africa; and (3) the VMB sub-study of the Healthy Life in an Urban Setting (HELIUS) study in Amsterdam, the Netherlands.

The Rwanda VMB study screened HIV-negative, non-pregnant, pre-menopausal women at high risk of sexually transmitted infections (STIs) for BV (van de Wijgert et al., 2020a). Women with BV were treated with metronidazole for seven days, and when cured of BV and other urogenital infections, were randomized to no intervention, or intermittent use of oral metronidazole or two different lactobacilli-containing vaginal probiotics for 2 months. The lactobacilli contained in the vaginal probiotics did not include any naturally occurring vaginal lactobacilli. Women were sampled at screening (start of BV/urogenital infection treatment, if applicable), enrollment (start of the interventions), Day 7, Month 1, Month 2 (cessation of the interventions), and Month 6. The study found that all three interventions were safe and affected naturally occurring lactobacilli and BV-anaerobes (in favor of the lactobacilli, particularly *L. iners*), but not pathobionts. However, to avoid any bias in this meta-analysis due to exposure to interventions, we conducted analyses that included lactobacilli and BV-anaerobes levels on VMB data that were not influenced by the interventions ($N = 366$ of 629 samples): data from samples collected in all randomization groups at the screening visit prior to any treatments (if applicable) and at the Month 6 visit (4 months after cessation of the interventions), as well as samples collected in the no intervention group at the Month 1 and Month 2 visits.

The VMB sub-study of the HARP study was a nested case-control study within a prospective cohort study conducted in Johannesburg, South Africa (van de Wijgert et al., 2020b). The study enrolled HIV-positive women and investigated the associations of VMB composition with high-risk human papillomavirus (hrHPV) and cervical intraepithelial neoplasia (CIN) acquisition, clearance, and/or persistence. All but one participant were of sub-Saharan African origin. Samples for VMB analyses were collected at baseline ($N = 445$) and at endline ($N = 414$), a median of 16 months later. The study concluded that hrHPV infection (and/or increased sexual risk-taking) likely causes anaerobic vaginal dysbiosis, but that a bidirectional relationship is also possible. Furthermore, in this population, dysbiosis did not increase CIN2+ risk, but CIN2+ increased dysbiosis risk. Since the study did not include an intervention, we used all available VMB data for the analyses presented in this paper.

The HELIUS study is a large, multi-ethnic cohort study in Amsterdam, the Netherlands (Snijder et al., 2017). Sampling was stratified by ethnic group and included the six largest ethnic groups in the city (Dutch, African Surinamese, South-Asian Surinamese, Turkish, Moroccan, and Ghanaian). In a sub-sample, a cross-sectional study on the association of ethnicity with VMB composition was performed (Borgdorff et al., 2017). For this sub-study, vaginal samples of 546 pre-menopausal women were sequenced. The most prevalent VMB composition in ethnically Dutch women was a *L. crispatus*-dominated VMB, in African Surinamese and Ghanaian women a polybacterial *G. vaginalis*-containing VMB, and in the other ethnic groups a *L. iners*-dominated VMB. This study did not include an intervention either, and we therefore used all available VMB data for the analyses presented in the current paper.

Sequencing and Other Laboratory Methods

All three studies extracted DNA from vaginal swabs and conducted 16S rRNA gene sequencing of the V3–V4 variable regions on Illumina platforms (San Diego, CA, USA) as described by Fadrosh et al. (2014). Standard diagnostic tests were used to test for STIs (all three studies), and BV and vulvovaginal candidiasis (Rwanda VMB and HARP studies only). There were some differences in sequencing and diagnostic methods used, as outlined in **Table 1**. Because of these differences, we conducted all analyses on each study separately as well as the three studies combined.

The BactQuant 16S rRNA gene qPCR (Liu et al., 2012) was only done in the Rwanda VMB study ($N = 379$, of which 158 samples were not influenced by the interventions). The 16S rRNA gene concentration per sample was used to convert the relative abundances of that sample into estimated concentrations as previously described (van de Wijgert et al., 2020a). We used both relative abundances as well as estimated concentrations for the Rwanda VMB study, but only had relative abundances for the other two studies.

Sequencing Data Processing

The 16S rRNA gene sequencing and initial data processing yielded two-dimensional tables with samples and bacterial taxa on the axes, and relative abundances in the cells, for each of the three studies. DADA2 assigns amplicon sequence variants (ASVs) to taxa (in the Rwanda VMB study) and Swarm and USEARCH assign them to operational taxonomic units (OTUs; in the HARP and HELIUS studies, respectively). Details on quality control and cleaning of reads, taxonomic assignments, conversion of read counts into relative abundances, and rarefaction are summarized in **Table 1** and explained in the original publications. The three study-specific relative abundance tables were combined into a single table, and all subsequent data processing steps were redone for the combined table (i.e., are slightly different from the original publications) to ensure that they were identical for the three studies.

Data reduction for biostatistical modeling was done in three different ways. First, the Simpson diversity index (1-D) was calculated for each sample, ranging from 0 (no diversity) to 1 (infinite diversity). Second, each ASV/OTU was assigned to one of four "bacterial groups" based on the published literature (**Supplementary Material 1**) as follows: (1) lactobacilli; (2) BV-anaerobes; (3) pathobionts; and (4) a rest group called "other bacteria" (which contained mostly skin and Bifidobacteria). Pathobionts were defined as all bacterial taxa that have been reported in the literature as having been associated with invasive disease, and are not typically associated with BV; we also included STI pathogens in this category because their mean relative abundances were too low to justify a separate bacterial

TABLE 1 | Main study characteristics of the three studies.

	VMB Rwanda[a]	HARP South Africa[a]	HELIUS Netherlands[a]
Number of women	162 (68 randomized)	455	546
Number of baseline samples	162	445	546
Total number of samples	629 (366 not influenced by interventions)	869	546
Years samples collected	2015–2016	2011–2014	2011–2013
Year samples sequenced	2017	2016	2014
Study location	Kigali	Johannesburg	Amsterdam
Study population	HIV-negative women with high sexual risk	HIV-positive women on cART (2/3) or not on cART (1/3)	Random samples of city population, stratified by ethnic group
Sequencing methods			
Sequencing laboratory	University of Liverpool, Center for Genomic Research	University of Liverpool, Center for Genomic Research	Amsterdam University Medical Center, location VUmc
Type of vaginal samples	Vaginal swab frozen dry the same day at −80°C and shipped to Liverpool on dry ice.	Vaginal swab in Boonfix, stored and shipped to Liverpool at room temperature.	Vaginal swab frozen dry at −20°C after at most 6 days at 2–8°C.
DNA extraction method	Lysozyme lysis with bead-beating, followed by Qiagen DNeasy Blood and Tissue kit	Lysozyme lysis (no bead-beating) followed by Qiagen DNeasy Blood and Tissue kit	Lysozyme, mutanolysin, lysostaphin lysis with bead-beating followed by proteinase K/RNase A and ChemaGen extraction robot
16S sequencing platform	Illumina HiSeq (rapid mode; 2 × 300bp)	Illumina HiSeq (rapid mode; 2 × 300bp)	Illumina MiSeq (2 × 300bp)
16S variable region	V3–V4	V3–V4	V3–V4
Taxonomic assignment and unit of analysis	DADA2 v1.4.0 ASV	Swarm v2.1.13 OTU	USEARCH v5.2.236 OTU
Reference databases	Silva v128, NCBI, Vaginal 16S rDNA Reference Database by Srinivasan et al.	Silva v128, NCBI	GreenGenes v13.8, NCBI, Vaginal 16S rDNA Reference Database by Srinivasan et al.
Rarefaction	At 1,111 reads	At 1,039 reads	None, but all samples with <100 reads discarded
Mean read count per sample	374,543	122,490	25,392
Unique ASVs/OTUs[b]	401 (177 non-minority)	1,981 (246 non-minority)	455 (141 non-minority)
BactQuant assay done[c]	Yes	No	No
Diagnostic tests			
Bacterial vaginosis	Nugent, Amsel	Nugent	Not done
Yeasts	Wet mount microscopy	Gram stain microscopy	Not done
Trichomonas vaginalis	Wet mount microscopy and InPouch culture	APTIMA Combo 2 PCR	APTIMA PCR
Chlamydia trachomatis and *Neisseria gonorrhoeae*	Presto or GeneXpert real-time PCR	APTIMA Combo 2 PCR	APTIMA Combo 2 PCR
Syphilis	Spinreact RPR + TPHA	Immutrep RPR + TPHA	Not done
HIV-1	National algorithm (serology)	National algorithm (serology)	Not done
Herpes simplex type 2	Kalon IgG2 ELISA	Kalon IgG2 ELISA	Not done
HPV	Not done	Digene HC-II, CareHPV, INNO-LiPA HPV Genotyping Extra	SPF10-PCR-DEIA/LiPA25 system version 1

ASV, Amplicon Sequence Variant; cART, combination antiretroviral therapy for HIV; ELISA, enzyme-linked immunosorbent assay; HIV, human immunodeficiency virus; HPV, human papillomavirus; IgG, Immunoglobulin G; NCBI, National Center for Biotechnology Information; OTU, Operational Taxonomic Unit; PCR, polymerase chain reaction; RPR, Rapid Plasma Reagin test; TPHA, Treponema pallidum Hemagglutination Assay.

[a]Details of procedures and supplies/databases used can be found in the original publications (Borgdorff et al., 2017; van de Wijgert et al., 2020a,b).

[b]Non-minority is defined as at least 1% in at least one sample. The number of minority OTUs was higher in the HARP study than in the other two studies because OTUs matching to the same or overlapping taxa were not merged. This has, however, not affected the analyses in this paper, which were based on bacterial groups and a select number of non-minority taxa.

[c]BactQuant is a commercial assay that quantifies 16S genes in a sample by quantitative PCR (Liu et al., 2012).

group. For each sample, relative abundances of ASVs/OTUs belonging to the same bacterial group were summed. This resulted in four relative abundances (one for each bacterial group) per sample, which sum to one in total. For example, one sample could contain 0.5 (50%) lactobacilli reads, 0.4 (40%) BV-anaerobes reads, 0.08 (8%) pathobionts reads, and 0.02 (2%)

other bacteria reads. Third, we classified samples into nine VMB types (with each sample assigned to only one VMB type): (1) *Lactobacillus iners*-dominated (Li; ≥75% relative abundance of lactobacilli of which *L. iners* was the most common); (2) *L. crispatus*-dominated (Lcr; also ≥75% lactobacilli of which *L. crispatus* was the most common); (3) dominated by other *Lactobacillus* species (Lo; also containing ≥75% lactobacilli); (4) lactobacilli and anaerobes (LA; ≥25% lactobacilli with the remainder BV-anaerobes); (5) high diversity BV-anaerobes with ≥10% *G. vaginalis* presence (BV_GV); (6) high diversity BV-anaerobes with <10% *G. vaginalis* presence (BV_noGV); (7) *G. vaginalis*-dominated (GV; *G. vaginalis* ≥50%); (8) substantial presence of pathobionts (PB; ≥20% pathobiont taxa); and (9) *Bifidobacterium*-dominated (BD; ≥50% Bifidobacteria).

Statistical Analyses and Figures

Statistical analyses were performed using Stata version 13 (StataCorp, College Station, TX, USA) and R version 3.2.3 (R foundation for Statistical Computing 2016, Vienna, Austria). All analyses were cross-sectional, sometimes including samples collected at baseline only (one sample per woman) and sometimes including all samples (in case of the Rwanda VMB and HARP studies, more than one sample per woman). Women in the Rwanda VMB study were exposed to antibiotic and/or probiotic interventions, and samples that could potentially have been influenced by these interventions were excluded from most analyses as described above and as indicated in the tables and text. Unadjusted differences between groups of interest were tested by Fisher's exact test for binary variables, Chi-squared test for categorical variables, and Kruskal-Wallis test for continuous variables. Pathobiont levels (relative abundances or estimated concentrations) were correlated with those of other bacterial groups or taxa by Spearman's rank correlation when all samples were included and by Pearson's correlation coefficient when samples with <1% pathobionts were excluded. To assess sociodemographic, behavioral, and clinical determinants of pathobionts detection (≥1% compared to <1%) and levels, we used unadjusted logistic regression models for analyses including one sample per woman, and Kruskal-Wallis tests for analyses of pathobionts levels that included all samples. The heatmap was made with the *gplots* package in R (Warnes et al., 2016), bar charts in Stata, and correlation matrices with the *corrplot* package in R (Taiyun, 2019).

RESULTS

Participant Characteristics

The median age in the three studies combined was 30 years (interquartile range 26–34) and most women were non-pregnant by design (**Table 2**). The majority of women in the Rwanda VMB study were at high risk of HIV/STI by design: 93.2% reported two or more partners in the month prior to the baseline visit and 76.6% reported no or inconsistent condom use. Much lower proportions of women in the HARP study reported these current sexual risk behaviors but they were all HIV-positive by design. Women in the HELIUS study were not selected based on sexual risk or HIV-status. The proportions of women reporting sexual risk behaviors could be considered average for a young, urban, Dutch population, but with differences by ethnic group: proportions were highest in women of Dutch origin, followed by Dutch women of sub-Saharan African (African Surinamese or Ghanaian) origin, and Dutch women of Turkish, Moroccan, or South Asian Surinamese origin. Current hormonal contraceptive use varied substantially between studies and ethnic groups, as did current smoking habits. Almost half of the women (39.4–45.1%; not assessed in the HARP study) reported current urogenital symptoms, but none of them had sought care for them. Laboratory-confirmed viral and bacterial STI prevalences were high in the Rwanda VMB study and low in the HELIUS study, whereas viral STI prevalences were high and bacterial STI prevalences low in the HARP study (consistent with high past but low current sexual risk). Antibiotic use in the 2 weeks prior to baseline was rare.

Overall Vaginal Microbiota Characteristics

A heatmap of key taxa for all 2,044 samples from all three studies combined is shown in **Supplementary Material 2**, **Table S1**. In **Table 3**, **Figure 1**, VMB study samples were stratified by exposure to interventions, and HELIUS study samples by ethnic group. Mean Simpson diversity indexes and mean relative abundances of bacterial groups, and key taxa within these groups, differed significantly between the three studies and these pre-specified strata within studies (**Table 3**, **Figure 1A**). By far the most common bacterial groups in all studies and strata were the lactobacilli and BV-anaerobes, with mean relative abundances ranging from 0.46 to 0.73 and 0.25 to 0.49, respectively. The differences between studies and strata were as expected, with lower lactobacilli and higher BV-anaerobes proportions in women with higher sexual risk profiles and/or STI exposures and in women of sub-Saharan African ethnicities. In contrast, Rwanda VMB study participants who had recently been exposed to antibiotic/probiotic interventions had higher lactobacilli and lower BV-anaerobes proportions. The differences in *L. crispatus* mean relative abundance were especially striking, ranging from only 0.03 in the Rwanda VMB study samples that were not influenced by interventions to 0.38 in the HELIUS samples from women of Dutch origin. Mean relative abundances for pathobionts and the "other bacteria" group were low in all studies and strata, ranging from 0.01 to 0.07 and 0 to 0.05, respectively. The mean pathobionts relative abundance did not show a clear pattern between studies and strata but was lowest in the HELIUS women of Dutch origin. Estimated concentrations were only available for the Rwanda VMB study, and mean estimated concentrations in $\log_{10}$ cells/μl were 5.12 for lactobacilli (mostly consisting of *L. iners*), 5.17 for BV-anaerobes, 2.18 for pathobionts, and 1.92 for other bacteria in samples not influenced by interventions (**Table 3**). The mean pathobionts estimated concentration was therefore 871 times lower than the mean lactobacilli estimated concentration, and 977 times lower than the mean BV-anaerobes estimated concentration.

The VMB types for all samples combined (N = 2,044) were distributed as follows: Li 31.6%, Lcr 10.5%, Lo 2.4%, LA 15.8%, BV_GV 20.3%, BV_noGV 5.5%, GV 8.3%, PB 5.2%,

TABLE 2 | Baseline characteristics by study, and by ethnic group within the HELIUS study.

	VMB Rwanda (*N* = 162)	HARP South Africa (*N* = 455)	HELIUS Netherlands Sub-Saharan African origin[a] (*N* = 183)	HELIUS Netherlands Turkish, Moroccan, South-Asian origin[b] (*N* = 264)	HELIUS Netherlands Dutch origin (*N* = 99)	All studies (*N* = 1,163)	*P*-value[c]
Age (median, IQR)	30 (27–34)	34 (30–39)	26 (22–30)	27 (23–31)	26 (22-30)	30 (26–34)	<0.001
Currently pregnant (*n*%)[d]	6 (3.7)	0	0	0	0	6 (0.5)	<0.001
Contraceptive use if not pregnant (*n*%)	*N = 156*					*N = 1,154*	
- None or condom use only	64 (41.0)	340 (74.7)	97 (53.6)	148 (56.3)	27 (27.7)	676 (58.6)	<0.001
- Oral contraception[e]	16 (10.3)	23 (5.1)	61 (33.7)	88 (33.5)	46 (46.5)	234 (20.3)	
- Progestin-only injectable	45 (28.9)	86 (18.9)	1 (0.6)	0	1 (1.0)	133 (11.5)	
- Progestin-only implant	28 (18.0)	6 (1.3)	1 (0.6)	5 (1.9)	0	40 (3.5)	
- Any intrauterine device[f]	3 (1.9)	0	20 (11.1)	20 (7.6)	20 (20.2)	63 (5.5)	
- Contraceptive ring	0	0	1 (0.6)	2 (0.8)	5 (5.1)	8 (0.7)	
Currently using hormonal contraception or is pregnant (*n*%)[g]	95 (58.6)	115 (25.3)	*N = 161* 64 (39.8)	*N = 243* 95 (39.1)	*N = 79* 52 (65.8)	*N = 1,100* 421 (38.3)	<0.001
Current smoker (*n*%)	NA[h]	25 (5.5)	27 (14.8)	68 (25.9)	30 (30.3)	*N = 999* 150 (15.0)	<0.001
Used any antibiotic in past 14 days (*n*%)	0	NA	8 (4.4)	7 (2.7)	4 (4.0)	*N = 706* 19 (2.7)	0.024
Reported any type of vaginal cleansing (n%)[i]	*N = 64* 11 (17.2)	179 (39.3)	47 (25.8)	72 (27.7)	18 (18.2)	*N = 1,064* 327 (30.7)	<0.001
Number of sex partners in period prior to sampling[j]							
- None	0	77 (17.0)	54 (29.7)	103 (39.0)	23 (23.2)	257 (22.1)	<0.001
- One	11 (6.8)	352 (77.5)	100 (55.0)	142 (53.8)	51 (51.5)	656 (56.4)	
- Two or more	151 (93.2)	25 (5.5)	28 (15.4)	19 (7.2)	25 (25.3)	248 (21.3)	
Frequency of condom use (*n*%)[k]							
- Never	9 (5.6)	24 (5.3)	60 (32.0)	99 (37.6)	43 (43.4)	235 (20.2)	<0.001
- Inconsistent	115 (71.0)	129 (28.4)	42 (23.1)	39 (14.8)	26 (26.3)	351 (30.2)	
- Consistent	38 (23.5)	221 (48.6)	26 (14.3)	22 (8.4)	7 (7.1)	314 (27.1)	
- NA (no sexual partner)	0	81 (17.8)	54 (29.7)	103 (39.2)	23 (23.2)	262 (22.5)	
Reported any urogenital symptoms (*n*%)	73 (45.1)	NA	73 (39.9)	117 (44.3)	39 (39.4)	*N = 708* 302 (42.7)	0.644
Reported unusual vaginal discharge (*n*%)	21 (13.0)	NA	25 (13.7)	57 (21.6)	16 (16.2)	*N = 708* 119 (16.8)	0.069
Positive HIV test (*n*%)	16 (9.9)	455 (100)	0	0	0	471 (40.5)	<0.001
Yeasts by microscopy (*n*%)	*N = 140* 14 (10.0)	*N = 445* 34 (7.6)	NA	NA	NA	*N = 585* 48 (8.2)	0.380
Trichomonas vaginalis by culture/NAAT (*n*%)	*N = 138* 17 (12.3)	71 (15.6)	6 (3.3)	1 (0.4)	0	*N = 1,139* 95 (8.3)	<0.001
Chlamydia trachomatis by NAAT (*n*%)	*N = 139* 30 (21.6)	24 (5.3)	10 (5.5)	6 (2.3)	2 (2.0)	*N = 1,140* 72 (6.3)	<0.001

(Continued)

TABLE 2 | Continued

	VMB Rwanda (*N* = 162)	HARP South Africa (*N* = 455)	HELIUS Netherlands Sub-Saharan African origin[a] (*N* = 183)	HELIUS Netherlands Turkish, Moroccan, South-Asian origin[b] (*N* = 264)	HELIUS Netherlands Dutch origin (*N* = 99)	All studies (*N* = 1,163)	*P*-value[c]
Neisseria gonorrhoeae by NAAT (*n*%)	*N = 139* 18 (13.0)	10 (2.2)	0	0	0	*N = 1,140* 28 (2.5)	<0.001
Mycoplasma genitalium by NAAT (*n*%)	NA	38 (8.4)	NA	NA	NA	NA	NA
Active syphilis by serology (*n*%)	13 (8.0)	*N = 452* 3 (0.7)	NA	NA	NA	*N = 614* 16 (2.6)	<0.001
Herpes simplex virus type 2 by serology (*n*%)	109 (67.3)	*N = 453* 432 (95.4)	NA	NA	NA	*N = 615* 541 (88.0)	<0.001
Any high-risk HPV by PCR (*n*%)	NA	363 (79.8)	56 (30.6)	66 (25.0)	41 (41.4)	*N = 1,001* 526 (52.6)	<0.001

HPV, human papilloma virus; IQR, inter-quartile range; NA, not applicable; NAAT, nucleic acid amplification test; PCR polymerase chain reaction.

The unit of analysis is one sample (collected at baseline) per woman.

[a] *Included Dutch women of African Surinamese and Ghanaian origin.*

[b] *Included Dutch women of South-Asian Surinamese, Moroccan, and Turkish origin.*

[c] *Using the Fisher's exact test for binary variables, the Chi-squared test for categorical variables, and Kruskal-Wallis test for continuous variables.*

[d] *Pregnant women were not eligible for enrollment in any of the studies, but six women screened for the Rwanda VMB study were pregnant when the baseline vaginal swabs were taken, prior to enrollment.*

[e] *Includes combined and progestin-only oral contraception.*

[f] *In the VMB and HARP studies, only copper intrauterine devices were used. In the HELIUS study, women may have used either a copper or hormone-containing intrauterine device. One HELIUS participant used both an intrauterine device and a pill and she is included here.*

[g] *Excluding HELIUS participants who used intrauterine devices (including the participant who used an intrauterine device and a pill).*

[h] *This question was not asked in the Rwanda VMB study but we know from previous studies in the same population that women rarely smoke.*

[i] *In the Rwanda VMB study, only participants who were subsequently randomized to the interventions were asked this question.*

[j] *The recall period was 1 month in the Rwanda VMB study, 3 months in the HARP study, and 6 months in the HELIUS study. In the Rwanda VMB study, the frequencies were as follows for 12 months recall: no partners 0%, one partner 2.5%, and two or more partners 97.5%.*

[k] *The recall period was 2 weeks in the Rwanda VMB study, 3 months in the HARP study, and 6 months in the HELIUS study.*

TABLE 3 | Overview of VMB composition characteristics by study, stratified by intervention exposure (VMB Rwanda) and ethnic group (HELIUS).

	VMB Rwanda (not influenced by interventions)[a]	VMB Rwanda (influenced by interventions)[a]	HARP South Africa	HELIUS Sub-Saharan African origin[b]	HELIUS Turkish, Moroccan, South-Asian origin[c]	HELIUS Dutch origin	All groups	*P*-value[d]
Samples with relative abundance data available (*N*)	366	263	869	183	264	99	2,044	NA
Samples with estimated concentration data available (*N*)	158	221	0	0	0	0	379	
Nugent score category (n %)[e]:	*N = 231*	*N = 227*	*N = 445*	NA	NA	NA	*N = 903*	
- 0–3	79 (34.2)	111 (48.9)	151 (33.9)				341 (37.8)	<0.001
- 4–6	27 (11.7)	40 (17.6)	102 (22.9)				169 (18.7)	
- 7–10	125 (54.1)	76 (33.5)	192 (43.2)				393 (43.5)	
Simpson diversity index (1-D; mean, 95% CI)[f]	0.53 (0.50–0.56)	0.40 (0.36–0.44)	0.54 (0.52–0.56)	0.39 (0.35–0.43)	0.34 (0.31–0.38)	0.32 (0.27–0.37)	0.47 (0.46–0.48)	<0.001
VMB types (*n* %)[g]:								
- *L. iners*-dominated (Li)	119 (32.5)	128 (48.7)	237 (27.3)	49 (26.8)	89 (34.6)	24 (24.5)	646 (31.6)	<0.001
- *L. crispatus*-dominated (Lcr)	10 (2.7)	7 (2.7)	65 (7.5)	33 (18.0)	61 (23.7)	38 (38.8)	214 (10.5)	
- Dominated by other lactobacilli (Lo)	18 (4.9)	10 (3.8)	5 (0.6)	5 (2.7)	5 (2.0)	6 (6.1)	49 (2.4)	
- Lactobacilli plus anaerobes (LA)	45 (12.3)	41 (15.6)	188 (21.6)	12 (6.6)	28 (10.9)	8 (8.2)	322 (15.8)	
- Polybacterial *G. vaginalis*-containing (BV_GV)	102 (27.9)	36 (13.7)	215 (24.7)	38 (20.8)	15 (5.8)	8 (8.2)	414 (20.3)	
- Polybacterial with little *G. vaginalis* (BV_noGV)	21 (5.7)	2 (0.8)	75 (8.6)	4 (2.2)	10 (3.9)	0	112 (5.5)	
- *G. vaginalis*-dominated (GV)	26 (7.1)	15 (5.7)	49 (5.6)	35 (19.1)	32 (12.5)	13 (13.3)	170 (8.3)	
- Pathobionts-containing (PB)	25 (6.8)	24 (9.1)	33 (3.8)	7 (3.8)	17 (6.6)	1 (1.0)	107 (5.2)	
Relative abundance of VMB bacterial groups (mean, 95% CI):								
- Total lactobacilli	0.46 (0.42–0.51)	0.63 (0.59–0.68)	0.47 (0.44–0.50)	0.52 (0.45–0.58)	0.64 (0.59–0.69)	0.73 (0.65–0.80)	0.53 (0.51–0.55)	<0.001
- *L. iners*	0.36 (0.32–0.40)	0.53 (0.48–0.58)	0.37 (0.34–0.39)	0.30 (0.25–0.36)	0.34 (0.29–0.39)	0.28 (0.20–0.35)	0.37 (0.36–0.39)	<0.001
- *L. crispatus*	0.03 (0.02–0.05)	0.04 (0.02–0.06)	0.08 (0.07–0.10)	0.18 (0.13–0.23)	0.24 (0.19–0.28)	0.38 (0.29–0.46)	0.11 (0.10–0.12)	<0.001
- Other lactobacilli	0.07 (0.05–0.09)	0.07 (0.04–0.09)	0.02 (0.01–0.02)	0.04 (0.02–0.06)	0.06 (0.04–0.08)	0.08 (0.04–0.11)	0.04 (0.04–0.05)	<0.001
- Total BV-anaerobes	0.48 (0.44–0.52)	0.30 (0.25–0.34)	0.49 (0.46–0.52)	0.44 (0.37–0.50)	0.27 (0.22–0.31)	0.25 (0.17–0.32)	0.42 (0.40–0.44)	<0.001
- *G. vaginalis*	0.18 (0.16-0.20)	0.15 (0.12–0.17)	0.17 (0.15–0.18)	0.23 (0.19–0.27)	0.16 (0.12–0.19)	0.16 (0.10–0.21)	0.17 (0.16–0.18)	<0.001
- *A. vaginae*	0.03 (0.02-0.04)	0.01 (0.01–0.02)	0.03 (0.02–0.03)	0.09 (0.07–0.11)	0.04 (0.03–0.05)	0.06 (0.03–0.08)	0.04 (0.03–0.04)	<0.001
- *Prevotella* species	0.07 (0.06–0.08)	0.03 (0.02–0.04)	0.06 (0.06–0.07)	0.02 (0.01–0.03)	0.02 (0.01–0.03)	0.01 (0–0.01)	0.05 (0.04–0.05)	<0.001
- Other BV-anaerobes	0.20 (0.18–0.23)	0.10 (0.08–0.12)	0.23 (0.22–0.25)	0.10 (0.07–0.12)	0.05 (0.04–0.06)	0.03 (0.02–0.04)	0.16 (0.15–0.17)	<0.001
- Total pathobionts	0.05 (0.03–0.07)	0.07 (0.04–0.09)	0.03 (0.02–0.04)	0.03 (0.01–0.05)	0.04 (0.02–0.06)	0.01 (0–0.03)	0.04 (0.03–0.04)	<0.001
- *Streptococcus* species	0.04 (0.02–0.05)	0.05 (0.03–0.07)	0.02 (0.01–0.03)	0.02 (0.01–0.04)	0.03 (0.02–0.04)	0 (0–0.01)	0.03 (0.02–0.03)	<0.001
- *Staphylococcus* species	0 (0–0.01)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0.01 (0–0.03)	0 (0–0)	<0.001
- *Escherichia/Shigella* species	0.01 (0–0.02)	0.01 (0–0.02)	0 (0–0)	0 (0–0.01)	0 (0–0.01)	0 (0–0)	0 (0–0.01)	<0.001
- Other pathobionts[h,i]	0 (0–0)	0.01 (0–0.01)	0 (0–0)	0 (0–0)	0.01 (0–0.01)	0 (0–0)	0 (0–0)	<0.001
- Total other bacteria	0 (0–0)	0 (0–0.01)	0.01 (0.01–0.01)	0.01 (0.01–0.02)	0.05 (0.03–0.07)	0.01 (0.00–0.03)	0.01 (0.01–0.02)	<0.001

(Continued)

TABLE 3 | Continued

	VMB Rwanda (not influenced by interventions)[a]	VMB Rwanda (influenced by interventions)[a]	HARP South Africa	HELIUS Sub-Saharan African origin[b]	HELIUS Turkish, Moroccan, South-Asian origin[c]	HELIUS Dutch origin	All groups	*P*-value[d]
Relative abundance total pathobionts categorical (n %):								
<1% of reads	467 (74.2)	190 (72.2)	726 (83.5)	162 (88.5)	203 (76.9)	87 (87.9)	1,645 (80.5)	<0.001
1%–<10%	87 (13.8)	36 (13.7)	88 (10.1)	14 (7.7)	42 (15.9)	10 (10.1)	241 (11.8)	
10%–<20%	26 (4.1)	13 (4.9)	22 (2.5)	0	2 (0.8)	1 (1.0)	51 (2.5)	
20%–<50%	24 (3.9)	10 (3.8)	18 (2.1)	2 (1.1)	10 (3.8)	0	54 (2.6)	
50% or more	25 (4.0)	14 (5.3)	15 (1.7)	5 (2.7)	7 (2.7)	1 (1.0)	53 (2.6)	
Estimated concentration of VMB bacterial groups in log_{10} cells/μl (mean, 95% CI):								
- Total lactobacilli	5.12 (4.97–5.27)	4.84 (4.58–5.10)	NA	NA	NA	NA	5.32 (5.15–5.49)	0.005
- *L. iners*	4.78 (4.60–4.97)	4.56 (4.25–4.87)					4.95 (4.72–5.17)	0.051
- *L. crispatus*	0.64 (0.48–0.80)	0.44 (0.23–0.65)					0.78 (0.56–1.01)	0.049
- Other lactobacilli	2.29 (2.08–2.50)	1.73 (1.40–2.06)					2.69 (2.43–2.96)	<0.001
- Total BV-anaerobes	5.17 (4.99–5.35)	5.74 (5.48–6.01)					4.76 (4.53–4.99)	<0.001
- *G. vaginalis*	4.48 (4.25–4.71)	5.08 (4.75–5.40)					4.05 (3.75–4.35)	<0.001
- *A. vaginae*	2.80 (2.53–3.07)	3.97 (3.56–4.38)					1.96 (1.64–2.29)	<0.001
- *Prevotella* species	3.00 (2.75–3.25)	4,01 (3.65–4.38)					2.28 (1.97–2.59)	<0.001
- Other BV-anaerobes	4.30 (4.09–4.51)	5.08 (4.79–5.37)					3.75 (3.48–4.02)	<0.001
- Total pathobionts	2.18 (1.96–2.40)	2.04 (1.69–2.38)					2.28 (1.99–2.57)	0.318
- *Streptococcus* species	1.59 (1.38–1.81)	1.47 (1.14–1.80)					1.68 (1.39–1.97)	0.326
- *Staphylococcus* species	0.46 (0.35–0.58)	0.41 (0.24–0.58)					0.50 (0.34–0.66)	0.400
- *Enterococcus* species	0.17 (0.09–0.25)	0.06 (0–0.13)					0.24 (0.12–0.37)	0.055
- *Escherichia/Shigella* species	0.50 (0.37–0.63)	0.35 (0.17–0.54)					0.61 (0.42–0.79)	0.030
- *Campylobacter* species	0.12 (0.05–0.19)	0.17 (0.04–0.29)					0.09 (0.01–0.17)	0.239
- *Haemophilus* species	0.07 (0.02–0.12)	0.08 (0–0.16)					0.07 (0–0.13)	0.860
- Other pathobionts[i,j]	0.36 (0.18–0.53)	0.40 (0.25–0.55)					0.38 (0.27–0.49)	0.579
- Total other bacteria	1.92 (1.72–2.11)	1.92 (1.61–2.31)					1.92 (1.66–2.17)	1.00

A, Atopobium; BV, bacterial vaginosis; CI, confidence interval; G, Gardnerella; L, Lactobacillus; NA, not assessed/applicable; VMB, vaginal microbiota.

The unit of analysis is one sample. Cells contain at most five missing values unless otherwise indicated.

[a] *Samples collected at the screening and Month 6 visits in all randomization groups, and at the Month 1 and Month 2 visits in the no–intervention group, were considered not influenced by the interventions.*

[b] *Included Dutch women of African Surinamese and Ghanaian origin.*

[c] *Included Dutch women of South–Asian Surinamese, Moroccan, and Turkish origin.*

[d] *Using the Chi-squared test for categorical variables and the Kruskal-Wallis test for continuous variables.*

[e] *Nugent scoring of Gram stains was performed during the all scheduled study visits in the VMB study, the first study visit in the HARP study, and not at all in the HELIUS study.*

[f] *Based on the rarefied sequencing data set of each of the studies.*

[g] *The HARP and HELIUS studies also identified samples that had significant abundance of Bifidobacteria (n = 2 in HARP and n = 8 in HELIUS).*

[h] *These pathobiont genera were uncommon (mean relative abundance lower than 1% for each of the genera).*

[i] *Also includes reads assigned to the pathogens Chlamydia, Neisseria, and Treponema genus.*

[j] *Individual pathobionts in this rest group were detected at a mean estimated concentration of at most 0.02 log_{10} cells/μl in the Rwanda VMB study.*

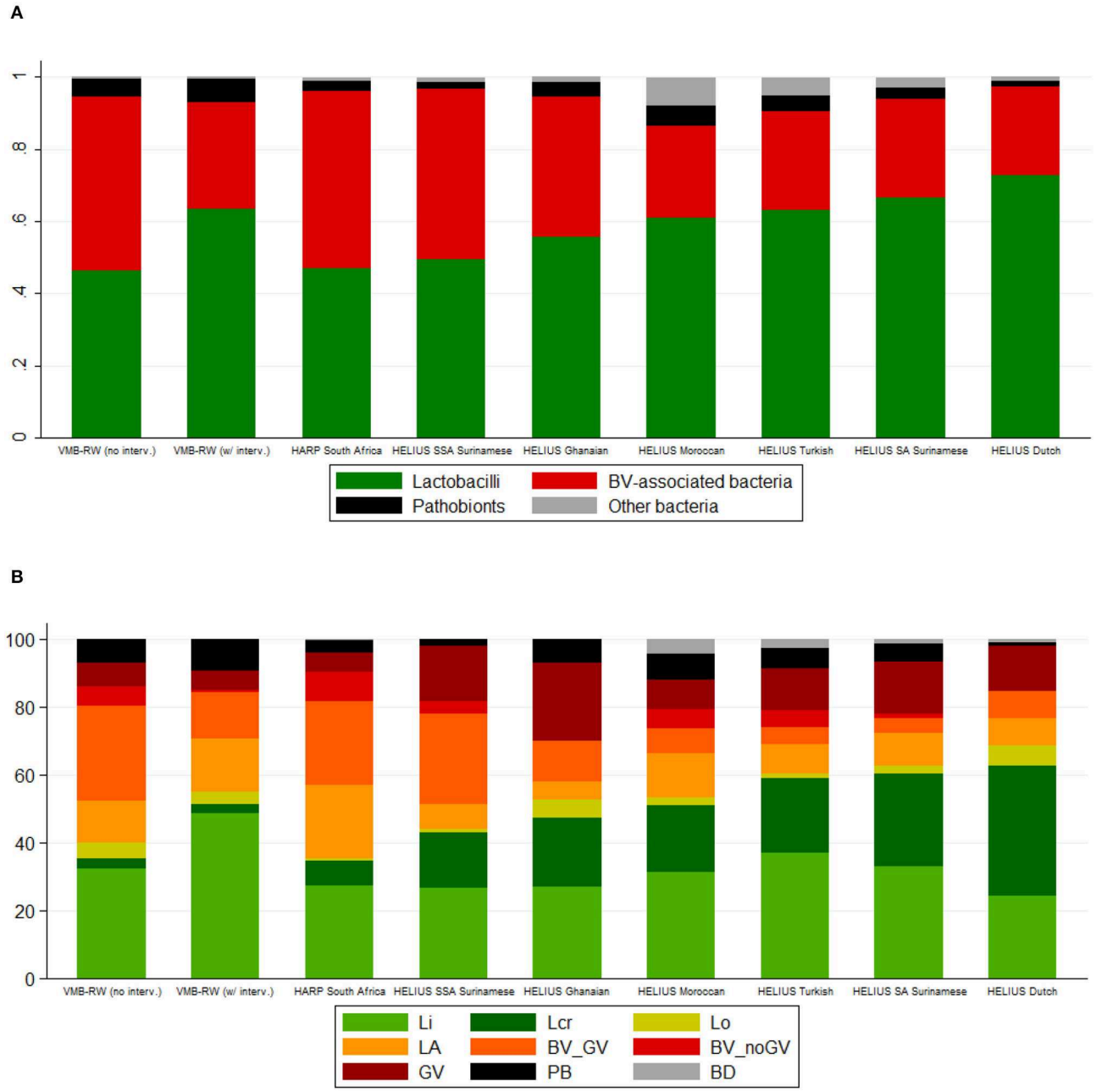

FIGURE 1 | Bar charts by study, intervention exposure (VMB Rwanda) and ethnic group (HELIUS). **(A)** Mean relative abundance of bacterial groups. **(B)** Cumulative percentage of women with a specific VMB type. *BV,* bacterial vaginosis; *BD, Bifidobacterium*-dominated; *BV_GV,* polybacterial *Gardnerella vaginalis*-containing; *BV_noGV,* polybacterial but low *G. vaginalis*; *CI,* confidence interval; *GV, G. vaginalis*-dominated; *interv*, (study product) interventions; *LA,* lactobacilli and anaerobes; *Lcr, L. crispatus*-dominated; *Li, L. iners*-dominated; *Lo,* other lactobacilli-dominated; *NA,* not applicable; *PB,* pathobionts-containing; *SA,* South-Asian; *SSA,* sub-Saharan African; *VMB,* vaginal microbiota; *VMB-RW*, Rwanda VMB study. Rwanda VMB study samples collected at the screening and Month 6 visits in all randomization groups, and at the Month 1 and Month 2 visits in the no-intervention group, were considered not influenced by the interventions.

and BD 0.5%. The latter VMB type included only 10 samples and was therefore not included in subsequent comparisons. Consistent with the bacterial group findings, the VMB types characterized by lactobacilli-domination (Li, Lcr, and Lo; 44.5%) or by anaerobic dysbiosis (LA, BV_GV, BV_noGV, GV; 49.9%) were much more common than the VMB type characterized by ≥20% pathobionts (5.2%). VMB type distributions differed significantly between the studies and strata, following the same patterns as described above for the bacterial group findings (**Table 3**, **Figure 1B**).

Identification of Common Vaginal Pathobionts

We identified 40 different pathobiont taxa in all 2,044 samples combined (**Supplementary Material 1**; reported at species level if only one species was identified, genus level if multiple species and/or the genus was identified, and family or class level if only that level was identified). However, 20 of these were never a non-minority taxon (defined as present at a relative abundance of at least 1% in at least one sample) in any of the studies. Only six taxa were a non-minority taxon in all three studies: *Streptococcus, Staphylococcus, Enterococcus*, *Escherichia/Shigella, Haemophilus*, and *Campylobacter*. *Chlamydia* was consistently detected in all three studies but only as a non-minority taxon in HELIUS, and *Neisseria* and *Treponema* were detected in the two African studies only. The remaining 11 taxons varied in their detection (yes vs. no) and relative abundance (minority vs. non-minority) status between the three studies. More than half (54%) of all pathobiont sequencing reads were assigned to *Streptococcus* genus/species and 24% of the *Streptococcus* reads were assigned to *S. agalactiae* or *S. agalactiae/pyogenes*. Seventeen percent of all women (196/1,153) at baseline, and 19.5% (399/2,044) of all samples, had at least 1% pathobionts in their VMB; these proportions were 12.7% (147/1,153), and 14.9% (304/2,044) for at least 1% *Streptoccoccus*. Among samples with ≥20% pathobionts ($N = 107$; **Supplementary Material 2**, **Table S1**), 33 contained *Streptococcus* genus/species as the only pathobionts (relative abundances of 0.53–0.98) and an additional 52 contained multiple pathobionts including substantial relative abundances of *Streptococcus* genus/species (0.13–0.73). The other 22 samples contained other pathobionts (most commonly staphylococci, *Escherichia/Shigella* species, *Haemophilus* species, and/or enterococci), with <5% *Streptococcus*.

Of note, the total estimated bacterial concentration differed significantly per VMB type (**Table 4**; data available for the Rwanda VMB study only). The mean total bacterial estimated concentration of women with the PB VMB type (5.85 $\log_{10}$ cells/μl) was comparable to those of women with *Lactobacillus*-dominated VMB types (5.13–5.83 $\log_{10}$ cells/μl) but lower than those of women with VMB types associated with anaerobic dysbiosis (6.11–6.87 $\log_{10}$ cells/μl). When samples were stratified by the proportion of pathobionts in the VMB (<1%, 1-<10%, 10%-<20%, 20-<50%, and ≥50%), the mean estimated concentration of pathobionts increased as expected, but reached a plateau at proportions of 10% or more. The mean estimated concentration of total bacteria remained stable but declined somewhat when the pathobionts proportion reached above 20%. Results were similar when only samples not influenced by interventions were included in these analyses (**Table 4**).

Correlations Between Vaginal Pathobionts and Other VMB Characteristics

We next investigated correlations between pathobionts levels (relative abundances or estimated concentrations), lactobacilli levels, and BV-anaerobes levels for samples not influenced by interventions (**Table 5**). With increasing pathobionts proportion (from <1% to 1-<10% to 10%-<20%, etc.), the mean relative abundance of lactobacilli declined significantly ($\rho = -0.1851$; 95% confidence interval (CI) −0.2286 to −0.1416). The same applied to estimated concentrations, but this trend was not significant ($\rho = -0.0132$; 95% CI −0.1891 to 0.1627). We could not detect a pathobionts proportion threshold: the weak negative effect on lactobacilli was detectable even in the lowest pathobionts proportion categories. By contrast, the mean relative abundance of BV-anaerobes remained stable initially, and only declined when pathobionts made up 30% or more of the VMB. The mean BV-anaerobes estimated concentration significantly increased with increasing estimated pathobionts concentration and did not reach a plateau. In all pathobionts concentration categories, BV-anaerobes outnumbered pathobionts.

Correlation matrixes for samples not influenced by interventions confirmed that relative abundances of BV-anaerobes and lactobacilli were strongly negatively correlated ($\rho = -0.9234$; **Figure 2A**), but showed that their estimated concentrations were not correlated ($r = 0.0031$; **Figure 2B**; for correlation coefficients with 95% confidence intervals, see **Supplementary Material 2**, **Table S2**). Pathobionts and lactobacilli relative abundances were also negatively correlated, albeit less strongly ($\rho = -0.2076$), and their estimated concentrations were not ($r = 0.0436$). Pathobionts and BV-anaerobes relative abundances were not correlated ($\rho = 0.0160$) and their estimated concentrations were weakly positively correlated ($r = 0.1938$). Pathobionts also correlated positively with the "other bacteria" rest group ($\rho = 0.3831$ for relative abundances and $r = 0.3388$ for estimated concentrations). At individual genus level, the estimated concentrations of the six pathobionts that were a non-minority genus in all three studies correlated positively with one another except for *Campylobacter* with the other five taxa, and *Haemophilus* and *Escherichia/Shigella*.

Correlates of Vaginal Pathobionts Detection

Finally, we investigated the correlates of pathobionts detection (≥1% vs. <1%), relative abundance, and estimated concentration for all studies combined (**Table 6**) and for each study separately (**Supplementary Material 2**, **Tables S3A–C**). The mean relative abundance and mean estimated concentration of pathobionts decreased with increasing age (except in the HELIUS study), and with ethnicities other than Dutch. The data consistently showed strong associations with Nugent score categories in both studies that assessed these (the Rwanda VMB and HARP studies): the likelihood of detection (OR = 5.29; 95% CI 2.82–9.90), mean relative abundance, and mean estimated concentration of pathobionts were highest for Nugent score category 4–6 (intermediate), followed by 7–10 (BV-positive), and 0–3 (BV-negative). Positive associations between pathobionts detection or levels and hormonal contraceptive use, smoking, antibiotic use in the 14 days prior to sampling, HIV status, and the presence of STI pathogens were found in at least one but not in all studies. Associations with sexual risk-taking and unusual vaginal discharge reporting were inconsistent between studies,

TABLE 4 | Estimated bacterial concentration per VMB type and by proportion of pathobionts (Rwanda VMB study only, multiple samples per woman).

VMB type		Estimated total bacterial concentration in log_{10} cells/μl		
	N	All samples Mean (95% CI)[b]	N	Not influenced by interventions[a] Mean (95% CI)[c]
Lactobacillus iners-dominated (Li)	144	5.81 (5.69–5.92)	34	5.99 (5.75–6.22)
L. crispatus-dominated (Lcr)	9	5.36 (4.99–5.72)	3	5.43 (4.51–6.35)
Other lactobacilli-dominated (Lo)	10	5.13 (4.63–5.64)	3	5.59 (4.13–7.05)
Lactobacilli and BV-anaerobes (LA)	57	6.31 (6.11–6.51)	21	6.34 (5.96–6.73)
Polybacterial *Gardnerella vaginalis*-containing (BV_GV)	90	6.87 (6.72–7.02)	57	6.91 (6.72–7.11)
Polybacterial but low *G. vaginalis* (BV_noGV)	16	6.29 (5.75–6.82)	14	6.15 (5.57–6.73)
G. vaginalis-dominated (GV)	27	6.11 (5.77–6.46)	16	6.14 (5.64–6.64)
Pathobionts-containing (PB)	26	5.85 (5.43–6.27)	10	5.76 (4.76–6.76)
Proportion pathobionts		**Estimated total bacterial concentration in log_{10} cells/μl**		
<1%	289	6.17 (6.07–6.27)	124	6.47 (6.31–6.62)
1%–<10%	50	6.14 (5.89–6.38)	19	6.05 (5.69–6.42)
10%–<20%	14	6.33 (5.89–6.78)	5	6.29 (5.02–7.56)
20%–<50%	15	6.04 (5.49–6.59)	8	6.18 (5.22–7.15)
≥50%	11	5.59 (4.84–6.34)	2	4.06 (−5.20–13.33)
Proportion pathobionts		**Estimated pathobionts concentration in log_{10} cells/μl**		
<1%	289	1.36 (1.16–11.56)	124	1.32 (0.99–1.65)
1%–<10%	50	4.38 (4.12–4.63)	19	4.21 (3.84–4.58)
10%–<20%	14	5.25 (4.80–5.69)	5	5.21 (4.00–6.41)
20%–<50%	15	5.40 (4.85–5.94)	8	5.51 (4.51–6.50)
≥50%	11	5.41 (4.71–6.12)	2	4.00 (−6.02–14.02)

BV, bacterial vaginosis; CI, confidence interval; VMB, vaginal microbiota.

[a] *Estimated concentrations were only available for the Rwanda VMB study. Samples collected at the screening and Month 6 visits in all randomization groups, and at the Month 1 and Month 2 visits in the no-intervention group, were considered not influenced by the interventions.*

[b] *The Kruskall Wallis p-values comparing the relevant categories are <0.001 for VMB types and estimated total bacterial concentration, 0.606 for pathobionts proportion and estimated total bacterial concentration, and <0.001 for pathobionts proportion and estimated pathobionts concentration.*

The Kruskall Wallis p-value comparing the relevant categories are <0.001 for VMB types, 0.061 for pathobionts proportion and estimated total bacterial concentration, and <0.001 for pathobionts proportion and estimated pathobionts concentration.

and we did not find associations with detection of vaginal yeasts by microscopy.

DISCUSSION

Seventeen percent of this highly diverse group of women from Africa and Europe had a VMB containing at least 1% pathobionts, and 5.2% had a VMB containing at least 20% pathobionts. *Streptococcus* was most common (54% of the pathobionts sequencing reads), but *Staphylococcus, Enterococcus*, *Escherichia/Shigella, Haemophilus*, and *Campylobacter* were also detected as non-minority genera in all three studies. Mean relative abundances and estimated concentrations of pathobionts were much lower than those of lactobacilli and BV-anaerobes, but the pathogenic potential may be higher, and these levels may therefore be clinically relevant.

The meta-analysis confirmed that the VMB of many women contains both lactobacilli and BV-anaerobes, but that the BV-anaerobes concentration is low in "healthy" women with lactobacilli-domination. Our relative abundance, estimated concentration, and correlation data may be best explained by the following hypothesis. BV-anaerobes are present or frequently introduced into the vagina of most women, and may start to expand in response to a trigger, such as recent sex or menses (Jespers et al., 2017). When the BV-anaerobes concentration increases, the lactobacilli concentration does not seem to decline much initially, but instead, the total bacterial concentration increases. The lactobacilli relative abundance therefore does decline. We cannot test this hypothesis directly because our analyses were cross-sectional, but the strong negative correlation between lactobacilli and BV-anaerobes relative abundances ($\rho = -0.9234$) but not estimated concentrations ($r = 0.0031$), and the higher overall bacterial load of the anaerobic dysbiotic VMB types (means 6.11–6.87 log_{10} cells/μl) compared to the lactobacilli-dominated VMB types (means 5.13–5.83 log_{10} cells/μl) fit this hypothesis.

By contrast, a much smaller proportion of women in our study carried pathobionts in their VMB (17% if a 1% relative abundance is used as a cut-off). Our relative abundance, estimated concentration, and correlation data may be best explained by the following hypothesis. Pathobionts are occasionally introduced into the vagina from the gut, urinary

TABLE 5 | VMB correlates by pathobionts relative abundance category, all samples from all studies not influenced by interventions ($N = 1,781$)[a].

Cells: mean relative abundance (95% CI)	**Total pathobionts relative abundance category**							**ρ (95% CI)**	**r (95% CI)**
	<1%	**1%–<10%**	**10%–<20%**	**20%–<30%**	**30%–<40%**	**40%–<50%**	**≥50%**	**All [b]**	**If ≥1%[c]**
	($n = 1,445$)	**($n = 205$)**	**($n = 38$)**	**($n = 15$)**	**($n = 15$)**	**($n = 14$)**	**($n = 39$)**	**($N = 1,781$)**	**($n = 326$)**
Total pathobionts	0 (0–0)	0.03 (0.03–0.03)	0.15 (0.14–0.16)	0.25 (0.23–0.27)	0.35 (0.33–0.37)	0.46 (0.44–0.47)	0.81 (0.76–0.85)	NA	NA
Total lactobacilli	0.55 (0.53–0.57)	0.45 (0.39–0.50)	0.25 (0.14–0.36)	0.21 (0.05–0.36)	0.24 (0.10–0.39)	0.15 (0.05–0.25)	0.07 (0.03–0.10)	−0.1851 (−0.2286, −0.1416)	−0.3756 (−0.4340,−0.3172)
Total BV-anaerobes	0.44 (0.42–0.46)	0.46 (0.41–0.51)	0.52 (0.41–0.64)	0.53 (0.38–0.68)	0.33 (0.20–0.46)	0.35 (0.24–0.46)	0.10 (0.06–0.13)	−0.0012 (−0.0453, 0.0429)	−0.3223 (-0.3946,−0.2501)
Total other bacteria	0.01 (0.00–0.01)	0.06 (0.04–0.09)	0.08 (0.01–0.15)	0.01 (0.00–0.02)	0.08 (0.00–0.15)	0.04 (0.00–0.07)	0.02 (0.01–0.04)	0.3894 (0.3464, 0.4323)	−0.0866 (−0.1356, −0.0376)
Lactobacillus iners	0.37 (0.35–0.39)	0.32 (0.27–0.36)	0.21 (0.11–0.32)	0.18 (0.04–0.32)	0.17 (0.04–0.31)	0.08 (-0.01–0.16)	0.04 (0.01–0.07)	−0.1564 (−0.2020, −0.1106)	−0.3069 (-0.3584,−0.2553)
L. crispatus	0.13 (0.12–0.15)	0.10 (0.07–0.13)	0.03 (−0.01–0.08)	0.01 (−0.01–0.03)	0.02 (0.00–0.04)	0.05 (−0.01–0.12)	0.02 (0.00–0.04)	0.0470 (0.0022, 0.0919)	−0.1484 (−0.1953, −0.1061)
Other lactobacilli	0.04 (0.04–0.05)	0.03 (0.02–0.04)	0.01 (0.00–0.01)	0.02 (0.00–0.03)	0.05 (−0.03–0.14)	0.02 (−0.01–0.06)	0.01 (−0.01–0.03)	0.0397 (−0.0272, 0.5989)	−0.0700 (−0.1290, −0.0010)
Gardnerella vaginalis	0.18 (0.17–0.19)	0.17 (0.14–0.20)	0.20 (0.12–0.28)	0.29 (0.12–0.45)	0.17 (0.05–0.29)	0.15 (0.06–0.25)	0.04 (0.01–0.07)	0.0038 (−0.0408, 0.0485)	−0.1759 (−0.2390, −0.1129)
Atopobium vaginae	0.04 (0.04–0.05)	0.02 (0.01–0.03)	0.02 (0.00–0.03)	0.04 (−0.02–0.11)	0.00 (0.00–0.01)	0.01 (0.00–0.02)	0 (0–0.01)	−0.1102 (−0.1560, −0.0643)	−0.1032 (−0.1567, −0.0496)
Prevotella species	0.05 (0.04–0.05)	0.07 (0.05–0.08)	0.09 (0.04–0.14)	0.03 (0.00–0.05)	0.05 (0.00–0.09)	0.04 (0.01–0.06)	0.01 (0.00–0.02)	0.0551 (0.0077, 0.1025)	−0.1961 (−0.2490, −0.1433)
Other BV-anaerobes	0.17 (0.16–0.19)	0.20 (0.17–0.24)	0.21 (0.15–0.28)	0.18 (0.06–0.30)	0.11 (0.04–0.19)	0.15 (0.05–0.25)	0.04 (0.02–0.06)	0.0504 (0.0040, 0.0968)	−0.2382 (−0.2994, −0.1771)
Cells: mean estimated concentration in log_{10} cells/μL (95% CI)[d]	**$<10^3$**	**10^3-$<10^4$**	**10^4-$<10^5$**	**10^5-$<10^6$**	**$\geq 10^6$**			**ρ (95% CI) All[b]**	**r (95% CI) If ≥1%[c]**
	($n = 89$)	**($n = 33$)**	**($n = 21$)**	**($n = 10$)**	**($n = 5$)**			**($n = 158$)**	**($n = 34$)**
Total pathobionts	0.25 (0.09–0.40)	3.59 (3.48–3.70)	4.51 (4.37–4.65)	5.40 (5.23–5.56)	6.50 (5.92–7.09)			NA	NA
Total bacteria	6.30 (6.10–6.49)	6.12 (5.83–6.42)	6.45 (6.00–6.91)	7.04 (6.43–7.65)	7.46 (7.18–7.74)			0.1219 (−0.0374, 0.2811)	0.8117 (0.7200, 0.9034)
Total lactobacilli	4.98 (4.70–5.26)	4.68 (4.12–5.24)	4.66 (3.71–5.62)	5.22 (3.68–6.76)	3.47 (−0.62–7.67)			−0.0132 (−0.1891, 0.1627)	0.0366 (−0.3937, 0.4669)
Total BV-anaerobes	5.60 (5.21–5.98)	5.55 (5.02–6.08)	5.88 (5.20–6.57)	6.68 (5.77–7.60)	7.22 (6.69–7.76)			0.1009 (−0.0596, 0.2615)	0.5778 (0.3391, 0.8166)
Total other bacteria	1.43 (1.05–1.81)	2.67 (2.05–3.28)	2.37 (1.41–3.33)	2.76 (0.99–4.52)	2.19 (−1.60–5.98)			0.2652 (0.1026, 0.4278)	0.0689 (−0.3669, 0.5048)
L. iners	4.65 (4.28–5.03)	4.31 (3.65–4.98)	4.54 (3.51–5.58)	5.22 (3.68–6.76)	3.29 (−0.60–7.19)			−0.0115 (−0.1817, 0.1588)	0.0891 (−0.3009, 0.4791)
L. crispatus	0.47 (0.19–0.74)	0.53 (0.02–1.05)	0.20 (−0.22–0.63)	0 (0–0)	1.28 (−2.28–4.85)			0.0325 (−0.1103, 0.1753)	0.2864 (−0.2120, 0.7849)

(Continued)

TABLE 5 | Continued

Cells: mean relative abundance (95% CI)	Total pathobionts relative abundance category							ρ (95% CI)	r (95% CI)
	<1%	1%–<10%	10%–<20%	20%–<30%	30%–<40%	40%–<50%	≥50%	All [b]	If ≥1% [c]
	(*n* = 1,445)	(*n* = 205)	(*n* = 38)	(*n* = 15)	(*n* = 15)	(*n* = 14)	(*n* = 39)	(*N* = 1,781)	(*n* = 326)
Other lactobacilli	1.56 (1.14–1.98)	1.98 (1.25–2.72)	2.24 (1.24–3.24)	0.97 (−0.49–2.43)	2.38 (−1.69–6.44)			0.0975 (−0.0672, 0.2622)	0.0767 (−0.3394, 0.4927)
G. vaginalis	4.98 (4.54–5.43)	5.03 (4.42–5.64)	5.13 (4.17–6.10)	5.82 (4.18–7.45)	5.41 (1.63–9.20)			0.0657 (−0.1036, 0.2350)	0.1127 (−0.3010, 0.5264)
A. vaginae	4.36 (3.86–4.86)	3.60 (2.72–4.47)	3.21 (1.84–4.58)	3.87 (1.45–6.30)	2.81 (−1.97–7.58)			−0.1391 (−0.3162, 0.0380)	−0.0221 (−0.3908, 0.3464)
Prevotella species	4.14 (3.67–4.62)	3.68 (2.92–4.44)	3.33 (2.05–4.61)	4.77 (2.69–6.84)	5.15 (3.88–6.41)			−0.0384 (−0.2086, 0.1317)	0.4183 (0.1589, 0.6778)
Other BV-anaerobes	4.87 (4.43–5.31)	4.87 (4.34–5.41)	5.34 (4.68–6.01)	6.25 (5.41–7.09)	6.70 (5.85–7.54)			0.1090 (−0.0496, 0.2675)	0.6760 (0.4868, 0.8653)

BV, bacterial vaginosis; CI, confidence interval; NA, not applicable; VMB, vaginal microbiota.

[a] *VMB study samples collected at the screening and Month 6 visits in all randomization groups, and at the Month 1 and Month 2 visits in the no-intervention group, were considered not influenced by the interventions.*

[b] *Spearman's rank correlation coefficient calculations included all samples not influenced by interventions.*

[c] *Pearson's correlation coefficient calculations only included samples not influenced by interventions and containing at least 1% pathobionts.*

[d] *Estimated concentrations are available for Rwanda VMB study samples only.*

tract, and perineum, or from the male partner external genitalia, but are usually cleared or remain at low levels. If they do persist and expand, lactobacilli decline somewhat, and BV-anaerobes expand alongside the pathobionts. As before, we cannot test this hypothesis directly, but the modest positive correlations between estimated concentrations of pathobionts and BV-anaerobes, the declines in estimated concentration/relative abundance of lactobacilli with increasing pathobionts level, and an overall bacterial load of the ≥20% pathobionts VMB type (5.85 $\log_{10}$ cells/μl) that is similar to that of the lactobacilli-dominated VMB types (means 5.13–5.83 $\log_{10}$ cells/μl) fit this hypothesis. Pathobionts also correlated positively with the "other bacteria" group, which contains non-pathogenic skin bacteria such as *Corynebacterium*. The pathobionts and non-pathogenic skin bacteria may have been introduced into the vagina from the woman's perineum or the skin of the external genitalia of her male partner, but specimen contamination via the hands of specimen handlers cannot be ruled out.

Gram stain Nugent scoring is the current gold standard for BV diagnosis (Nugent et al., 1991). In this method, Gram stained slides are viewed under a microscopy, and three bacterial morphotypes are scored: Gram-positive rods (presumed to be lactobacilli), small Gram-variable rods (presumed to be *G. vaginalis*), and curved Gram-variable rods (presumed to be *Mobiluncus*). A Nugent score of 0–3 is considered BV-negative, 4–6 intermediate microbiota, and 7–10 BV-positive. In this meta-analysis, the likelihood of detection, mean relative abundance, and mean estimated concentration of pathobionts were consistently highest for Nugent score category 4–6, followed by 7–10, and 0–3. These findings also fit the above-mentioned hypotheses, and provide a partial explanation for what a Nugent score of 4–6 signifies. A Nugent score of 4–6 should, however, not be used to diagnose pathobionts presence because another significant proportion of these samples likely contain lactobacilli plus BV-anaerobes.

Positive associations between pathobionts detection and/or levels and young age, non-Dutch origin, hormonal contraceptive use, smoking, antibiotic use in the 14 days prior to sampling, HIV status, and the presence of STI pathogens were found in at least one study. All of these factors are also risk factors for anaerobic dysbiosis, except for hormonal contraceptive use. Hormonal contraception, and especially methods containing estrogen, protects women from anaerobic dysbiosis (van de Wijgert et al., 2013). Authors have hypothesized that estrogen increases vaginal glycogen, which is converted into lactic acid by lactobacilli. This keeps BV-anaerobes at bay but perhaps not pathobionts. Streptococci, for example, can tolerate low vaginal pH very well (Shabayek and Spellerberg, 2017). Sexual risk-taking is an important proven risk factor for anaerobic dysbiosis (van de Wijgert et al., 2014), but associations with pathobionts detection and/or levels were inconsistent in this meta-analysis. This could be due to the fact that women in the two African cohorts were recruited based on sexual risk or HIV-status, and women in the Dutch cohort were not, thereby introducing collinearity between study/ethnic group and sexual risk. Associations between pathobionts detection/levels and unusual vaginal discharge reporting were also inconsistent

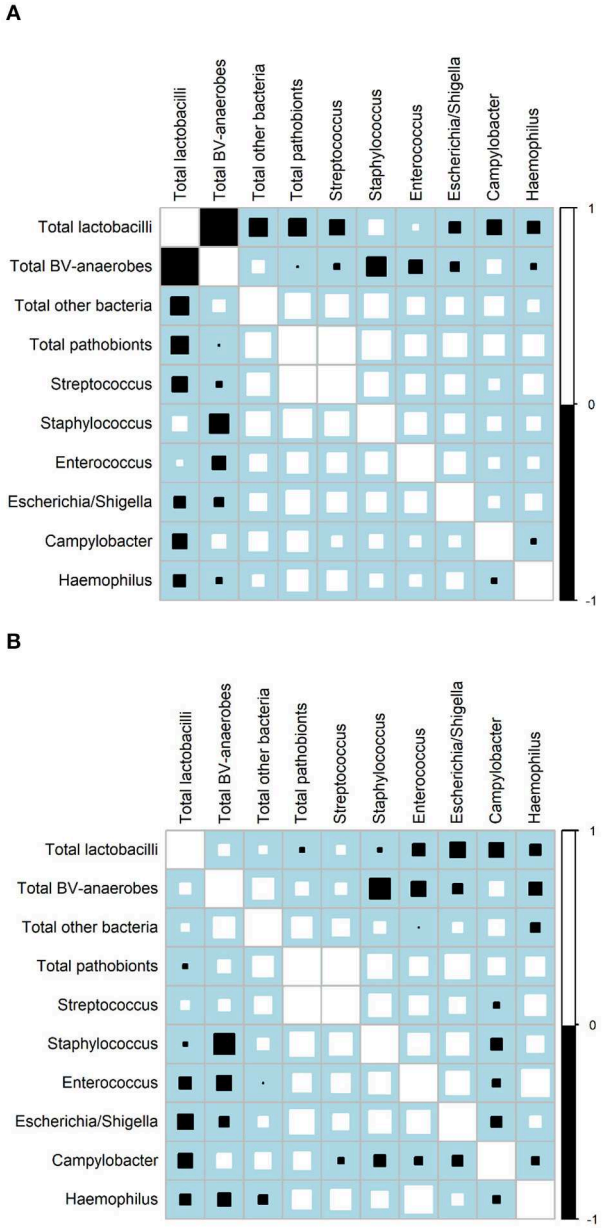

FIGURE 2 | Correlation matrices. **(A)** Relative abundances, all three studies (*N* = 1,781 samples)[a]. **(B)** Estimated concentrations, VMB study only (*N* = 158 samples)[a]. *BV,* bacterial vaginosis.[a]Only includes VMB samples not influenced by the interventions, collected at Screening, Month 6, and Month 1 and 2 in the no-intervention group. Squares indicate the Spearman's rank correlation between −1 and +1. Positive correlations are shown in white and negative correlations in black. The size of each square is loosely proportionate to the magnitude of the correlation. For the actual correlation coefficients, (see **Supplementary Material**, **Tables S2C,D**).

between studies. In our experience, vaginal symptom-reporting rarely correlates well with the actual presence of a vaginal infection or vaginal dysbiosis (Verwijs et al., 2019b). None of the women in the three studies had severe symptoms, such as those associated with desquamative inflammatory vaginitis, and we could therefore not test the association between pathobionts levels and such symptoms.

A limitation of our study is that each of the three studies used slightly different sequencing-related laboratory and initial data processing methods (**Table 1**). However, we took this into account by stratifying most analyses by study. Another limitation is that we did not detect and quantify individual pathobiont species and genera by quantitative PCR. Past *S. agalactiae* prevalence studies using selective culture have shown average rectovaginal detection rates of 22% in sub-Saharan Africa and 19% in Europe (Kwatra et al., 2016), and a recent study using quantitative PCR found a vaginal detection rate of 20% in Kenyan women and 23% in South African women (Cools et al., 2016). Detection of vaginal *Streptococcus* was lower in our meta-analysis when a relative abundance cut-off of 1% was used (12.7% of all women at baseline; about a quarter of those were *S. agalactiae*). It is currently not known how rectovaginal and vaginal selective cultures, PCR, and sequencing results relate to one another, but it is possible that pathobionts are not only under-detected in sequencing studies due to the bioinformatics used, but also due to DNA extraction, amplification, and other biases. For example, the detection rate in the HARP study was especially low, which may have been due to the fact that we did not use bead-beating during DNA extraction in that study (Gill et al., 2016). Third, some of the standard diagnostic tests that we used are known to have lower sensitivity than NAAT-based tests (e.g., culture for vulvovaginal candidiasis and *T. vaginalis*), and not all women in all three studies were screened for all STI pathogens.

We also report some limitations related to our statistical analyses. Correlating variables derived from relative abundance data is problematic because they are not independent (Knight et al., 2018); estimated concentrations of these same variables are independent and did indeed provide new insights as described above. However, we only had estimated concentration data for the Rwanda VMB study. Furthermore, our analyses were cross-sectional, and some of them had limited statistical power. Our findings are therefore hypothesis-generating and the hypotheses should be tested in well-powered longitudinal studies that assess the VMB quantitatively. We did not exclude all women who had recently used antibiotics, but reported antibiotic use in the last 2 weeks was rare. A strength of our study is the inclusion of women and samples from three world regions and multiple ethnic groups, and with different behaviors and STI pathogen exposures. The variability in VMB compositions that we observed reflects this wide variety of study participants.

CONCLUSION

While substantial presence of pathobionts in the VMB was less common than anaerobic dysbiosis, the pathogenic potential of pathobionts is higher than that of BV-anaerobes, and modest levels could therefore be clinically relevant. The most frequently used VMB types, and analyses limited to relative abundance, are inadequate. We recommend that future etiologic and intervention studies quantify the most common vaginal pathobiont genera, as well as lactobacilli and BV-anaerobes.

TABLE 6 | Correlates of pathobionts detection, relative abundance, and estimated concentration.

Independent variables[a]	Rwanda VMB study screening, HARP baseline, and HELIUS samples				All samples			
	<1% (% of *N* = 957)	≥1% (% of *N* = 196)	OR (95% CI)[b]	*P*[c]	Mean relative abundance (95% CI); *N* = 2,044	*P*[c]	Mean estimated concentration $\log_{10}$ cells/μl (95% CI); *N* = 379	*P*[c]
Potentially influenced by interventions:[d]								
- Yes	NA[d]	NA[d]	NA[d]	NA[d]	0.07 (0.04–0.09)	**0.032**	2.28 (1.99–2.57)	0.318
- No					0.03 (0.03–0.04)		2.04 (1.69–2.38)	
Age categories:								
- 18–24	19.8	21.4	Reference	0.719	0.08 (0.06–0.11)	**<0.001**	3.09 (2.56–3.62)	**<0.001**
- 25–29	25.7	27.6	0.99 (0.63–1.54)		0.04 (0.03–0.05)		2.29 (1.80–2.78)	
- 30–34	31.5	29.6	0.87 (0.56–1.34)		0.03 (0.02–0.03)		2.01 (1.66–2.35)	
- 35–44	20.5	17.9	0.80 (0.49–1.31)		0.03 (0.02–0.04)		1.63 (1.19–2.06)	
- 45–50	2.6	3.6	1.26 (0.51–3.11)		0.02 (0.01–0.04)		NA	
Study or ethnicity:								
- VMB Rwanda	14.2	13.3	1.39 (0.66–2.89)	**0.013**	0.06 (0.04–0.07)	**<0.001**	2.18 (1.96–2.40)	NA
- HARP South Africa	38.6	38.8	1.49 (0.78–2.87)		0.03 (0.02–0.04)		NA	
- HELIUS sub-Saharan African	16.9	10.7	0.94 (0.44–2.00)		0.03 (0.01–0.05)		NA	
- HELIUS Turkish, Moroccan, South-Asian	21.2	31.1	2.18 (1.12–4.25)		0.04 (0.03–0.06)		NA	
- HELIUS Dutch	9.1	6.1	Reference		0.01 (0.00–0.03)		NA	
Contraceptive use:	*N = 954*				*N = 1,768*		*N = 252*	
- None or condom use only	58.0	61.2	Reference	0.309	0.03 (0.02–0.03)	**0.026**	1.53 (1.11–1.95)	**0.014**
- Any oral contraception	20.7	19.4	0.89 (0.60–1.33)		0.03 (0.02–0.04)		2.89 (1.93–3.86)	
- Progestin-only injectable	11.9	8.2	0.65 (0.37–1.14)		0.05 (0.03–0.07)		2.37 (1.86–2.88)	
- Progestin-only implant	3.1	5.6	1.74 (0.84–3.58)		0.04 (0.02–0.06)		2.58 (2.00–3.17)	
- Any IUD (copper or hormonal)	5.7	4.6	0.76 (0.37–1.59)		0.01 (0.00–0.03)		1.55 (0.05–3.04)	
- Contraceptive ring	0.6	1.0	1.53 (0.30–7.66)		0.02 (−0.02–0.05)		NA	
- NA (currently pregnant)	0.6	0	ND		0 (0–0)		1.27 (−4.21–6.75)	
Any hormonal contraception or pregnant:	*N = 902*	*N = 188*			*N = 1,768*		*N = 252*	
- Yes	38.4	35.6	0.87 (0.63–1.21)	0.415	0.04 (0.03–0.05)	**0.026**	2.50 (2.15–2.85)	**<0.001**
- No	61.6	64.4	Reference		0.03 (0.02–0.03)		1.53 (1.13–1.93)	
Current smoker:	*N = 819*	*N = 170*			*N = 1,413*			
- Yes	15.5	13.5	0.85 (0.53–1.38)	0.508	0.01 (0.00–0.03)	0.960	NA	NA
- No	84.5	86.5	Reference		0.03 (0.02–0.04)			
Sample taken:[e]					*N = 462*		*N = 310*	
- During or within 7 days after menses	NA	NA	NA	NA	0.05 (0.02–0.09)	0.236	2.12 (1.63–2.62)	0.606
- Not during or within 7 days after menses					0.08 (0.06–0.10)		2.25 (1.97–2.53)	
Any type of vaginal cleansing:	*N = 871*	*N = 190*			*N = 1,320*		*N = 310*	
- Yes	30.7	27.9	0.88 (0.62–1.24)	0.462	0.03 (0.01–0.04)	0.805	2.47 (1.82–3.11)	0.417
- No	69.4	72.1	Reference		0.04 (0.03–0.05)		2.18 (1.92–2.44)	

(Continued)

TABLE 6 | Continued

Independent variables[a]	Rwanda VMB study screening, HARP baseline, and HELIUS samples				All samples			
	<1% (% of *N* = 957)	**≥1% (% of *N* = 196)**	**OR (95% CI)[b]**	***P*[c]**	**Mean relative abundance (95% CI); *N* = 2,044**	***P*[c]**	**Mean estimated concentration log_{10} cells/μl (95% CI); *N* = 379**	***P*[c]**
Number of sex partners prior to sampling:					*N = 1,775*		*N=249*	
- None	21.4	25.6	Reference	0.379	0.04 (0.02–0.06)	**0.008**	1.81 (−21.19–24.82)	0.571
- One	57.1	52.3	0.77 (0.53–1.11)		0.03 (0.01–0.03)		1.82 (1.21–2.43)	
- Two or more	21.4	22.1	0.86 (0.55–1.35)		0.04 (0.02–0.05)		2.17 (1.87–2.48)	
Frequency of condom use:					*N = 1,473*		*N=369*	
- Never	20.5	18.4	Reference	0.659	0.03 (0.01–0.04)	**0.006**	2.04 (1.19–2.89)	0.955
- Inconsistent	30.8	28.6	1.04 (0.66–1.64)		0.03 (0.02–0.04)		2.19 (1.90–2.47)	
- Consistent	26.8	27.6	1.15 (0.72–1.82)		0.03 (0.02–0.05)		2.17 (1.76–2.58)	
- NA (no sexual partner)	21.9	25.5	1.30 (0.81–2.09)		0.04 (0.02–0.06)		NA	
Any antibiotic use in past 14 days:	*N = 587*	*N = 119*			*N = 1,173*			
- Yes	2.4	4.2	1.80 (0.63–5.08)	0.293	0.10 (0.06–0.14)	**0.048**	2.32 (1.94–2.69)	0.363
- No	97.6	95.8	Reference		0.04 (0.03–0.05)		2.10 (1.83–2.38)	
Current urogenital symptom:	*N = 588*	*N = 120*			*N = 1,044*			
- Yes	42.7	42.5	0.99 (0.67–1.48)	0.970	0.03 (0.02–0.05)	0.655	2.33 (1.71–2.95)	0.481
- No	57.3	57.5	Reference		0.04 (0.03–0.05)		2.15 (1.91–2.39)	
Current unusual vaginal discharge:	*N = 588*	*N = 120*			*N = 1,044*			
- Yes	17.7	12.5	0.66 (0.37–1.19)	0.153	0.02 (0.00–0.04)	0.371	2.65 (1.21–4.08)	0.386
- No	82.3	87.5	Reference		0.04 (0.03–0.05)		2.16 (1.94–2.38)	
Tested HIV-positive:[f]					*N = 1,641*		*N = 126*	
- Yes	40.0	39.8	0.99 (0.72–1.36)	0.953	0.03 (0.02–0.03)	**<0.001**	1.91 (−22.4–26.2)	0.983
- No	60.0	60.2	Reference		0.03 (0.02–0.04)		1.84 (1.45–2.22)	
Nugent score categories:[f]	*N = 477*	*N = 96*			*N = 893*		*N = 364*	
- 0–3	38.2	17.7	Reference	**<0.001**	0.01 (0.01–0.02)	**<0.001**	1.61 (1.29–1.94)	**0.001**
- 4–6	16.6	40.6	5.29 (2.82–9.90)		0.06 (0.04–0.09)		2.78 (2.16–3.40)	
- 7–10	45.3	41.7	1.98 (1.09–3.62)		0.02 (0.01–0.03)		2.25 (1.92–2.59)	
Yeasts by microscopy:[f]	*N = 477*	*N = 98*			*N = 905*		*N = 374*	
- Yes	8.0	8.2	1.03 (0.46–2.27)	0.948	0.02 (0.00–0.04)	0.846	2.21 (1.33–3.08)	0.912
- No	92.0	91.8	Reference		0.03 (0.03–0.04)		2.18 (1.94–2.41)	
Trichomonas vaginalis by culture/NAAT:[f]	*N = 935*	*N = 194*			*N = 1,462*		*N = 373*	
- Yes	7.7	11.3	1.53 (0.93–2.54)	0.108	0.02 (0.01–0.04)	0.806	2.92 (1.89-3.95)	0.133
- No	92.3	88.7	Reference		0.03 (0.03–0.04)		2.15 (1.92-2.37)	
Chlamydia trachomatis by NAAT:[f]	*N = 936*	*N = 194*			*N = 1,195*		*N = 126*	
- Yes	6.5	4.6	0.70 (0.34–1.43)	0.307	0.02 (0.00-0.05)	0.930	2.74 (1.81-3.67)	**0.019**
- No	93.5	94.4	Reference		0.03 (0.02-0.03)		1.60 (1.19-2.01)	
Neisseria gonorrhoeae by NAAT:[f]	*N = 936*	*N = 194*			*N = 1,195*		*N = 126*	
- Yes	2.2	2.6	1.15 (0.43–3.10)	0.781	0.01 (0–0.03)	0.650	2.78 (1.67–3.89)	**0.043**
- No	97.8	97.4	Reference		0.03 (0.02–0.03)		1.68 (1.28–2.08)	

(Continued)

TABLE 6 | Continued

Independent variables[a]	Rwanda VMB study screening, HARP baseline, and HELIUS samples				All samples			
	<1% (% of *N* = 957)	**≥1% (% of *N* = 196)**	**OR (95% CI)[b]**	***P*[c]**	**Mean relative abundance (95% CI); *N* = 2,044**	***P*[c]**	**Mean estimated concentration log_{10} cells/μl (95% CI); *N* = 379**	***P*[c]**
Mycoplasma genitalium by NAAT:[f]	*N = 369*	*N = 76*			*N = 445*			
- Yes	8.9	6.6	0.72 (0.27–1.90)	0.489	0.02 (0.00–0.03)	0.896	NA	NA
- No	91.1	93.4	Reference		0.03 (0.02–0.04)			
Herpes simplex virus type 2 by serology:[f]	*N = 503*	*N = 102*			*N = 627*		*N = 87*	
- Yes	87.1	91.2	1.53 (0.74–3.19)	0.232	0.02 (0.01–0.03)	**0.054**	2.03 (1.33–2.74)	0.410
- No	12.9	8.8	Reference		0.02 (0.00–0.04)		1.67 (1.02–2.33)	
Active syphilis by serology:[f]	*N = 502*	*N = 102*			*N = 668*		*N = 126*	
- Yes	2.8	2.0	0.70 (0.16–3.12)	0.622	0.01 (−0.01–0.03)	0.293	1.87 (−1.33–5.07)	0.918
- No	97.2	98.0	Reference		0.02 (0.02–0.03)		1.84 (1.45–2.22)	
High-risk HPV by PCR:[f]	*N = 821*	*N = 170*			*N = 1,415*			
- Yes	52.5	50.6	0.93 (0.67–1.29)	0.650	0.03 (0.02–0.03)	0.107	NA	NA
- No	47.5	49.4	Reference		0.03 (0.02–0.04)			

CI, confidence interval; HPV, human papilloma virus; IUD, intrauterine device; NA, not applicable; NAAT, nucleic acid amplification test; ND, not determinable; OR, odds ratio; PCR, polymerase chain reaction.

[a] *Refer to the footnotes of Table 2 for other details regarding the independent variables tested in these logistic regression models.*

[b] *Logistic regression analysis with total pathobionts relative abundance (≥1 vs. <1%) as the outcome. All models contained the outcome and one independent variable.*

[c] *By Kruskall–Wallis test, comparing mean pathobionts relative abundances or estimated concentrations between independent variable categories. For age, Spearman's rank correlation was used, correlating age as a continuous variable with pathobionts relative abundances or estimated concentrations as continuous variables.*

[d] *VMB study samples collected at the screening and Month 6 visits in all randomization groups, and at the Month 1 and Month 2 visits in the no-intervention group, and all HARP and HELIUS samples, were considered not influenced by interventions.*

[e] *Menses data are only available for follow-up visits in the Rwanda VMB study.*

[f] *Includes samples from all study visits at which this outcome was tested (excluding invalid results, if applicable).*

Furthermore, the various detection and quantification methods (culture, PCR, and sequencing) should be rigorously compared to one another to facilitate interpretation of clinical study results.

DATA AVAILABILITY STATEMENT

The three studies included in this meta-analyses were governed by different institutions and ethics committees. Data availability therefore differs for each study. The three original publications, which are referenced in this publication, include data availability statements for each respective study. Additional data unique to this publication are provided in **Supplementary Material 1**.

ETHICS STATEMENT

The Rwanda VMB study was sponsored by the University of Liverpool, approved by the Rwanda National Ethics Committee and the University of Liverpool Research Ethics Subcommittee for Physical Interventions, and registered on ClinicalTrials.gov (NCT02459665). The South African portion of the HARP study was approved by the ethics committees of the University of Witwatersrand in Johannesburg and the London School of Hygiene and Tropical Medicine. The HELIUS study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam (protocol number: 10/100; amendment 10/100# 10.17.1729; NL32251.018.10). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JW was the Principal Investigator of all three VMB sequencing studies. PM was the Principal Investigator of the HARP parent study. MV, AG, HB, and CV contributed to the VMB sequencing laboratory work, processed the initial sequencing data, and compiled the metadata datasets. MV and JW wrote the data analysis plan. MV compiled the combined dataset and conducted the analyses presented in this paper. JW and MV wrote the manuscript. All authors commented on and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00129/full#supplementary-material

Supplementary Material 1 | List of taxa identified in the three studies and classification into bacterial groups.

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REVIEW
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Gardnerella vaginalis as a Cause of Bacterial Vaginosis: Appraisal of the Evidence From *in vivo* Models

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Koch's postulates dictate the use of experimental models to illustrate features of human disease and provide evidence for a singular organism as the cause. The underlying cause(s) of bacterial vaginosis (BV) has been debated in the literature for over half a century. In 1955, it was first reported that a bacterium now known as *Gardnerella vaginalis* may be the cause of a condition (BV) resulting in higher vaginal pH, thin discharge, a fishy odor, and the presence of epithelial cells covered in bacteria. Here we review contemporary and historical studies on BV with a focus on reports of experimental infections in human or animal models using *Gardnerella vaginalis*. We evaluate experimental evidence for the hypothesis that *G. vaginalis* is sufficient to trigger clinical features of BV or relevant health complications associated with the condition. Additionally, we evaluate *in vivo* models of co-infection employing *G. vaginalis* together with other bacterial species to investigate evidence for the hypothesis that *G. vaginalis* may encourage colonization or virulence of other potential pathogens. Together, these studies paint a complex picture in which *G. vaginalis* has both direct and indirect roles in the features, health complications, and co-infections associated with BV. We briefly review the current taxonomic landscape and genetic diversity pertinent to *Gardnerella* and note the limitations of sequence-based studies using different marker genes and priming sites. Although much more study is needed to refine our understanding of how BV develops and persists within the human host, applications of the experimental aspects of Koch's postulates have provided an important glimpse into some of the causal relationships that may govern this condition *in vivo*.

Keywords: ***Gardnerella vaginalis*, animal model, bacterial vaginosis, dysbiosis, co-infection**

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INTRODUCTION

Bacterial vaginosis (BV) is a dysbiosis—a condition of the vaginal microbiome that has been associated with a wide variety of adverse health outcomes. The condition is characterized by low levels of "healthy" lactobacilli and overgrowth of diverse bacteria from other taxonomic groups, including *Gardnerella, Atopobium, Mobiluncus, Prevotella, Bacteroides, Anaerococcus, Peptostreptococcus, Sneathia, Leptotrichia*, and members of the class *Clostridia*, among others (Ravel et al., 2011; Srinivasan et al., 2012). BV has been associated with higher risk of sexually transmitted infections (Wiesenfeld et al., 2003; Brotman et al., 2010; Van De Wijgert, 2017), urinary tract infections (Harmanli et al., 2000; Hillebrand et al., 2002), post-surgical complications (Watts et al., 1990), infertility (Spandorfer et al., 2001), pregnancy losses (Ralph et al., 1999), preterm birth (Svare et al., 2006), intrauterine (Di Paola et al., 2017; Ádám et al., 2018) and intraamniotic infections (Silver et al., 1989), as well as cervical infections, dysplasia, and cancer (Di Paola et al., 2017; Laniewski et al., 2018; Brusselaers et al., 2019). These conditions and health complications associated with BV and its member bacteria can be caused not only by bacteria, but also by eukaryotic (e.g., trichomoniasis) (Jarrett et al., 2019) and viral (e.g., HIV, HSV, HPV) pathogens. In addition, women with diverse BV-like microbiomes are more likely to exhibit signs of genital inflammation (Lennard et al., 2017) and vaginal colonization by other potential pathogens such as beta-hemolytic streptococci (Cherpes et al., 2005) and *Fusobacterium nucleatum* (Hillier et al., 1993; Hill, 1998; Hitti et al., 2001). BV is characterized by reduced numbers of lactobacilli and overgrowth of a polymicrobial consortium often containing large numbers of *Gardnerella vaginalis* (Shipitsyna et al., 2013; Balashov et al., 2014). Despite all of this, many/most women do not report symptoms of BV to their providers, even in settings where the clinical signs and/or inflammatory markers are evident (Masson et al., 2019).

First reported as "*Haemophilus vaginalis,*" the organism now known as *Gardnerella vaginalis* was reported to be the sole cause of clinical signs and symptoms now used to diagnose BV (then referred to as non-specific vaginitis, NSV) (Gardner and Dukes, 1954, 1955). However, controversies surrounding the clinical significance of *Gardnerella* have abounded since its debut in the literature in the 1950s. Today the pendulum has swung in the opposite direction. While some believe that *G. vaginalis* may be a sole causal contributor to BV (Schwebke et al., 2014b), others have been skeptical of this and consider *G. vaginalis* to be one of many within the BV consortium (Hickey and Forney, 2014; Schwebke et al., 2014a). Recently, a more complex model has been proposed, taking into account recent efforts to build animal models for BV and arguing that relationships between multiple microbes, including *G. vaginalis,* may underpin the condition (Muzny et al., 2019a).

Here we review and consider the implications of contemporary and historical findings from studies reporting experimental infections *in vivo* using *Gardnerella vaginalis*. From the early experimental studies in women, to more recent attempts to replicate clinical signs and associated health complications of BV in small animal models, the existing studies paint a complex picture that reflects the biology of the mammalian vagina and the organisms that colonize it. In this review, we will evaluate the evidence from experimental inoculations in humans and other animals that *G. vaginalis* (on its own, or together with other organisms) has a causal role in generating features or complications that have been linked with BV. We also review and discuss the interpretations of some of the earlier experiments in light of our current appreciation about *Gardnerella* and BV, specifically considering the genetic diversity among *Gardnerella* strains (Ahmed et al., 2012; Janulaitiene et al., 2018; Vaneechoutte et al., 2019).

HISTORICAL CONTEXT AND CURRENT CONTROVERSIES

Originally the diagnostic term "non-specific vaginitis" (NSV) was used to describe vaginal symptoms of unknown etiology, but Gardner and Dukes suggested in the 1950's that >90% of NSV cases were caused by a single organism-*G. vaginalis* (Catlin, 1992). A substantial body of clinical literature links both NSV (and as it later became known BV) to adverse health outcomes. Although the term "vaginitis" suggests otherwise, NSV was not typically associated with canonical signs of inflammation such as redness or swelling. Even in the 1950s, Gardner and Dukes realized that "argument may be advanced against calling this infection an inflammatory disease as the term vaginitis implies; however, it must continue to be included in this category until such time as a more suitable term has been proposed and accepted" (Gardner and Dukes, 1959). It would be decades later before "bacterial vaginosis" (BV) started to be used in the literature as a diagnostic term that more accurately represented the condition in question. Around the time "BV" started to be used, Amsel et al. outlined a set of clinical criteria, now known as the Amsel criteria, to diagnose NSV (Amsel et al., 1983). Today, the Amsel criteria is still used clinically for the diagnosis of BV (C.f.D.C.a. Prevention, 2015). Three of four of the following (Amsel) criteria are generally considered to support the diagnosis of BV: (1) a thin, homogenous discharge, (2) higher than normal pH (>4.5), (3) the presence of "clue" cells in wet mount (epithelial cells that appeared to be coated in bacteria), and (4) the detection of a fishy odor, with or without treatment of the sample with 10% potassium hydroxide. BV can also be diagnosed via the Nugent scoring method. Although high interobserver variability of the Nugent score has been noted in some studies (Forsum et al., 2008), it remains the current gold standard for laboratory-based BV diagnosis used in clinical research. First published by Nugent et al. (1991), this method does not rely on clinical signs of BV, but instead uses Gram-stained smears of vaginal fluid to evaluate the balance of bacterial morphotypes present in the genital tract, resulting in a score from 0 to 10 (Nugent et al., 1991). A Nugent score of 0–3 indicates a normal *Lactobacillus*-dominant microbiota, 4–6 indicates an "intermediate microbiota," and a score of 7–10 indicates BV (Nugent et al., 1991; Amegashie et al., 2017).

Additional characteristics of BV include lower viscosity vaginal fluids, a shift in fermentation products and other metabolites, presence of the diamines putrescine and cadaverine,

detectable levels of the enzyme sialidase and the liberation and overall depletion of mucosal sialic acids (Briselden et al., 1992; Catlin, 1992; McGregor et al., 1994; Olmsted et al., 2003; Lewis et al., 2013; Chappell et al., 2014; Srinivasan et al., 2015; Nelson et al., 2018). BV is still clinically considered a non-inflammatory condition, as it is not typically associated with edema of vaginal tissue (Mitchell and Marrazzo, 2014) nor increased numbers of neutrophils in the cervicovaginal space (Giraldo et al., 2012). However, several studies have reported increased levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and IP-10 (among others) in vaginal specimens from women with BV as compared to "healthy" controls (Cauci et al., 2002, 2008; Hemalatha et al., 2012). The apparent discrepancy between clinical findings (lack of neutrophils/overt inflammation) is not fully understood (Forsum et al., 2005). High resolution studies evaluating relationships between chemokine/cytokines and specific types of communities or member bacterial taxa associated with BV have found that larger proportions of *Prevotella* and other microbes were associated with a more pro-inflammatory phenotype and increased risk of adverse health outcomes (Anahtar et al., 2015; Gosmann et al., 2017; Lennard et al., 2017; Shannon et al., 2017; Fettweis et al., 2019). However, in general, high levels of neutrophils present in vaginal secretions is indicative of other conditions such as "desquamative inflammatory vaginitis" or "aerobic vaginitis" (Donders et al., 2002; Mason and Winter, 2017). Finally, we emphasize that not all cases BV are characterized by the same signs/symptoms or the presence of clue cells and no single species is a universal marker of BV (Srinivasan et al., 2012).

Gardnerella vaginalis has had a long history of controversy related to its taxonomy and clinical significance, some of which continues to this day. Around the same time the organism was isolated by Gardner and Dukes from the vaginas of women with nonspecific vaginitis (NSV) (Gardner and Dukes, 1954, 1955), it was also identified as a "*Haemophilus*-like" bacterium found in patients with cervicitis and prostatitis (Leopold, 1953). These first reports classified it as a *Haemophilus* species because they found it negative by Gram staining and unable to grow on agar without blood (Turovskiy et al., 2011). However, later studies revealed that *H. vaginalis* did not require hemin or nicotinamide adenine dinucleotide (NAD) for growth, making it clear that the organism was not actually a member of the *Haemophilus* genus. Additionally, the bacterium appeared to sometimes have a positive Gram staining reaction. For these reasons *H. vaginalis* was reassigned to the *Corynebacterium* genus, and renamed *Corynebacterium vaginale* (Catlin, 1992). This name too proved to be inaccurate, as unlike *Corynebacterium* species, the bacterium was catalase negative and did not have arabinose in its cell wall (Turovskiy et al., 2011). In 1980, two large taxonomic studies used DNA-hybridization, electron microscopy, and a variety of biochemical methods to show that the bacterium lacked close similarity with any previously established genera (Greenwood and Pickett, 1980; Piot et al., 1980; Catlin, 1992). This lead to the proposal of a new genus, *Gardnerella*, and the name *Gardnerella vaginalis* for the bacterium (Greenwood and Pickett, 1980).

Despite early confusion about the taxonomy of what came to be known as *Gardnerella*, Gardner and Dukes believed they had found the sole etiological agent of most cases of NSV. The 1955 paper reported that among the (Caucasian) women from Gardner and Dukes gynecology practices, *H. vaginalis* was isolated from the lower genital tract of 127 out of 138 women diagnosed with NSV (92%), but none of the 78 healthy women examined. Gardner and Dukes argued that the vast majority of NSV cases were really cases of *H. vaginalis* infection (Gardner and Dukes, 1955). But, not long after *H. vaginalis* was discovered as a potential causative agent of NSV, it became a point of controversy. This topic remains controversial to this day, with some recent studies using sensitive molecular techniques suggesting that most women (including >60% without BV) have *Gardnerella* colonizing the vagina, although women without BV had ∼4 orders of magnitude lower levels compared to women with BV (Balashov et al., 2014; Cox et al., 2015). Of note, primer sets that target variable regions 1 and 2 of the gene encoding 16S often see lower relative levels of *Gardnerella* in women with BV when compared to studies that target variable regions 3 and/or 4 or that use culture-based studies (Graspeuntner et al., 2018). It has been recently emphasized that 16S gene sequences cannot differentiate the genetically divergent subsets of *Gardnerella* and that other methods (e.g., cpn60 sequencing) are more effective in this regard (Hill et al., 2019). Regardless of the method used to detect *Gardnerella* in women, whether the organism is a causal factor in BV cannot be determined by observational study. A number of experimental studies in humans and other animals, which we review here, have been conducted in an attempt to address this question.

Methodology in brief: The purpose of this review is to evaluate the experimental evidence for *G. vaginalis* as a causal factor in generating the features or health complications that have been associated with BV. Thus, in general, we sought to focus on publications that had a similar experimental intent. While this was not intended to be a systematic review, we first searched PubMed using the search string: [("*Gardnerella*" or "*Haemophilus vaginalis*") AND ("murine" OR "mouse" OR "mice" OR "*in vivo*" OR "animal" OR "model")]. Some papers, especially older literature, could not be found using these specific search terms. To find additional papers relevant to the topic, we also carefully reviewed several published reviews on BV and/or *Gardnerella* that addressed animal models (Catlin, 1992; Turovskiy et al., 2011; Herbst-Kralovetz et al., 2016). Once the papers were located, they were screened for inclusion in this review based on the following criteria: (1) The papers were available in English, (2) They described an *in vivo* inoculation of *G. vaginalis* into the urogenital tract of an animal (including humans), and (3) After inoculation, experimental hosts were assessed for one or more features or health complications previously associated with BV. Based on these criteria, we excluded papers in which *G. vaginalis* was isolated from animals but never experimentally inoculated, inoculation of *G. vaginalis* was not into the urinary or reproductive tract, or the sole intent was to introduce an intervention to eliminate *G. vaginalis* colonization rather than to understand the causal or mechanistic role of *G. vaginalis* interaction with the experimental host.

EXPERIMENTAL STUDIES *IN VIVO* USING *Gardnerella* ALONE

Vaginal Inoculation in Women

The earliest *in vivo* experiments using *G. vaginalis* were performed in women with the intent of demonstrating "proof of pathogenicity." Gardner and Dukes suggested that *G. vaginalis* could trigger "bacterial vaginitis" in otherwise healthy women. Gardner and Dukes asserted that *G. vaginalis* was the sole causative agent in most cases of the condition (Gardner and Dukes, 1955). In their experimental infections, they vaginally inoculated 13 healthy female volunteers with "pure cultures" of several different strains of *G. vaginalis* that had been isolated from women with BV (although specific strains or culture conditions were not reported, see **Table 1**). Of the inoculated women, 10 of 13 tested negative for *G. vaginalis* after inoculation. Two women had positive cultures for *G. vaginalis* for 2–3 months, but showed none of the clinical signs of NSV previously described by Gardner and Dukes (vaginal pH >5, the presence of clue cells, thin homogenous discharge, and a decrease in vaginal lactobacilli). Only one of the 13 women was both culture-positive for *G. vaginalis* and displayed clinical signs (Gardner and Dukes, 1955).

A second experiment was performed with 15 healthy female volunteers, but the women were inoculated directly with vaginal material from patients with NSV rather than cultured *G. vaginalis*. This inoculation resulted in NSV signs in 11 of 15 women. In all of the patients that displayed clinical symptoms, *G. vaginalis* was recovered by culture. Gardner and Dukes attributed the low-incidence of vaginitis upon infection with pure *G. vaginalis* cultures compared to direct inoculation with vaginal material, to a loss of viability during serial passaging of the bacteria (i.e., repeated culturing of the strain in the laboratory) (Gardner and Dukes, 1955). However, looking back from a modern perspective, these findings have other potential explanations. For example, culture conditions are not stated and viability of the inoculum used to infect women was not evaluated; limited viability could have been due to growth phase rather than serial passage. Alternatively, perhaps this was an early indication that other organisms in addition to *G. vaginalis* may play a role in BV. Consistent with the reproducible initiation of BV by exposure to vaginal material (Gardner and Dukes, 1955), a number of modern investigations have shown that specific sexual practices that would result in a similar exposure to vaginal material are linked with higher risks of BV, including the sharing of sex toys between women who have sex with women (Bradshaw et al., 2014; Olson et al., 2018; Muzny et al., 2019b).

Criswell et al. (1969) reported another attempt to induce NSV in women via inoculation with pure cultures of *G. vaginalis*. Twenty-nine pregnant volunteers were vaginally inoculated with one of four different *G. vaginalis* strains (about 2×10^7 CFU), which had been grown for either 12 or 24 h in liquid media. The women were observed for development of NSV (referred to as "*H. vaginalis* vaginitis"). Only two of the 20 subjects inoculated with a 24-h culture of *G. vaginalis* developed symptoms, but five of the nine women that were inoculated with a 12 h culture showed clinical signs of *H. vaginalis* (see **Table 1**). The primary conclusion by Criswell et al. was that these results confirmed the earlier human experiments and hypotheses put forward by Gardner and Dukes, namely that *G. vaginalis* infection alone could trigger NSV. Their findings suggested that the age of the inoculum affected the ability of *G. vaginalis* to colonize the host resulting in clinical signs of NSV (Criswell et al., 1969). We will explore this concept further in Section Discussion.

Vaginal Inoculation in Non-human Primates

In 1984, Johnson et al. published an attempt to model NSV in non-human primates (Johnson et al., 1984) (see **Table 1**). Twelve pig-tailed macaques, six tamarins, and four chimpanzees were vaginally inoculated with one of four different *G. vaginalis* strains. These strains had been collected from women diagnosed with NSV at a London clinic. The authors were unable to recover *G. vaginalis* from any tamarins or chimpanzees 5–7 days after inoculation. Only the pig-tailed macaques appeared susceptible to colonization with the strain used in this setting, as all of the inoculated animals remained culture-positive for *G. vaginalis* for 11–39 days. It was noted that the *G. vaginalis* strain used for inoculation of chimpanzees was different than the one used in macaques, and the former isolate had been extensively passaged in the laboratory, whereas the latter had only been passaged once. One of the two control macaques receiving PBS (vehicle alone) had detectable *G. vaginalis* later in the experiment, which the authors attributed to cross-contamination from a colonized animal during vaginal washing (Johnson et al., 1984).

The authors examined the infected pig-tailed macaques for clinical signs of NSV. No clue cells were observed in the vaginal smears, regardless of whether the animal was successfully colonized with *G. vaginalis*. Women with NSV had been previously shown to exhibit higher vaginal pH and a higher ratio of succinate to lactate compared to women without NSV (Amsel et al., 1983). However, the authors also did *not* see an increase in vaginal pH or higher succinate to lactate ratio in the colonized animals. This may be explained by differences in vaginal physiology and the endogenous microbes between humans and macaques. *Lactobacillus*-dominant vaginal microbiomes in women produce lactate, generating the characteristically low vaginal pH (3.8–4.5) seen in most women (Boskey et al., 1999, 2001). The authors noted that compared to these "normal" women, macaques at baseline already seemed to possess NSV-like characteristics, including a high pH (6.0–7.0) and a high succinate to lactate ratio (Johnson et al., 1984). The authors also noted a high baseline vaginal pH in the chimpanzees (5.5–6.0), and tamarins (7.0). These data suggest that non-human primates have a fundamentally different composition of microbes with different metabolic properties compared to women. More recent studies using molecular approaches have confirmed that macaque and other non-human primate vaginal microbiomes typically reflect a scarcity of lactobacilli accompanied by diverse microbial taxa, some of which are also found in human BV (Uchihashi et al., 2015; Zhu et al., 2015; Obiero et al., 2016; Miller et al., 2017). A 2010 study identified *G. vaginalis* via 16S sequencing in the vaginal microbiome of Rhesus macaques. However, *G. vaginalis* was found in only 2 of the 11 macaques and

TABLE 1 | Experimental studies of *G. vaginalis* infection in humans and other primates.

References	Host species	Bacterial strain(s)	Culture media	Culture condition	Culture time (h)	Dose	Route	N	n colonized by *G.v.*	Positive for features/complications of BV
Gardner and Dukes, 1955	Human (pregnant and non-pregnant)	NR	NR	Limited oxygen	NR	NR	Vaginal	13	3/13	1/13 positive for *G.v.* & clinical signs: homogenous, odorous discharge; pH>5.0
		"Material from the vagina"	N/A	N/A	N/A	N/A	Vaginal	15	11/15	11/15 positive for *G.v.* & clinical signs: homogenous, odorous discharge; pH>5.0
Criswell et al., 1969	Human (pregnant)	*G.v.* 3299, 3309, 3310	Biphasic. Bacto Casman's Agar + 5% rabbit serum	10% CO_2	24	2×10^{10}	"Poured [broth cultures] into the vagina"	15	2/15	2/15 *G.v.* positive & clinical signs: gray, thin, homogenous, odorous discharge; pH >5.0, clue cells, Gram neg rods in smears
		G.v. 594 (ATCC 14018)			24			5	0/5	0/5 *G.v.* positive; no clinical signs (as above)
					12			9	5/9	5/9 *G.v.* positive & clinical signs (as above)
Johnson et al., 1984	Pig-tailed macaque	*G.v*. 584, 614	Peptone-starch-dextrose broth + 10% horse serum	NR	24	5×10^6- 1×10^7	Intravaginal (catheter or pipette)	10	10/10	0/10 had clue cells; increased pH; non-volatile fatty acids
	Tamarin	*G.v.* 584				3×10^6		4	0/4	0/4 had increased pH
	Chimpanzee	*G.v.* 812 and 958	Bordet-Gengou agar	NR	48	5×10^7- 1×10^8		3	0/3	0/3 had clue cells; increased pH
Mårdh and Møller, 1984	Grivet monkeys	*G.v.* L824 LCR L100 (long curved rod) SCR L1599 (short curved rod)	NR	Anaerobic or 10% CO2	48	2×10^9	Intravaginal (swab)	8	2/8	Profuse, thin gray discharge observed in 2/2 animals infected with *G.v.* + LCR, 0/3 mono-infected animals. *G.v.* recovered only from *G.v.* + LCR infected animals. No clue cells, increased odor, or elevated vaginal pH observed in any animals

was present at relatively low levels—unlike the high abundance and prevalence of *G. vaginalis* in human BV (Spear et al., 2010).

DNA as a Component of *G. vaginalis* Biofilms

BV can be treated with antibiotics (most often metronidazole and clindamycin) and antiseptics (e.g., dequalinium chloride) (Mendling et al., 2016). However, though initially effective in ∼80% of cases, the rates of recurrence following antibiotic treatment are extremely high; >50% of women will have recurrent episode(s) within 6–12 months (Bradshaw and Brotman, 2015). One proposed explanation for these high rates of recurrence is the presence of *G. vaginalis* biofilms. Dense biofilms containing *G. vaginalis* have been identified on the vaginal epithelium of women with BV (Swidsinski et al., 2005), and these biofilms can sometimes re-form after oral metronidazole treatment (Swidsinski et al., 2008). In 2013, Hymes et al. demonstrated *in vitro* that *G. vaginalis* biofilms formed on polystyrene plates contain extracellular DNA. The biofilms could be disrupted by DNase application, resulting in a ∼5-fold reduction of bacterial titers measured in biofilms. Furthermore, using a vaginal *G. vaginalis* infection model in C57BL/6 mice, the authors also demonstrated that *in vivo* DNAse treatment resulted in a >10-fold reduction in *G. vaginalis* titers in the mouse vagina after 48 h (see **Table 2** for comparison to other rodent colonization models). However, the authors did not evaluate whether *G. vaginalis* forms biofilms in the mouse vagina, and colonization density was low at 48 h, even in untreated mice (Hymes et al., 2013). Other than the presence of *G. vaginalis* in the vagina, this study did not examine or report the presence of other features of BV.

Vaginal Infection With *G. vaginalis* in Mice Results in Several Clinical Features of BV

In 2013, Gilbert et al. described a model of *G. vaginalis* vaginal inoculation into C57BL/6 mice (6-8 weeks of age) using a bacterial strain that had been recently isolated from a woman with BV (see **Table 2**) (Gilbert et al., 2013). The mice were treated with 17-β estradiol to bring them in proestrus, a technique that had previously been used to model vaginal infection with *Neisseria gonorrhoeae* (Jerse et al., 2011). *G. vaginalis* colonized the mouse vagina and ascend into the uterine horns. *G. vaginalis* titers in vaginal washes and vaginal homogenates were strongly correlated, allowing for monitoring of *G. vaginalis* colonization in vaginal washes prior to the study's endpoint. Uterine and vaginal titers were also significantly correlated, suggesting that the degree of vaginal colonization was an important factor in ascending infection by the bacterium. In addition to the presence of *G. vaginalis* in vaginal fluid, several other BV-like features were detected in the model. There was a strong correlation in vaginal washes between the amount of live *G. vaginalis* and levels of sialidase activity, an enzyme that has been used as the basis for a point of care BV diagnostic test (Myziuk et al., 2003; Bradshaw et al., 2005). Mice inoculated with *G. vaginalis* also exhibited clue-like cells in vaginal washes, which were confirmed to be coated with *G. vaginalis* that were fluorescently labeled prior to inoculation. Although the literature often refers to epithelial exfoliation as an explanation for the presence of clue cells, this manuscript provided the first measurements of exfoliated epithelial cells, both in the mouse model and comparing women with and without BV. Significantly higher levels of exfoliated cells were observed in animals infected with *G. vaginalis* compared to mock-infected controls. Interaction between *G. vaginalis* and mouse epithelial cells was visualized by fluorescence microscopy (**Figure 1**). Likewise, women with Nugent-defined BV had significantly higher levels of exfoliated cells in Gram-stained slides compared to women without BV. Finally, as in women, the *G. vaginalis* vaginal colonization did not seem to induce classical signs of inflammation, as evidenced by the lack of neutrophil infiltration or edema (Gilbert et al., 2013). However, the lack of inflammation could also be a result of estradiol administration.

Gardnerella vaginalis IN ASCENDING INFECTION

G. vaginalis and other BV-associated bacteria are commonly isolated from human endometrial, placental, intraamniotic and perinatal infections (Berardi-Grassias et al., 1988; Hillier et al., 1988; Silver et al., 1989; Watts et al., 1990; Gibbs, 1993; Goldenberg et al., 1996; DiGiulio et al., 2010; DiGiulio, 2012; Petrina et al., 2019). In this section, we examine the ability of *G. vaginalis* to cause intrauterine infections after vaginal inoculation or elicit health complications associated with ascending infection during experimental infection.

Intrauterine Inoculation in Pregnant Rabbits

Two studies have used a rabbit intrauterine infection model to investigate the impact of *G. vaginalis* in the upper reproductive tract during pregnancy (see **Table 2**). In this model, *G. vaginalis* (strain ATCC14018) was transcervically administered directly into each uterine horn by threading a cannula through the cervix (Field et al., 1993). The stated goal of these experiments was to determine if intrauterine infection by *G. vaginalis* would lead to preterm birth, fetal abnormalities, or maternal morbidity. The results indicated a significantly lower live-birth rate in the *G. vaginalis* inoculated rabbits compared to the saline-inoculated controls; however, there was not a greater incidence of preterm birth among animals in the infected group. In 88% of the infected animals, *G. vaginalis* could be detected in the amniotic fluid. Additionally, the fetal and placental weights on the viable fetuses were lower in the *G. vaginalis* infected group. The saline-inoculated uterine horns did not appear inflamed, but clinical signs of inflammation and histological deciduitis were observed in the uterine horns that received *G. vaginalis*. There was also significantly more evidence of neuropathology, including severe brain injury, in the *G. vaginalis* exposed fetuses. From these results, the authors conclude that *G. vaginalis* in the upper reproductive tract has pathophysiological consequences for both maternal and fetal tissues (Field et al., 1993).

TABLE 2 | Experimental studies of *G. vaginalis* infection in rodent models.

References	Host species	Bacterial Strain(s)	Culture Media	Culture Condition	Culture time (h)	Dose	Route	N	n colonized by *G.v.*	Positive for features/ complications of BV
Field et al., 1993	New Zealand & Calif. White Rabbits (pregnant)	*G.v.* ATCC 14018	V-selective agar	Increased CO2	48–72	2×10^4-2×10^6	Uterine (transvag/ cervical cannula)	17	N/A	17/17 *G.v.* deciduitis; 15/17 *G.v.* intraamniotic infection; 10/17 severe neuronal injury (0 in controls); 2/17 had preterm labor; infected group had lower weight & high fetal mortality
McDuffie et al., 2002	New Zealand White Rabbits (pregnant)	*G.v.* NR	NR	NR	NR	10^7		16	11/16	*G.v* intraamniotic (9/16), uterine (11/16), blood (7/16), fetal brain (10/16), fetal heart (6/16), and fetal lung (8/16) infection.
Gilbert et al., 2013	C57BL/6 (inbred) mice	*G.v.* JCP8151B	NYC III	Anaerobic chamber	NR (16–18)	5×10^7	Intravaginal (pipette)	39	36/39	Sialidase activity; epithelial exfoliation; clue-like cells, mucus degradation, uterine infection; absence of histologic inflammatory response
Hymes et al., 2013	C57BL/6 (inbred) mice	*G.v.* ARG37 (mouse-passaged ATCC14018)	NR	5% CO2	NR	5×10^6	Intravaginal (5% gelatin)	10	10/10	DNase treatment reduced *G.v.* titers >10-fold
Sierra et al., 2018	CD-1(outbred) mice (pregnant)	*G.v.* ATCC14019	Tryptic Soy Broth + 5% Horse Serum	5% CO2	NR	2.5×10^7 $^-2.5 \times 10^9$ (twice)	Intravaginal (pipette)	50-60	NR	*G.v.* detected in cervicovaginal fluid; increased cervical pro-inflammatory cytokines; increased IL-6 in amniotic fluid, cervical remodeling but no increase in preterm birth
Gilbert et al., 2019	C57BL/6 (inbred) mice	*G.v.* JCP8151B; *P. bivia* ATCC2903	NYC III (*G.v.)* CDC Anaerobe media + 10% laked sheep's blood (*P.b.*)	Anaerobic chamber	NR (16-18)	*G.v.* 8×10^7 *P. bivia* 1-2 $\times 10^7$	Inravaginal (pipette)	31	23/31 (24hr)	Sialidase activity; epithelial exfoliation; increased vaginal *P. bivia* titers in presence of *G.v.*; increased uterine infection by *P. bivia* in presence of *G.v*; absence of inflammatory response
Gilbert et al., 2017	C57BL/6 (inbred) mice	*G.v.* JCP8151B; *E. coli* UTI89	NYC III (*G.v.)*	Anaerobic chamber	18	*G.v.* $\sim 10^8$ (twice) *E. coli* 10^7	Transurethral (catheter)	46	N/A	*G.v.* exposure triggered exfoliation of urothelial cells; emergence of *E. coli* reservoirs; *G.v.* kidney infection, kidney inflammation, more severe *E. coli* kidney infections
Trinh et al., 2011	ICR (outbred) mice	KCTC5096	BHI broth + yeast extract, maltose, glucose, 10% horse serum or general anaerobic medium	Sealed anaerobic jar	"Up to 36 hours"	1.2×10^5	Intravaginal	6	NR	Increased vaginal TNF-α, IL-1β, and IL-6. Decreased IL-10. Increased iNOS, COX-2, and myeloperoxidase acitivity. Histological vaginal inflammation
Joo et al., 2011 Jang et al., 2017					48 h	1×10^6				Increased vaginal TNF-α, IL-1β, IL-17a, COX-2, iNOS, and myeloperoxidase. Decreased IL-10. Histological vaginal inflammation
Kim et al., 2019	C57BL/6 (inbred) mice	NR	General anaerobic medium	NR				7		Increased TNF-α and myeloperoxidase in vagina and uterus. Decreased IL-10 in uterus

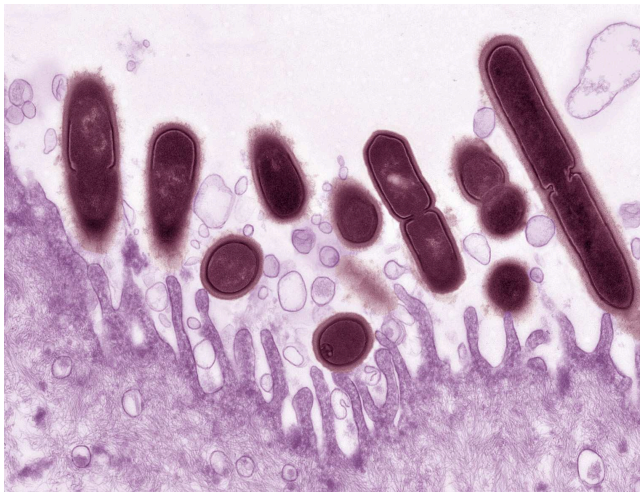

FIGURE 1 | *Gardnerella vaginalis* (maroon) associated with the surface of a mouse vaginal epithelial cell (purple). Epithelial cells were collected from estrogenized mice by vaginal lavage with phosphate buffered saline. Epithelial cells were centrifuged, washed three times to remove endogenous bacteria then incubated for 4 h at 37°C *ex vivo* with *G. vaginalis* strain JCP8151B. Uranyl acetate staining was followed by transmission electron microscopy. Photo credit: Wandy Beatty. This image illustrates how the use of a small animal model can provide new resolution to aspects of BV that we appreciate, but do not fully understand. The pictured interaction provides evidence that in mice, as in women, *G. vaginalis* has an affinity with the vaginal epithelium. More broadly, it also supports the use of mouse models in reflecting at least some of the physiology we believe to occur in women.

Nearly a decade later, another study used the same transvaginal cervical cannulation model of *G. vaginalis* infection in pregnant rabbits (McDuffie et al., 2002). In this study, the animals were sacrificed prior to parturition on days 4, 5, or 6 post-inoculation, and a wide array of fetal and maternal tissues were cultured to detect live *G. vaginalis*. *G. vaginalis* was detectable (though titers were not reported) in the fetal brain, heart, and lung of some infected animals, as well as in maternal blood, uterine tissue, and amniotic fluid (see **Table 2**). Histological evidence of fetal brain damage was greater in the infected group, although the authors did not state whether the fetuses showing signs of neuropathology were also those that had detectable *G. vaginalis* titers in the brain. Additionally, the authors do not report the strain of *G. vaginalis* used in this study, or from where it was isolated (McDuffie et al., 2002). Of note, *G. vaginalis* has been reported as a cause of bacteremia in pregnant women and in at least one case, in a preterm neonate (Monif and Baer, 1976; Venkataramani and Rathbun, 1976; Reimer and Reller, 1984; Boggess et al., 1996; Agostini et al., 2003; Chen et al., 2018).

An important limitation of these two studies (Field et al., 1993; McDuffie et al., 2002) was that they bypassed the normal cervical barriers to ascending infection. As with many of the experimental studies of *G. vaginalis* infection, this study used a relatively high inoculum (see **Tables 1**, **2**); it is unclear what amount of *G. vaginalis* exposure to the upper reproductive tract is physiologically relevant. It would have been informative if one of these studies had included an experimental control group in which "beneficial" vaginal bacteria (e.g., *Lactobacillus crispatus*) were introduced in the same manner (transvaginal cervical cannulation) to evaluate whether a non-pathogenic organism would be culturable from tissues or stimulate maternal and fetal immune responses when introduced in this manner. It is notable that other BV bacteria (e.g., *Prevotella bivia*) have been delivered using the same model, resulting in maternal fever and preterm delivery in up to 33% of animals (Gibbs et al., 2004). Intrauterine, intraperitoneal, or intravenous exposures to bacterial products such as lipopolysaccharide (Gram negative bacteria) (Fidel et al., 1998) or lipoteichoic acid (Gram positive bacteria) (Kajikawa et al., 1998) have also been used as models of preterm birth in pregnant mice.

Vaginal Inoculation of *Gardnerella vaginalis* in Pregnant Mice Induces Cervical Remodeling

BV has been linked to increased risk of preterm birth, although the mechanisms linking such associations are still unclear (Svare et al., 2006). Sierra et al. (2018) used a pregnant mouse model of *G. vaginalis* infection to investigate the connection between BV and preterm birth. Timed-pregnant CD-1 mice were vaginally inoculated twice (on embryonic (E) days 12 and 13) with either 2.5×10^7 or 2.5×10^9 CFU of *G. vaginalis*. Animals were then sacrificed 48 h after the second dose (E15) or allowed to proceed to parturition. The authors did not observe a significant increase in preterm birth (defined as delivery any day before E18) in the *G. vaginalis* infected animals. There was also not a significant difference in pup weight or litter size between the control and infected animals. However, in those dams sacrificed on E15, the authors observed signs of inflammation and cervical remodeling following *G. vaginalis* inoculation. *G. vaginalis* was found in the lower reproductive tract at sacrifice, as determined by PCR. Although it is unclear what the detection limit was in these experiments, the authors were unable to detect the bacterium in the uterine horns, placenta, or fetal membranes. Histology revealed increased mucicarmine staining (interpreted as increased mucin, but other acidic glycans could also explain this result). The authors conclude that increased mucin production in pregnancy may reflect enhanced protection against ascending infection. We note that the ATCC14018 strain used in these experiments is negative for sialidase and does not encode the genes recently shown to be responsible for sialidase activity in cultured isolates (Robinson et al., 2019). Thus, it is also possible that this strain lacks the ability to cause ascending infection due to an inability to engage in mucin degradation. There was a significant increase in IL-6 protein levels in the cervicovaginal fluid of infected animals as compared to the controls. Interestingly, there was also increased IL-6 in the amniotic fluid (AF) of infected animals, despite the apparent lack of upper-reproductive tract colonization by *G. vaginalis*. Infected mice also had increased soluble E-cadherin in the cervicovaginal space, a biomarker of cervical epithelial remodeling, and increased expression of Tff-1 gene (Sierra et al., 2018), which is also thought to be important in the remodeling process (Akgul et al., 2014). There was also increased

transcript levels of the pro-inflammatory cytokines IL-8, IL-1β, and IL-10 in the cervices of infected mice. The authors also observed dispersion of collagen fibers in the cervices of infected dams. Biomechanical testing showed that cervices of *G. vaginalis* infected animals displayed significantly lower modulus and higher maximum strain, but displayed no difference from the control group in tissue cross sectional area, maximum load, stiffness, or maximum stress. These data provide evidence in support of the conclusion that cervical softening may be "occurring faster/earlier" in response to *G. vaginalis* (Sierra et al., 2018). While remodeling of the cervical epithelium is a normal part of pregnancy as the body prepares for parturition (Timmons et al., 2010), early induction of this remodeling by *G. vaginalis* could contribute to preterm birth.

Gardnerella Infection and Vaginal Inflammation

As previously discussed, there is some controversy surrounding BV's classification as an inflammatory or non-inflammatory condition. Most cases of BV lack clinical signs of overt inflammation such as swelling and redness (Mitchell and Marrazzo, 2014), which seems at odds with studies that report increased levels of inflammatory cytokines (Cauci et al., 2002, 2008; Hemalatha et al., 2012). To the best of our knowledge, cytokine responses to *G. vaginalis* infection have not been extensively studied using animal models. One exception is the study just discussed by Sierra et al. in which authors found elevated transcripts of several inflammatory cytokines in *G. vaginalis* infected mice (Sierra et al., 2018). Another notable exception is a group of studies published by a single group, which investigate probiotic strategies to treat BV using a mouse model of *G. vaginalis* infection (Joo et al., 2011; Trinh et al., 2011; Jang et al., 2017; Kim et al., 2019). These studies assessed immune markers of vaginal inflammation in response to inoculation with *G. vaginalis*, to determine if these markers were lower in animals receiving probiotics. These papers performed intravaginal inoculation with the *G. vaginalis* strain KCTC5096 to model BV in β-estradiol treated mice (Joo et al., 2011; Trinh et al., 2011; Jang et al., 2017) [note: bacterial strain not reported in] (Kim et al., 2019). The mouse strains used were ICR in two of the papers (Joo et al., 2011; Trinh et al., 2011), and C57Bl/6 in the others (Jang et al., 2017; Kim et al., 2019).

Overall, all four studies report finding increased levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (determined by ELISA) in the vaginal tissue of infected mice as compared to non-infected controls (Joo et al., 2011; Trinh et al., 2011; Jang et al., 2017; Kim et al., 2019). The authors also reported that IL-10, often regarded as an anti-inflammatory cytokine (Couper et al., 2008), was consistently present at lower levels in *G. vaginalis* infected mice compared to controls. In addition to cytokines, the authors examined evidence of *G. vaginalis*-induced inflammation by assessing myeloperoxidase, iNOS, and COX-2 levels in the vaginal tissues of infected mice. The authors report that mice inoculated with *G. vaginalis* had higher levels of these inflammatory markers compared to uninfected controls. Additionally, when histology was performed on vaginal tissue, there appeared to be substantial edema and immune cell infiltrate into the superficial mucosal layers (Joo et al., 2011; Trinh et al., 2011).

A caveat to the findings of these studies is that some aspects of the methodology are obscure. For example, it is not always clear if the control group of uninfected mice were given β-estradiol treatment. If they were not, it becomes impossible to compare the infected vs. non-infected groups, as any differences in inflammatory markers or histopathology could simply be the result of only one group receiving hormone treatment. Additionally, in at least one of the studies, the inocula of *G. vaginalis* seems to be inconsistent between figures (Jang et al., 2017). None of the data reported in these four publications show individual data points to represent the mice in each group and the routine use of means and standard deviation might make these data more sensitive to skewing by outliers. Finally, several of the reported interpretations of increased vaginal inflammation rely on immunoblots that were not quantified and histological images that were not scored (Joo et al., 2011; Trinh et al., 2011; Jang et al., 2017).

The question of BV and inflammation remains a tricky one. Future *in vivo* studies aimed at untangling this mystery will need to be rigorous about having appropriate controls and reporting the data and methods in clear detail.

UNDERSTANDING HOW *G. vaginalis* MAY INFLUENCE THE GROWTH OR PATHOGENESIS OF OTHER BACTERIA

Despite Gardner and Dukes early assertions that *Gardnerella* was the sole causative agent of BV, this is not a universally accepted hypothesis (Hickey and Forney, 2014; Schwebke et al., 2014a,b). Another hypothesis is that multiple species of bacteria are needed to generate the features and complications that have been associated with the condition (Muzny et al., 2019a). There is also evidence, however, that *G. vaginalis* may still have a role in the development of features that it does not directly cause, and may do so by impacting the abundance or pathogenesis of other organisms.

The Characteristic Fishy Odor of BV Cannot Be Solely Attributed to *G. vaginalis*

In 1979 Chen et al. identified seven amines in the vaginal fluid of women with BV (then NSV) that were absent in the fluid of healthy controls. The authors theorized that these amines likely contribute to the "fishy" odor associated with the condition, and showed that the amines could be produced *in vitro* by the vaginal bacteria isolated from NSV patients. However, while these mixed vaginal communities were able to produce amines, *G. vaginalis* isolates alone did not do so under the conditions studied, suggesting that one of the major diagnostic features of BV cannot be solely attributed to the presence of *Gardnerella* (Chen et al., 1979). This finding is supported by a more recent report from Nelson et al. (2015), which sought to identify the vaginal bacteria capable of producing the BV-associated amines. The authors looked for homologs of characterized biogenic

amine-synthesizing proteins in the genomes of common vaginal bacterial taxa. They found no evidence of any predicted genes encoding biogenic amine-synthesizing proteins in the four *G. vaginalis* strains they investigated (Nelson et al., 2015).

If *G. vaginalis* is unable to produce the amine species characteristic of BV, then it is necessary to re-examine interpretations from some of the first *Gardnerella* infection models. The early human experiments by Criswell et al. (1969) found that vaginal inoculation of pure *G. vaginalis* cultures could replicate the clinical symptoms of "*H. vaginalis* vaginitis" as described by Gardner and Dukes. These symptoms included the "characteristic offensive odor" of the vaginal discharge (Criswell et al., 1969). Assuming that the authors are describing the same "fishy" odor as the Amsel criteria, this means that inoculation with *G. vaginalis* was able to lead to the presence of various amines in vaginal fluid. If *G. vaginalis* itself is unable to produce these amines, this would suggest that colonization of the vaginal tract by *G. vaginalis* also encourages the growth of amine-producing bacterial species. Even at the time, Chen et al. (1979) noted a possible symbiosis between *G. vaginalis* and amine-producing bacteria, observing that while *G. vaginalis* cannot produce amines *in vitro*, it does release large concentrations of pyruvic acid and amino acids during growth. Conversely, amino acids and pyruvic acid were depleted in the media of the NSV mixed vaginal communities that were able to produce amines, indicating the possibility that metabolites produced by *G. vaginalis* can be consumed by amine-producing vaginal bacteria (Chen et al., 1979).

Currently the BV field has identified several vaginal bacterial taxa that have either been shown to produce amines *in vitro* or have predicted amine-synthesizing proteins, including *Prevotella*, *Dialister,* and *Eggerthella* species (Pybus and Onderdonk, 1997; Nelson et al., 2015). Srinivasan et al. (2012) showed that the presence of these taxa in the vaginal microbiome was associated with the Whiff test, lending creed to their potential role as amine producers. Notably, Srinivasan et al. (2012) also found that the Whiff test was associated with the presence of *G. vaginalis*. This finding seems to provide additional evidence to the possible symbiosis between *G. vaginalis* and amine-producers in the vaginal environment (Srinivasan et al., 2012).

G. vaginalis Encourages Ascending Uterine Infection by *Prevotella bivia* in Non-pregnant Mice

In 2019, Gilbert et al. described an estradiol-treated mouse co-infection model with *G. vaginalis* and *Prevotella bivia* (Gilbert et al., 2019). *Prevotella* species are prevalent in the vaginal microbiome of women with BV (Srinivasan et al., 2012). Additionally, like a number of other BV-associated organisms, vaginal colonization by *P. bivia* has been linked to higher rates of preterm birth (Krohn et al., 1991). In the reported model, both *G. vaginalis* and *P. bivia* were able to colonize mouse vaginas on their own, but co-inoculation with *G. vaginalis* led to higher *P. bivia* vaginal titers 1-day post-infection, compared to animals that were inoculated with *P. bivia* alone. Consistent with the idea of reciprocal interactions between *Gardnerella* and *Prevotella* (as discussed in section Interaction Between Gardnerella and Curved Rods Generates Features of BV in Grivet Monkeys above), the titers of the two organisms during vaginal colonization were highly correlated (Spearman r = 0.9636). Additionally, *P. bivia* titers in the uterine horns of co-infected animals were ∼20 times higher than in mono-infected animals at 2 days post-infection. This finding suggests that *G. vaginalis* enhances the ability of *P. bivia* to cause ascending infection of the reproductive tract. In this model, *G. vaginalis* titers in the vagina and uterine horns were not significantly different between mono and co-infected groups. Consistent with the *G. vaginalis* inoculation model reported in 2013, no clinical signs of inflammation were observed in the vaginas or uterine horns of mono or co-infected animals (Gilbert et al., 2013, 2019).

Interaction Between *Gardnerella* and Curved Rods Generates Features of BV in Grivet Monkeys

In the 1980s it was reported that women with BV often had motile, anaerobic curved rods (CR) in their vaginas. In 1984, Mardh et al. vaginally inoculated grivet monkeys with both *G. vaginalis* and CR isolates to determine if the clinical features of BV could be replicated in a co-infection primate model (see **Table 1** for comparison to other human and non-human primate experiments). This group used a short morphotype (SCR) and long morphotype (LCR) (Mårdh and Møller, 1984). While we cannot say for certain, it seems highly likely that these CR morphotypes were in fact distinct species of *Mobiluncus* that are commonly present in women with BV. Two morphotypes of *Mobiluncus* can be distinguished by Gram-stain examination: small (1.7 um) Gram-variable cells that correspond to *Mobiluncus curtisii* and large (2.9 um) Gram-negative cells that correspond to *Mobiluncus mulieris* (Dworkin et al., 2006). Another possibility is that CR morphotypes on Gram stained slides may be BVAB1 (a member of the family *Lachnospiracea* and order *Clostridiales*) rather than *Mobiluncus* (Srinivasan et al., 2013). However, given that modern microbiologists have not yet succeeded in cultivating BVAB1, it seems more likely that what was isolated and used in the Grivet monkey model was in fact *Mobiluncus*.

In the Grivet model, *G. vaginalis* alone was unable to colonize, as the authors could not recover it from the mono-infected animals at 6 days post-infection. Conversely, both morphotypes of CR could be isolated back out of mono or co-infected animals for the entire 37-days observation period. One monkey that had been inoculated with LCR was still culture positive after 9 months. However, no mono-infection with any of the three organisms (SCR, LCR, or *G. vaginalis*) caused the animals to develop any clinical signs of BV (clue cells, increased vaginal discharge, or elevated vaginal pH). Co-infection with SCR and *G. vaginalis* also did not yield any clinical signs of BV, and while SCR could be recovered during the entire 37 days, *G. vaginalis* was never re-isolated. However, animals that were co-infected with *G. vaginalis* and LCR developed "profuse vaginal discharge" that began 5 days after infection, which lasted through the entire 37-days observation period. LCR could be isolated back

out of the co-infected animals during the entire 37 days, and *G. vaginalis* was recoverable until day 12. Co-infection with *G. vaginalis* and LCR was the only condition in which *G. vaginalis* was ever recoverable, and the only condition in which the profuse discharge was observed. No clue cells or vaginal inflammation were observed under any of the conditions (Mårdh and Møller, 1984).

G. vaginalis Triggers Kidney Injury and Recurrent Urinary Tract Infection by *Escherichia coli*

In addition to being present in the vagina, multiple studies have isolated *G. vaginalis* from urine, including those that used methods to collect urine that limit contamination by peri-urethral and vaginal bacteria (e.g., suprapubic needle aspiration or transurethral catheterization) (Pearce et al., 2014, 2015; Malki et al., 2016; Thomas-White et al., 2016; Gottschick et al., 2017; Kramer et al., 2018). Although most routine methods for urine culture will not detect *Gardnerella*, studies that have used appropriate isolation conditions suggest that the bladder is either transiently exposed to *G. vaginalis* or that the organism may colonize the bladder in some women [see Kline and Lewis (2016) and references therein]. In one study, *G. vaginalis* was cultured from (needle) aspirated urines from 20/33 (60%) patients with chronic atrophic pyelonephritis (a.k.a. reflux neuropathy) compared to only 2/35 healthy controls (9%) (Fairley and Birch, 1983). Of 61 aspirates from men that were tested, none yielded growth of *G. vaginalis*, while about 1/3 of women tested had *G. vaginalis* in aspirated urine. Interestingly, women with culture-positive (*G. vaginalis*, *U. urealyticum,* or other species) aspirated urine also had squamous epithelial cells covered in bacteria, bearing a close resemblance to clue cells seen in BV. Another study of hospital inpatients reported that individuals from whom *Gardnerella* was isolated from urine were more likely to have a history of recurrent UTI and current pyelonephritis (Josephson et al., 1988a). Together, these clinical data support further investigations of the effects of *Gardnerella* in the urinary tract.

Several reports suggest that women with BV are more likely to experience UTI (Harmanli et al., 2000; Hillebrand et al., 2002; Sharami et al., 2007; Sumati and Saritha, 2009; Amatya et al., 2013; Gautam et al., 2015). One recent study reported a mouse model to test the hypothesis that *G. vaginalis* interactions with the bladder may contribute directly to the association between BV and UTI (Gilbert et al., 2017). Mice first received transurethral inoculation of *Escherichia coli,* the most common cause of bladder and kidney infections. This resulted in the formation of intracellular reservoirs that persist following clearance of the bacteria from urine, as had been shown previously (Mysorekar and Hultgren, 2006). Mice were then inoculated twice with *G. vaginalis. G. vaginalis* did not establish lasting colonization in the bladder, and nearly all mice had undetectable titers by 12 h following exposure. However, scanning electron microscopy and histological analysis of the bladders, as well as urine cytology, revealed that even a transient exposure to *G. vaginalis* induced urothelial exfoliation. In the subsequent days following *G. vaginalis* exposure, *E. coli* re-emerged from latent reservoirs back into the urine to cause recurrent UTI that was accompanied by a neutrophilic response. This phenotype was not observed when the mice were transurethrally inoculated using identical methods in parallel, with similar numbers of *L. crispatus.* This condition was used to model bladder exposure to vaginal bacteria from a woman without BV. Additionally, bladder exposure to *G. vaginalis* increased the susceptibility of the mice to developing severe (kidney and systemic) *E. coli* infections. This is consistent with a clinical study which showed that kidney infection was more common among female inpatients who had detectable *G. vaginalis* in their urine (Josephson et al., 1988b). Gilbert et al. (2017) also demonstrated that transurethral exposure to *G. vaginalis* was sufficient to cause acute kidney injury that was a direct result of inflammatory signaling via the IL-1 receptor and occurred even in the absence of *E. coli* (Gilbert et al., 2017). These findings, in conjunction with clinical data, suggest that further investigation into the ability of *G. vaginalis* to induce urologic pathology is necessary. As with the other models discussed above, it will be interesting in future studies to learn if different species, subspecies, or strains of *Gardnerella* (see more below) are able to generate the different observed pathophysiologic features in the urinary tract.

DISCUSSION

Taxonomic Diversity and Pathogenesis of *Gardnerella*

Since the initial discovery of *G. vaginalis* (then *H. vaginalis*) (Gardner and Dukes, 1954), many efforts have been made to taxonomically sub-divide and identify isolates with the greatest virulence potential (Piot et al., 1980, 1984; Pleckaityte et al., 2012). Recent comparative genomic studies indicate that *G. vaginalis* comprises at least four distinct phylogenetic clades/subtypes (Ahmed et al., 2012; Malki et al., 2016). Recently, one manuscript proposed a few new species names within the genus *Gardnerella* (Vaneechoutte et al., 2019). However, the broader clade designations contain multiple proposed *Gardnerella* species, although names have only been proposed for some. Regardless of the exact taxonomic scheme used, the genomic diversity among *Gardnerella* isolates is substantial. Early reports have suggested that the core genome shared by all *G. vaginalis* isolates consists of only 25% of the total genes in the *G. vaginalis* pangenome (Ahmed et al., 2012). The fact that there are so many accessory genes among *G. vaginalis* clades supports the *hypothesis* that the clades may occupy distinct niches characterized by unique affiliations with other microbes, communities, or host factors. Although different studies have not always given consistent results, several clinical studies implicate clade 1 and/or clade 2 strains in BV status, recurrence, and other adverse outcomes linked with BV, such as preterm birth. Conversely, clades 3 and 4 lack certain predicted virulence factors and may be less pathogenic (Balashov et al., 2014; Callahan et al., 2017; Hilbert et al., 2017; Goltsman et al., 2018; Janulaitiene et al., 2018; Plummer et al., 2019). Interestingly, specific clades have

also been linked with sexual practices. For example, one recent study found that clade 1 was associated with higher numbers of recent sexual partners while clade 2 was associated with penile-vaginal intercourse and sharing of sex toys with female sex partners (Plummer et al., 2019).

The earliest suggestions that women with BV may harbor multiple subtypes of *G. vaginalis* came in 1990 when Briselden and Hillier tested vaginal specimens for different "biotypes" of *G. vaginalis* (Briselden and Hillier, 1990). Following the more recent availability of genome sequences and clade definitions, several literature reports have confirmed that multiple *G. vaginalis* clades often co-exist in the vagina in women with BV (Balashov et al., 2014; Janulaitiene et al., 2018; Plummer et al., 2019; Shipitsyna et al., 2019). Because of this complexity, it is difficult based on observations of clinical samples alone to make definitive assertions about which clades may be involved in different phenotypes or pathologies associated with BV.

Experimental models in animals (in the spirit of Koch's postulates) are needed to begin unraveling how each clade may interact with the host, other organisms, and/or each other to elicit features and health complications associated with BV. Clade identifications are only available for some of the strains used in previous experimental studies conducted to date. The most common strains used in animal models have been ATCC14018 (clade 1) and JCP8151B (clade 2, now designated *G. piotii*), both of which were sufficient to cause features and/or complications linked with BV in human and animal models (see **Tables 1**, **2**). Some of the limitations of these models and their conclusions are discussed in the paragraphs that follow.

Limitations of Animal Models

Mouse models of *G. vaginalis* infection have become much more prevalent over the last decade and have shed light on a number of interesting phenomena concerning *G. vaginalis* and BV. However, there are clear limitations with these models. As with the primate models, laboratory mice do not have a *Lactobacillus* dominant microbiome. Therefore, it is not surprising that the increase in vaginal pH associated with BV has not been achieved in these models (Miller et al., 2016). Additionally, there are likely to be differences in the endogenous vaginal microbiomes depending on the strain of mouse and the facility where they are raised. Differences in the mouse strain, housing conditions, and/or vaginal microbiome could strongly impact susceptibility and response to *G. vaginalis* infection. For example, Teixeira et al. (2012) observed "inflammatory lesions" upon *G. vaginalis* infection in their germ-free mice, while Gilbert et al. (2013, 2019) have specifically noted a lack of inflammatory response in their model using C57Bl/6 mice raised under conventional conditions (Teixeira et al., 2012; Gilbert et al., 2013, 2019). Additionally, many of the mouse models require β-estradiol administration to bring mice into pro-estrus for the duration of the experiment and avoid the PMN-rich post-ovulation period of the estrus cycle (Gilbert et al., 2013). A potential limitation is that normal immune responses to *G. vaginalis* infection may be altered by the use of β-estradiol. Another potential limitation of mouse models is that the vaginal epithelium is more highly keratinized in mice (during pro-estrus) than in humans, which may influence the normal course of *G. vaginalis* infection (Sierra et al., 2018). Finally, previous work had shown that the *G. vaginalis* toxin vaginolysin (a cholesterol-dependent cytolysin) is species-specific and requires the presence of human CD59 (Gelber et al., 2008). It is not clear whether this finding applies to other primate models, but nevertheless, the development of a humanized mouse expressing human CD59 might improve the *G. vaginalis* mouse infection model. Perhaps the biggest limitation of experimental studies in humans or animals is that they rarely consider that the presence of the endogenous vaginal microbiome is likely to discourage colonization by exogenous organisms that are introduced, a phenomenon known as colonization resistance. The use of non-human primates in research presents additional challenges, expense, and ethical considerations, but may be worthwhile to better understand the microbial ecology and interactions with the host mucosa in BV. It has been noted that the vaginal microbiomes of many of the species of non-human primates for whom such studies have been performed have communities that resemble BV (Uchihashi et al., 2015; Zhu et al., 2015; Obiero et al., 2016; Miller et al., 2017). Thus, while there are greater obstacles to their use, nonhuman primates may also offer important advantages over other model systems.

Ethics of Human Experimentation

When the early human infections with *G. vaginalis* were performed, the experimenters had little understanding of the pathology of BV. The risks and adverse health outcomes associated with BV were unknown at the time, and the condition was thought to be relatively benign. Indeed, in their hallmark 1955 paper, Gardner and Dukes express their belief that BV is "physically and esthetically objectionable," but that "the disease is not a serious one." Additionally, they wrote that "therapeutically, [BV] constitute[s] no difficult problem" (Gardner and Dukes, 1955) which is in clear contrast to more recent literature on the difficulty treating BV and the high rates recurrence (Bradshaw and Brotman, 2015). This erroneous belief in the banality of BV explains how groups in the 1950s and 60s were comfortable performing vaginal inoculations of *G. vaginalis* into women (Gardner and Dukes, 1955; Criswell et al., 1969). In light of our current understanding, it can be clearly argued that these experiments represented an unacceptable risk for the subjects that in today's scientific environment would need to be clearly articulated during informed consent and could result in outright rejection by institutional review boards. While these early papers provide useful insight into *G. vaginalis* and BV, their ethical dubiousness also act as a cautionary tale in the dangers of misguided assumptions in the pursuit of further understanding.

This lesson is an important one to remember, especially considering that human experimentation in BV research still occurs today. Now however, the experiments are aimed at directly treating BV and improving quality of life for those women afflicted. In 2019 Lev-Sagie et al. reported the first vaginal microbiome transplantation (VMT) performed in human women. The transplants were from three BV-negative donors with *Lactobacillus crispatus*-dominated vaginal microbiomes. The five recipients were women with intractable BV who experienced recurrent symptomatic episodes, despite antibiotic treatment, and so were good candidates for alternative therapy. The recipients all received antibiotic treatment for 5–7 days

immediately prior to the first VMT. Vaginal discharge from the donors was collected and introduced into the recipients, and the recipients received clinical follow-up to assess BV symptoms for 5–21 months following VMT. Four of the five recipients experienced full long-term remission (defined as absence of symptoms, an Amsel score of 0, and the presence of a *Lactobacillus* dominated microbiome), although three of the four required multiple rounds of VMT. The fifth woman experienced partial symptom improvement, although her vaginal microbiome was not lactobacilli-dominated at the end of the observation period (Lev-Sagie et al., 2019).

These results overall appear promising, and unlike in the human experiments of the 1950s and 60s, the authors of this study carefully screened donors for STIs and other important infections such as hepatitis and cytomegalovirus. However, the authors themselves also acknowledge that there is always the possibility of unforeseen risk with VMT—after all, we still know relatively little about the vast majority of the microbes that live in this niche. For this reason, Lev-Sagie et al. emphasize that VMT should be considered only as a last resort for intractable BV where quality of life is severely disrupted and multiple treatment options have failed (Lev-Sagie et al., 2019).

Finally, we note the need for consistency in diagnostic terms for BV that are used by academia and industry as well as the need to provide specific methods used to define such diagnoses.

Have Koch's Postulates Been Satisfied That *G. vaginalis* Is a Cause of BV?

Koch's original postulates established a microorganism is the cause of a disease if it was (1) found in all diseased, but not healthy individuals, (2) could be isolated and grown in pure culture, (3) caused disease when introduced into an experimental host, and (4) could be re-isolated and re-identified from the infected experimental host. Of course, arguments have been advanced by Koch himself, and many others since, in favor of revising the original postulates for biological and technical reasons as it became clear that they were too rigid in their original form (see below).

Is *Gardnerella* Found in All Cases of NSV/BV but Not Among Healthy Women?

Over the years, scientists have used different methods to detect and identify *Gardnerella* and different criteria to define BV, leading to somewhat different conclusions. In the first published studies, Gardner and Dukes found that *G. vaginalis* was detected in >90% of women with clinical signs we now recognize as BV, but never in apparently healthy control women (Gardner and Dukes, 1955). By the 1980's, the use of human blood bilayer media and other culture techniques had improved the ability to culture "*Gardnerella*" and led to a higher proportion of "normal" women appearing positive for *Gardnerella* (Totten et al., 1982; Hillier, 1993). However, culture-based studies in the 1980s and more recent quantitative PCR methods seem to agree that *Gardnerella* is found at orders of magnitude higher levels in women with BV compared to those without the condition (Sheiness et al., 1992; Balashov et al., 2014). This first of Koch's postulates has been modified for causative agents of some bacterial infections in light of findings that they can be harbored by a subset of individuals without causing symptoms or harm (e.g., *Haemophilus influenza, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus agalactiae*, etc.) (Fredricks and Relman, 1996; Duell et al., 2016; Krismer et al., 2017; Shabayek and Spellerberg, 2018; Weiser et al., 2018). In fact, Koch himself realized this upon the discovery that some individuals carried the causative agents of cholera and typhoid without apparent signs of disease (Evans, 1976). Thus, it is also possible that carrier states may exist for *Gardnerella*. Two alternate explanations for the presence of *Gardnerella* in women without BV could be that the strains in question may have fewer virulence factors (see the section above on *Gardnerella* taxonomy) or are kept "in check" by strains of beneficial bacteria with particularly powerful antimicrobial activities.

Can *Gardnerella* Be Isolated in Pure Culture and Re-Isolated From Infected Hosts?

Since its original description in the 1950s, the isolation and growth of *Gardnerella* in culture has been reported using several methodologies, including different culture media (see **Tables 1, 2**) and use of different atmospheric conditions (anaerobic, 5% CO_2). Broadly speaking, it is not known whether the reported culture conditions for preparing *G. vaginalis* inocula used in experimental studies result in expression of the factors needed for colonization/infection. Experimental studies in humans (Criswell et al., 1969), primates (Mårdh and Møller, 1984), and mice (Gilbert et al., 2013) showed that when signs of BV were present, *G. vaginalis* was recoverable. One exception to this was the finding in pig-tailed macaques that *G. vaginalis* was recoverable from 10/10 inoculated individuals but did not result in the appearance of clue cells, more basic pH, or change in non-volatile acids (Johnson et al., 1984). As discussed elsewhere, based on the absence of dominant lactobacilli in these animals, it is arguable that the latter two signs may not be possible to generate in this system.

Can *Gardnerella* Reproduce Features of BV in Experimental Models?

Only two studies in the literature inoculated *Gardnerella* into human volunteers. Although the first experiments from Gardner and Dukes (1955) claimed "proof of pathogenicity," these studies provide little evidence for this conclusion (see Section Vaginal Inoculation in Women) (Gardner and Dukes, 1955). It is hard to know what the authors did in these experiments since they report no detailed culture conditions or bacterial strain names. There was not a control group of women left uninoculated to determine if women might spontaneously develop signs of BV or become colonized with *Gardnerella*.

The second of the two experimental studies performed in humans was also performed by Gardner and Dukes, but involved another co-author (Criswell). One of the interesting questions evaluated by Criswell et al. (1969) was whether *Gardnerella* strains grown in culture for 12 or 24 h resulted in clinical signs of BV when "poured" into the vaginas of 29 women. About half of these women were inoculated with the strain ATCC14018, which caused clinical signs of BV in most of the women when the inoculum was prepared from a 12-h culture. In contrast,

the same strain grown for 24-h did not cause signs of BV in any of the inoculated individuals. Three other strains were used in this paper to inoculate another 15 women. Although clade designation of these strains is not known, 24-h cultures were used and only two of fifteen women were found to have signs of BV (Criswell et al., 1969). Although statistical analysis was not presented by the authors, our analysis of results from the 29 women inoculated shows that those receiving 12 h cultures were significantly more likely to develop signs of BV compared to those who received 24 h cultures (**Figure 2**). This raises the question of whether 24 h cultures of *Gardnerella* under the conditions used were viable for colonization.

Later studies that investigated signs of BV in primates inoculated with *Gardnerella* did not provide substantial evidence in support of *Gardnerella* as a causal factor in the development of BV, but had several important limitations. First, none of the strains used have survived to the present day so we cannot use molecular techniques to confirm their identity as *G. vaginalis* or determine to what clade they belong. Additionally, it is not always clear how the bacteria were cultured for preparation of the inoculum (medium or atmospheric conditions used were not fully reported). The Johnson et al. (1984) and Mårdh and Møller (1984) studies report using older cultures of *Gardnerella* (24 or 48 hour cultures) (Johnson et al., 1984; Mårdh and Møller, 1984). As stated above, although "increased pH" was one of the main foci of the investigations, the primates used in these studies typically already have more neutral vaginal pH at baseline (before inoculation). Thus, it may not be possible to make the vaginas of these animals more basic. Later studies have illustrated that non-human primates harbor microbiomes that contain diverse taxa and typically are not characterized by dominant lactobacilli (Stumpf et al., 2013). With that said, it is not always clear how interactions with humans may change the landscape of wild microbiomes, as recent studies in mice have revealed (Rosshart et al., 2017). It is noteworthy that 10/10 pig-tailed macaques developed colonization with *Gardnerella* in the Johnson et al. (1984) study, but did not develop signs of BV (Johnson et al., 1984). But, as we do not know what taxonomic lineage the strains used in this study belong to, the lack of BV signs could also reflect a less virulent isolate was used.

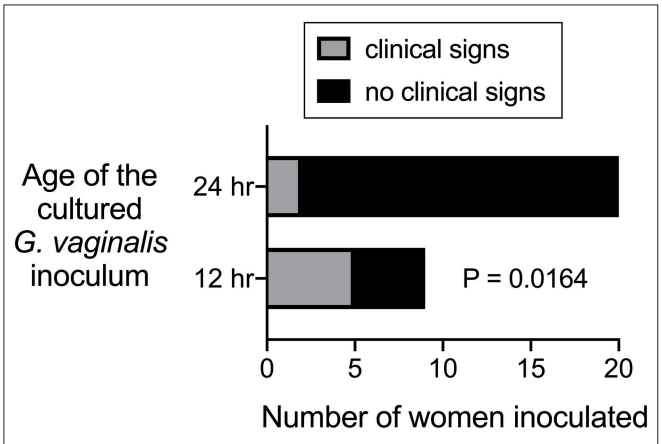

FIGURE 2 | Data from Criswell et al. (1969) plotted and analyzed to evaluate statistical difference between ability of 12 h vs. 24 h *G. vaginalis* cultures to cause signs of BV when inoculated into women.

We argue that the most definitive conclusions regarding whether *G. vaginalis* experimental administration reproduces features and complications associated with BV in women can be derived from experiments performed using three strains: ATCC14018, ATCC14019, and JCP8151B. Experimental studies performed using the clade 1 strain ATCC14018 showed that this strain yielded clinical signs of BV in 5/9 women when 12-h cultures were used for inoculation (Criswell et al., 1969) (see **Figure 2**). More than four decades later, one study may have provided an explanation by showing that extracellular DNA produced by *G. vaginalis* contributes importantly to the production of biofilms and is only present in the cell-free supernatant of early cultures (Hymes et al., 2013). Another *G. vaginalis* clade 1 strain, ATCC14019 was originally isolated by Gardner and Dukes and is nearly identical in genome sequence to ATCC14018. Administration of ATCC14019 vaginally into pregnant mice resulted in evidence of cervical remodeling and increased IL-6 in the vagina and amniotic fluid (Sierra et al., 2018), consistent with findings of elevated IL-6 in women with BV (Yudin et al., 2003; Campos et al., 2012) and associations of clade1/2 *G. vaginalis* with preterm birth (Callahan et al., 2017). In another mouse model, the clade 2 strain JCP8151B was shown to result in colonization and live recovery of *Gardnerella*, clue-like cells with adherent *Gardnerella*, an epithelial exfoliation response similar to that seen in humans, and higher levels of the hydrolytic enzyme sialidase together with biochemical evidence of mucus degradation, similar to that seen in BV (Gilbert et al., 2013; Lewis et al., 2013). The recent suggestions for new species names for subsets of *Gardnerella* strains seems to place the JCP8151B strain within the subset of clade 2 strains being referred to as *Gardnerella piotii* (Hill et al., 2019; Vaneechoutte et al., 2019). In short, the experimental evidence points to the conclusion that *G. vaginalis* and *G. piotii* (if this naming scheme is adopted by the field) can cause clinical signs of BV when introduced into human and animal hosts.

Can *Gardnerella* Influence the Pathogenesis of Other Organisms in Experimental Models?

Women with BV appear to be more susceptible to a wide variety of other infections, including infections of the lower reproductive tract (Brotman et al., 2010), the upper reproductive tract (Ádám et al., 2018), and the nearby urinary tract (Harmanli et al., 2000). However, we still know relatively little about which organisms in BV might confer these increased risks or the mechanisms leading to disease. In addition to findings that certain strains of *Gardnerella* can reproduce features of BV in experimental models, there is also evidence to support the conclusion that *Gardnerella* can influence whether other organisms cause pathophysiology. Specifically, vaginal colonization by the JCP8151B strain caused mice to experience higher titer ascending uterine infections by *Prevotella bivia* compared to animals that received only *P. bivia* (Gilbert et al., 2019). The JCP8151B strain was also able to trigger recurrent UTI caused by uropathogenic *E. coli* that re-emerged from long

lasting reservoirs inside epithelial cells in response to bladder exposure to *Gardnerella*, but not *Lactobacillus crispatus* (Gilbert et al., 2017).

In conclusion, we have only scratched the surface in understanding how microbes coordinate the biological processes that initiate and perpetuate BV. Nevertheless, experimental models have given us key insights into the causal relationship of *Gardnerella* with BV. Together, the studies reviewed here suggest that *Gardnerella vaginalis* can, under some conditions, recreate some of the features and complications that have been associated with BV. Although we do not yet understand the precise molecular mechanisms, additional experimental studies suggest that *Gardnerella vaginalis* may change the host landscape in a way that makes other organisms more likely to colonize or cause disease. Future studies should expand and optimize small animal models to further refine our understanding of how species, subspecies, and strains of BV bacteria act alone and in concert with other microbes to overcome the healthy microflora and lead to poor health outcomes.

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Impact of Symbiosis Between *Trichomonas vaginalis* and *Mycoplasma hominis* on Vaginal Dysbiosis: A Mini Review

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The protozoon *Trichomonas vaginalis* is responsible for trichomoniasis, a common sexually transmitted infection associated with an increased risk of HIV infection and adverse pregnancy outcomes. The protozoon has the surprising ability to establish a symbiotic relationship with other microorganisms. In fact, most *T.vaginalis* isolates intracellularly host the vaginal bacterium *Mycoplasma hominis* and can harbor up to four dsRNA viruses. Moreover, a novel *Mycoplasma* species named *Ca. Mycoplasma girerdii* has been recently described as associated with trichomonad cells. *Trichomonas vaginalis* colonizes the human vagina and its presence causes profound alterations of the resident microbiota, leading to dysbiosis. In healthy women, vaginal microbiota is characterized by the presence of a complex population of aerobic and anaerobic microorganisms living in a physiologically dynamic system dominated by bacteria of the genera *Lactobacillus*. The most common microbial vaginal imbalance is bacterial vaginosis, a polymicrobial disease associated with several adverse reproductive outcomes and increased risk of HIV infection. Here, we review the current knowledge regarding the interactions between both *T.vaginalis* and *M.hominis* and the vaginal microbiota, and we discuss the possibility of a cooperation between *T.vaginalis* and its symbionts in the development of vaginal dysbiosis.

Keywords: bacterial vaginosis, *Mycoplasma hominis*, *Trichomonas vaginalis*, vaginal dysbiosis, symbiosis

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INTRODUCTION

The protozoon *Trichomonas vaginalis* is a parasite of the human urogenital tract and is responsible for trichomoniasis, the most common non-viral sexually transmitted disease. Clinical presentation of trichomoniasis in women may vary from asymptomatic to severe vaginitis, while men tend to be mostly asymptomatic carriers of the protozoon (Petrin et al., 1998). *Trichomonas vaginalis* infection is associated with an increased risk of HIV infection and can lead to adverse pregnancy outcomes, such as preterm delivery and low birth weight. Moreover, trichomoniasis has been associated with an increased risk of cervical and prostate cancer (Stark et al., 2009).

Interestingly, the presence of the protozoon in females can profoundly alter the composition of the vaginal microbiota. The human vaginal ecosystem of women of childbearing age is characterized by the presence of a complex population of aerobic and anaerobic microorganisms that are able to establish interactions among each other and with the host in a physiologically

dynamic system. Development of "next-generation" sequencing technology led to the identification of five different vaginal microbiota, which we call community state types (CSTs) (Ravel et al., 2011). Bacteria of the genus *Lactobacillus* represent the predominant microbial community in four of them (CST-I, CST-II, CST-III and CST-V), while one, CST-IV, does not show a dominant species and is composed of a more complex microbial community. Lactobacilli exert a protective effect on the host by lowering vaginal pH through lactic acid production and by competing for nutrients. Growth of adverse microbiota is also inhibited by the production of antimicrobial compounds such as bacteriocins and hydrogen peroxide.

Endogenous and exogenous factors, such as age, pregnancy, sexual intercourse and antimicrobial treatments, can alter the dynamic equilibrium of the vaginal microbiome, leading to dysbiosis of the urogenital tract. The most common microbial vaginal imbalance that occurs during reproductive age is bacterial vaginosis (BV), which affects 10–30% of women worldwide (Morris et al., 2001). Bacterial vaginosis is characterized by abnormal and malodorous vaginal discharge, an increased vaginal pH and vaginal itching. Similar to *T.vaginalis,* BV is associated with several adverse reproductive outcomes, preterm birth and premature rupture of membranes. Moreover, bacterial vaginosis also represents a risk factor for the acquisition of sexually transmitted infections (STIs) (Mirmonsef et al., 2012). Although clinical manifestations and potentially associated microbiological agents have been described in several studies, a specific etiology remains elusive, suggesting that BV is not a specific microbiological process but a polymicrobial disease caused by changes within the bacterial community (Onderdonk et al., 2016). Several studies have highlighted the main characteristics that occur during BV: a reduction of number of protective Lactobacilli and an overgrowth of anaerobic bacteria. *Gardnerella vaginalis* is the bacterium most frequently associated with BV, but other genera, such as *Atopobium*, *Prevotella*, *Bacteroides*, and *Mycoplasma*, can overgrow in case of bacterial vaginosis (Fredricks et al., 2005; Onderdonk et al., 2016). Although it is clear that several bacterial species are present during BV, the role of each single microorganism in the pathogenesis of the disease has not yet been clarified. In fact, so far, it has been impossible to identify a lone bacterial species that fits Koch's postulates for bacterial vaginosis.

Intriguingly, *T.vaginalis* can establish a symbiosis with *M.hominis*, one of the bacteria implicated in bacterial vaginosis (Rappelli et al., 1998). The presence of *M.hominis* has been demonstrated in trichomonad isolates from different geographical areas, and their strict association is the first one described so far involving two obligate human pathogens. Even though both microorganisms are able to induce disease independently in the vagina, their association has been shown to have important consequences for the pathogenicity of each of them (Dessì et al., 2019).

In this review, we focus our attention on the interplay of *Trichomonas vaginalis* and its symbiont *Mycoplasma hominis* with vaginal microbiota, and we discuss their possible role in the onset of bacterial vaginosis.

Trichomonas Vaginalis and Vaginal Dysbiosis

During infection, *T.vaginalis* establishes complex interactions with the microbial community of the vagina, many of which are only partially known. An interesting study has evaluated the association between the different community state types and *T.vaginalis* and found that the 72% of women with trichomoniasis belonged to the CST-IV group (Brotman et al., 2013). This community state type is characterized by a reduced number of *Lactobacillus* species and a greater abundance of strict anaerobic bacteria, and is associated with bacterial vaginosis. Epidemiological data show that trichomoniasis is often associated with BV, but the prevalence of the two infections is probably underestimated, since both can be asymptomatic and share common features, such as the presence of vaginal discharge and inflammation (Fichorova et al., 2013). Nevertheless, the causal relationships between the two diseases are largely unknown: in other words, do particular types of vaginal bacterial communities predispose women to the acquisition of *T. vaginalis* or, on the contrary, does CST depend on the presence of the protozoon?

A longitudinal analysis showed a four- to nine-fold increased risk of trichomonad infection among sexually active women who had abnormal vaginal flora within a 3-month span, suggesting a causal role of altered microbiota on *T.vaginalis* infection (Rathod et al., 2011). *In vitro* experiments seem to support this hypothesis: in a very recent study, Hinderfeld and Simoes-Barbosa investigated the role of BV-associated bacteria on *T.vaginalis* adhesion, demonstrating that the biofilm produced by dysbiotic bacteria is able to enhance the adhesion of the protozoon to host cells, thus strengthening its pathogenic effect (Hinderfeld, 2020). Another recent study shows a cooperative interaction between *T.vaginalis* and CST-IV bacteria, resulting in an enhancement of the paracellular permeability of human ectocervical cells *in vitro* (Hinderfeld et al., 2019). Phukan et al. (2013) observed that some species of lactobacilli, and in particular *Lactobacillus gasseri,* can reduce *T.vaginalis* adhesion to vaginal cells. Since adherence to epithelial cells is a prerequisite for the cytopathic effect of the protozoon (Fiori et al., 1999), lactobacilli could exert a protective activity by reducing the pathogenicity of *T.vaginalis,* thus influencing its pathogenicity.

On the other hand, Fichorova et al. (2013) have demonstrated *in vitro* that *T. vaginalis* dramatically reduces the number of *Lactobacillus spp* associated with vaginal epithelial cells but has no effect on other species present in BV, such as *Prevotella bivia* and *Atopobium vaginae.* These findings, together with the fact that *T.vaginalis* is a phagocytic protozoon capable of ingesting and killing lactobacilli very efficiently (Juliano et al., 1991), support the hypothesis of a cause–effect relationship between trichomoniasis and bacterial vaginosis.

Another important aspect that characterizes trichomoniasis is a higher pH observed during infection than that of healthy vagina, normally ranging between 2.8 and 4.2 (McGrory et al., 1994). Since low pH is mainly due to the acidic metabolism of lactobacilli, the reduction of lactobacilli community associated with trichomoniasis leads to an increased pH, creating an environment more favorable for *T.vaginalis* growth and

pathogenicity (Petrin et al., 1998). In fact, the protozoon exerts its cytopathic effect through the release of pore-forming proteins, whose activity is strictly dependent on a pH with an optimum of 5.8 (Addis et al., 1997). In this respect, the reduction of *Lactobacilli* observed during trichomoniasis could be part of a strategy aimed at the creation of an environment that best suits protozoan needs.

Mycoplasma hominis and Vaginal Dysbiosis

The bacterium *Mycoplasma hominis* is an obligate parasite of the human urogenital tract belonging to the class Mollicutes and is characterized by the absence of a rigid cell wall and by one of smallest genomes among self-replicating organisms (Taylor-Robinson, 2017). *M.hominis* can colonize the human female urogenital tract of sexually mature females, and its prevalence greatly varies in studies, ranging from 1.3 to 51% (Diaz et al., 2010; Rumyantseva et al., 2019); it is influenced by several host factors, including age, sexual activity and pregnancy (Taylor-Robinson, 2017).

Although *M.hominis* can be found in both healthy and symptomatic women, several studies demonstrated that its presence is associated with vaginal flora alterations, including bacterial vaginosis and *Trichomonas vaginalis* infection. Rumyantseva et al. investigated the prevalence of *M.hominis* in 2,594 reproductive-aged women, both with healthy and altered vaginal flora, demonstrating a three-fold increase in *M.hominis* prevalence in BV patients (26.8%) compared to healthy women (8.9%) ($P < 0.001$) (Rumyantseva et al., 2019). Moreover, women with BV show a load of *M.hominis* in the vagina up to 7,500-fold higher than women without BV, suggesting that the microbiota characteristic of bacterial vaginosis may promote the growth of the bacterium. Interestingly, a synergistic relationship between *M.hominis* and the BV-associated microorganism *Gardnerella vaginalis* has been recently described. *Gardnerella vaginalis,* an anaerobic bacterium that is present in 95% of cases of BV, adheres to the vaginal epithelium and establishes a biofilm that acts as scaffold for other microorganisms contributing to the pathogenesis of the disease. Cox et al. (2016) demonstrated a higher co-infection rate of *M. hominis* and *Gardnerella vaginalis* in BV (60.7%) compared to non-BV (8.8%) women (Cox et al., 2016). Moreover, significantly higher loads of both *M.hominis* and *Gardnerella vaginalis* were detected in women with BV, suggesting a synergy between the two species. This hypothesis is corroborated by the fact that treatment of BV with metronidazole eliminates not only *G.vaginalis* and other sensitive bacteria but also *M.hominis*, that is metronidazole-resistant (Taylor-Robinson, 2017).

Other aspects characterizing vaginal dysbiosis are abundant vaginal discharge and a fishy odor, which are caused by the presence of amines (i.e., putrescine) that become malodorous in a high pH environment. The increased production of amine is caused by the great number of anaerobic bacteria that are able to produce the proteolytic enzymes that breakdown vaginal peptides (Huang et al., 2014). Recent articles showed that the presence of *M.hominis* in the vagina of women affected by BV is correlated with high levels of amine and short-chain fatty acids (SCFAs) (Vitali et al., 2015; Ceccarani et al., 2019). SCFAs include acetic, butyric and propionic acids and play an important role in a wide array of immune responses by inhibiting the production of proinflammatory cytokines, affecting immune cell migration and phagocytosis and inducing apoptosis in various cell types including neutrophils (Mirmonsef et al., 2011). These data, taken together, reinforce the hypothesis of the important role of *M.hominis* in BV condition.

Trichomonas vaginalis and its Symbionts: A Microbial Cooperation Influencing Vaginal Dysbiosis?

It is well established that both *T.vaginalis* and *M.hominis,* independently, interact with members of the resident vaginal microbiota in synergistic or antagonistic ways that may influence the course of infection. Less is known about the interplay between the vaginal microbiota and the two microorganisms when they live in symbiosis.

The symbiotic relationship between *Trichomonas vaginalis* and *Mycoplasma hominis* is the first and, so far, unique case involving two obligate human parasites that are able to cause independent diseases (Dessì et al., 2019). The presence of viable *M. hominis* within *T.vaginalis* has been widely demonstrated in clinical samples from people of different geographic origin with infection rates ranging from 5% to over 89% (Fichorova et al., 2017). *Mycoplasma hominis* replicates in a coordinated fashion with trichomonad cells, and its intracellular location has been demonstrated by gentamicin protection assays and confocal and electron microscopy. In this way, *T.vaginalis* provides the bacterium with an environment protected by antibiotics activity and host immune response (Dessì et al., 2005). Moreover, *M.hominis* can be transmitted from naturally mycoplasma-infected *T.vaginalis* strains to mycoplasma-free trichomonad cells and to human epithelial cells *in vitro* (Rappelli et al., 2001), suggesting that *T.vaginalis* plays a role in transmitting the bacterium to the human host. Our group demonstrated that not only *M.hominis* but also *T.vaginalis* benefits from the symbiosis. Experiments set up on isogenic *M.hominis*-free and *M.hominis*-infected trichomonad strains demonstrated that the presence of *M.hominis* within trichomonad cells enhances the protozoan pathogenicity *in vitro*. Interestingly, the presence of *M. hominis* upregulates the secretion of proinflammatory cytokines by monocytic cells in response to *T. vaginalis*. The presence of *M.hominis* within *T.vaginalis* cells might upregulate the proinflammatory response during trichomoniasis, thus further affecting conditions associated with inflammation, such as the increased risk of acquiring cervical and prostate cancer or HIV (Dessì et al., 2019). Furthermore, *Mycoplasma* is able to influence the metabolic biochemical pathway of the protozoon, promoting a mutual benefit (Margarita et al., 2016).

The parasite pathobiology may also be influenced by the presence of RNA viruses. In fact, *T.vaginalis* can harbor up to four species of dsRNA viruses named *Trichomonas vaginalis* virus (TVV1, TVV2, TVV3, and TVV4), which

have infection rates in protozoan isolates ranging from 40 to 100% (Fichorova et al., 2017). In a recent article, we showed that 51.28% of *T.vaginalis* isolates harboring *M.hominis* were infected by at least one type of TVV (Margarita et al., 2019). Interestingly, the presence of TVV can enhance (over 30-fold) the inflammatory reaction to *T.vaginalis in vitro* (Fichorova et al., 2013).

The surprising ability of *T.vaginalis* to establish symbiotic relationships with different organisms has been further confirmed by the recent discovery of a new bacterium within trichomonad cells named *Ca.M.girerdii* (Fettweis et al., 2014). This unculturable mycoplasma has been found by metagenomic studies only in women infected by *T. vaginalis,* with a vaginal microbiota characterized by a high abundance of *Prevotella* spp. and a reduced number of *Lactobacillus* species, and was associated with severe inflammation (Martin et al., 2013).

It is known that the presence of trichomonad endosymbionts may interfere with the pathogenicity of the protozoon and modify the immune response, but no data are available on its impact on the vaginal microbiota. To the best of our knowledge, no studies focusing on the effect of the consortium *T.vaginalis*/*M.hominis* on bacterial vaginosis were found, despite the findings reported above on the importance and implication of both pathogens on bacterial vaginosis. An intriguing aspect that requires further research is the role of consortium *T.vaginalis/M.hominis* on the production of vaginal biofilm during vaginal dysbiosis. The biofilm formation allows for the adhesion and successive colonization by pathogenic vaginal bacteria and confers antibiotic tolerance and resistance to host immune response. The main player in the formation of adherent biofilm on the vaginal epithelium of women with BV is *G.vaginalis* (Patterson et al., 2010), and a recent article has shown that the biofilm produced *in vitro* by *G.vaginalis* provides adhesion to the protozoan (Hinderfeld, 2020). In turn, the protozoan may provide advantages to *G. vaginalis,* since its symbiont *M. hominis* can act as a growth trigger for *G. vaginalis* during BV (Cox et al., 2016). The capability of *T.vaginalis* to establish endosymbiotic relationships with different microorganisms simultaneously (making a unique poly-microbial entity that might modify host response and parasite virulence) may have a profound impact on vaginal microbiota; this remains to be investigated.

Preterm Birth: An Important Sequaele of *M. hominis* and *T. vaginalis* Infections and Bacterial Vaginosis

The association between vaginal infections and adverse pregnancy outcomes, such as preterm birth (PTB) and preterm prelabor rupture of membranes (PPROM), has been confirmed by several studies in the last 20 years (Guaschino et al., 2006; Lamont, 2015; Cappelletti et al., 2016). Taken together, up to 40–50 % of preterm births are associated with microbes that are able to access to the amniotic cavity and the fetus through ascension from the lower reproductive tract to the placenta, fetal membranes and uterine cavity (Fettweis et al., 2019). Both *M.hominis* and *T.vaginalis* infections are associated with complications in pregnancy. *M.hominis* is a microbe frequently isolated from both placental membranes and amniotic fluid in women with PPROM, suggesting a potential direct effect of bacteria to initiate the synthesis of prostaglandins resulting in spontaneous preterm labor (Choi et al., 2012; Capoccia et al., 2013). In contrast, *T. vaginalis* limits its infection to the vagina and is unable to reach the amniotic fluid during pregnancy, and its role in adverse pregnancy outcomes seems to be limited to the induction of a massive local inflammation with production of proinflammatory cytokines that can indirectly lead to severe pregnancy complications (Fichorova, 2009; Dessì et al., 2019). Intriguingly, our group has recently demonstrated that 58% of *M.hominis* isolated from *T.vaginalis* carry the gene *goiC* (Thi et al., 2018), which is considered a virulent trait of bacterial strains and is significantly associated with amniotic infection and preterm labor risk (Allen-Daniels et al., 2015). *Trichomonas vaginalis* could represent an additional risk factor for adverse pregnancy outcomes, as it is able to transport and protect virulent *M. hominis,* which can reach and infect the amniotic fluid. Moreover, the inflammatory response to trichomonad infection can be synergistically enhanced by the presence of the symbionts *M.hominis* and TVV.

With respect to vaginal dysbiosis, it has been shown that bacterial vaginosis acquired during the first trimester of pregnancy is associated with a five- to seven-fold increased risk of spontaneous preterm labor and PTB (Taylor-Robinson and Lamont, 2011). Although infections of individual microorganisms and resulting inflammation are considered the major risk factors for pregnancy-adverse outcomes, several critical questions remain unanswered about the role of polymicrobial infections and the interplay between pathogens and vaginal microbiota during gestation.

CONCLUDING REMARKS

A healthy vaginal microbiota plays a major role in protecting the female genital tract against pathogenic organisms. Five different community state types have been identified based on the profile and complexity of microbiomes, and their relationships with single vaginal pathogens, including *T.vaginalis,* have been widely studied in the last years.

Nevertheless, the existence of symbiotic relations among the protozoan and the bacteria *M.hominis* and *Ca.M.girerdii* and the dsRNA viruses, leading to a complex pathogenic consortium, suggests that further studies that take into account their potential synergistic effects are necessary. In a context of vaginal dysbiosis, the microbiota, *T.vaginalis* and its endosymbionts make up a singular microbial entity that can lead to severe sequelae, including preterm delivery and the acquisition and transmission of HIV.

AUTHOR CONTRIBUTIONS

VM, PF and PR contributed to the conception and design of the study and wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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Vaginal Microbiota Evaluation and Lactobacilli Quantification by qPCR in Pregnant and Non-pregnant Women: A Pilot Study

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Pregnancy outcomes and women's health are directly affected by vaginal microbiota. This microbiota consists of a dynamic ecosystem of various microbes in different ratios, which in healthy conditions protect the vaginal epithelium from infections. However, cases of vaginal infection are regularly diagnosed in women of reproductive age, contributing to more severe outcomes. Therefore, our main goal was to determine the prevalence of bacterial vaginosis (BV), aerobic vaginitis (AV), and vulvovaginal candidiasis (VVC) among Ecuadorian pregnant and non-pregnant women. A cross-sectional study was conducted among 217 women between 13 and 40 years old seeking primary healthcare in Carlos Andrade Marin Hospital (HCAM), Gynecological-Obstetric Hospital Isidro Ayora (HGOIA) and Center for Teaching Health Cipriana Dueñas during October 2018 to February 2019. The classical characterization of the vaginal microbiota was performed through microscopy by the Nugent criteria to evaluate the presence of BV, healthy and intermediate microbiota, by the criteria of Donders to determine the presence of AV and by the Marot-Leblond criteria to diagnose VVC. DNA extraction from vaginal samples and Polymerase Chain Reaction (PCR) analysis was performed to characterize the presence of *Gardnerella* spp., *Mobiluncus mulieris*, *Escherichia coli*, *Enterococcus* spp., and *Lactobacillus* spp. Finally, quantification of the lactobacilli was performed by quantitative real-time PCR (qPCR) for samples from women with normal vaginal microbiota and women with AV. Our results showed 52% of women with healthy microbiota, 7% with intermediate microbiota, and 41% with vaginal dysbiosis, comprising 27% with AV, 8% with BV and 4% with VVC and 2% with co-infections or co-dysbiosis. Additionally, a higher amount of lactobacilli were found in pregnant women when compared to non-pregnant women, while AV cases were characterized by a significant drop of *Lactobacillus* spp., more precisely, between 1E3 and 1E5 colony forming units (CFU)/ml.

Finally, women with normal vaginal microbiota showed an average load of lactobacilli between 1E6 and 1E7 CFU/ml. This pilot study showed no statistically significant differences between pregnant and non-pregnant women, pointing to the possibility to use lactobacilli quantification for the prevention of future vaginal infections.

Keywords: vaginal microbiota, vaginal infection, bacterial vaginosis, aerobic vaginitis, pregnant, opportunistic pathogen, *Lactobacillus* spp.

INTRODUCTION

The normal vaginal microbiota plays a crucial role for the health of pregnant and non-pregnant women (Vaneechoutte, 2017b), preventing several urogenital diseases (Ling et al., 2013), including bacterial vaginosis (BV) (Dai et al., 2010; Ling et al., 2010, 2013; Gondo et al., 2011; Van De Wijgert et al., 2014), aerobic vaginitis (AV) (Donders et al., 2005, 2011; Fan et al., 2013; Jahic et al., 2013; Tansarli et al., 2013), urinary tract infections (UTI) (Cauci et al., 2002; Zhou et al., 2004; Borges et al., 2014), yeast vaginitis (Ringdahl, 2006; Dai et al., 2010; Xu et al., 2010), and sexually transmitted diseases (such as HIV) (Bolton et al., 2008; Srinivasan and Fredricks, 2008; Petrova et al., 2013; Van De Wijgert et al., 2014). In the context of this study, it is also important to mention that women and teenagers in Ecuador have a wide range of health care needs, in particular, related to sexual and reproductive health (Svanemyr et al., 2017). In Ecuador, a major concern is the high rate of adolescent pregnancy, i.e., pregnancy between ages 10 to 19. Several studies worldwide also demonstrated a higher risk of acquiring HIV, herpes simplex virus type 2 and other sexually transmitted infections in non-pregnant women with vaginal infections or intermediate vaginal microbiota (Li et al., 2012; Petrova et al., 2013; Datcu et al., 2014; Van De Wijgert et al., 2014). Thus, lactic acid-producing bacteria (such as *Lactobacillus* spp.) metabolize glycogen, increasing lactic acid and a normal acidic vaginal pH of 3.8–4.4 (Farage et al., 2010; Borges et al., 2014; Mendling, 2016; Vaneechoutte, 2017b).

The vaginal microbial community is a variable econiche that fluctuates between normal and dysbiotic microbiota (Vaneechoutte, 2017b), which could be influenced by several intrinsic and extrinsic factors (Mendling, 2016) and eventually leading to an increment of both aerobic and anaerobic microorganisms (Larsen and Monif, 2001; Ling et al., 2010; Fredricks, 2011; Ravel et al., 2011). However, the most predominant genus in a healthy vaginal microbiota is *Lactobacillus* (Borges et al., 2014; Vaneechoutte, 2017b). *Lactobacillus* genus is known to inhibit the adhesion and proliferation of opportunistic and primary pathogens (Bolton et al., 2008). The mechanisms by which vaginal lactobacilli provide colonization resistance is generally considered to be through production of several antimicrobial compounds such as hydrogen peroxide, lactic acid and/or bacteriocins (Aroutcheva et al., 2001; Alpay et al., 2003; Vaneechoutte, 2017b; Collins et al., 2018), as well as acting as biosurfactant on the vaginal epithelium (Boris and Barbés, 2000; Borges et al., 2014).

Although several species of *Lactobacillus* were already identified in vaginal microbiota, the most predominant species found in normal vaginal microbiota are *L. crispatus, L. gasseri*, *L. jensenii*, and *L. iners* (Farage et al., 2010; Borges et al., 2014; Oliveira et al., 2018). Also, other species could be detected in low amount among healthy vaginal microbiota such as *Atopobium*, *Enterobacter*, *Escherichia*, *Gardnerella*, *Mobiluncus*, *Prevotella*, *Staphylococcus, Shigella* (Hernández-Rodríguez et al., 2011; Gajer et al., 2012; Romero et al., 2014; Oliveira et al., 2018). These species can also behave as opportunistic pathogens (Gajer et al., 2012; Vaneechoutte, 2017a). Several factors can induce disruptions of the healthy microbiota equilibrium, establishing a microbial dysbiosis and, thus, future vaginal infections (Gajer et al., 2012; Johnson and Versalovic, 2012; Petrova et al., 2013; Vaneechoutte, 2017b).

According to previous studies, bacterial vaginosis (BV) is the most common vaginal dysbiosis among women of reproductive age (Cristiano et al., 1996; Nelson et al., 2009; Dai et al., 2010; Gondo et al., 2011), being characterized by lactobacilli replacement by anaerobes (Donders et al., 2011). *Gardnerella* spp., *Atopobium vaginae*, *Bacteroides* spp. and *Mobiluncus* spp. are the main pathogenic anaerobes associated with BV (Mendling, 2016), which is usually diagnosed by Nugent criteria (Nugent et al., 1991) or the Amsel criteria (Van De Wijgert et al., 2014). Besides BV, a condition designated aerobic vaginitis (AV) has also been recognized, characterized by the presence of aerobic bacteria in detriment of lactobacilli and by inflammation diagnosed a yellow-green discharge (Donders, 2007; Mendling, 2016). This vaginal infection is usually dominated by *Streptococcus* sp., *Enterococcus* sp., and/or Gram-negative bacteria of enteric origin (mainly, *Escherichia coli*). Finally, vulvovaginal candidiasis (VVC) is the most prevalent cause of vaginal infection by fungi, with at least 75% of healthy women suffering one episode of VVC during lifetime (Ringdahl, 2006) and whereby *Candida albicans* is the most important species (Marot-Leblond et al., 2009).

Our main goal of the present study was to evaluate the presence of vaginal infection among Ecuadorian women by classical and standard microbiological techniques or criteria (Fredricks, 2011) and to determine the dominance of different types of vaginal infection among pregnant and non-pregnant women. Also, the present study aimed to detect the presence of specific opportunistic pathogens (*E. coli, Enterococcus faecalis*, *Gardnerella* spp., and *Mobiluncus mulieris*) by Polymerase Chain Reaction (PCR) and quantified the number of lactobacilli through quantitative real-time PCR (qPCR). The analysis of the normal amount of lactobacilli in pregnant and non-pregnant women might enable to determine the lactobacilli threshold associated with the establishment of vaginal infection.

MATERIALS AND METHODS

Study Population, Design, and Subject Selection

The study was conducted in the Microbiology Institute at USFQ in collaboration with Hospital Carlos Andrade Marín (HCAM) and Universidad Central del Ecuador (UCE) from October 2018 to February 2019. The research team recruited 217 Ecuadorian female volunteers of Hispanic ethnicity but in reproductive age (13 and 40 years old), of which 111 were pregnant. Applicants were excluded from the study if they reported antimicrobial treatment in the last 3 months or any evidence of bleeding, and also if they had sexual intercourse within the previous 48 h. Also, a questionnaire was taken regarding demographic characteristics, sexual and health behavior of each patient, and each enrolled woman provided a usable vulvovaginal swab sample.

Ethics Statement

This study was approved by the Ethics Committee of Universidad San Francisco de Quito (USFQ) and the Ministry of Health of Ecuador (Protocol code: 2016-140M by MSP-SDM-10-2013-2019-O review board). The female participants were recruited to our study set, after having read and signed the informed consent or, in the case of underaged participants, from their parents or legal representatives.

Sample Collection

Samples were taken by a gynecologist using a sterile disposable vaginal speculum. The lateral vaginal walls were swabbed with a sterile swab to collect the cervical fluid, to prepare a smear on a microscope slide. Briefly, each vaginal smear was obtained by rolling the previous swab onto a glass slide, then heat-fixed and Gram-stained by using safranin as the counterstain. Following the Gram smear procedure, the swab was placed in 1 ml of phosphate buffer saline (PBS) and vortexed vigorously for ∼3 min. The remaining vaginal material was collected by centrifugation at 16,000 g for 5 min. The obtained pellet was suspended into an aliquot of 1 ml of saline (0.9% NaCl) which was used for culture of *Candida* spp. in different media (see section Culture of Candida spp.) and for wet mount microscopy for a better diagnosis of AV and VVC (see section Microbiological Classification of Vaginal Infections).

A second sample was taken by a cervical brush (Rovers Cervex Brush®) through endo and exo-cervical brushing, placed immediately in Cobas® Preservative Fluid, stored at 4°C until processing in the clinical laboratory of HCAM, and used for DNA extraction (see section DNA Extraction of Vaginal Swabs). Each sample was further used to culture of *Candida* spp.

Culture of *Candida* spp.

Candida spp. was cultured on different media from the saline aliquot (see section Sample Collection). Briefly, 100 μl of saline solution was plated onto Petri dishes containing 5% human blood agar (HBA), chocolate agar (heated human blood agar) or Sabouraud dextrose agar (SDA). The plates were incubated at 37°C for 48 h, under aerobic conditions, and colonies were analyzed and identified by gram staining, biochemical properties (catalase, oxidase, and hemolysis) and PCR (data not shown).

Microbiological Classification of Vaginal Infections

The Gram-stained vaginal smears were classified according to Nugent criteria for bacterial vaginosis (BV) (Nugent et al., 1991), the criteria of Donders et al. for aerobic vaginitis (AV) (Donders, 1999) and those of Marot-Leblond et al. for vulvovaginal candidiasis (VVC) (Marot-Leblond et al., 2009). The evaluation of several cell types present in each smear was performed for 10 to 15 microscopic fields under oil immersion at 1000 X magnification (Donders, 2007).

After an initial evaluation of the Gram-stained smears by the Nugent criteria, all samples were evaluated by means of phase-contrast microscopy (X400 magnification) of wet smears, according to Schröders classification (Donders et al., 2005) and the Marot-Leblond et al. (2009) criteria (see **Table 1**). The absence of *Lactobacillus* spp., presence of cocci or coarse bacilli in high numbers, presence of parabasal epithelial cells representing >10% of the epithelial cells, and/or presence of leucocytes were considered as indicative for AV (Donders et al., 2005). In addition, aggravated AV diagnosis was defined as the most extreme form of aerobic vaginitis under Donders evaluation from Schröders classification (Donders et al., 2005), where AV samples showed lactobacilli severely depressed or absent because of overgrowth of other bacteria (Cocci or chains), more than 10 leukocytes per epithelial cell present in the samples and more than 50% of the leukocytes had a toxic appearance. It is important to mention that leukocytes were also evaluated on their granular appearance due to abundant lysozyme activity ("toxic leukocytes") (Donders et al., 2005). Finally, VVC was assessed accordingly to Marot-Leblond and colleagues through at least one of the following criteria: positive Gram-stain preparation with budding yeasts in high numbers (five or more) in more than two microscopic fields, pseudohyphae, and/or hyphal forms in wet smears observation; and positive culture in Chocolate agar, HBA and/or SDA, along with negative microscopic examination results associated with eventual symptoms (thick, white vaginal discharge with no odor, vulvar and vaginal pruritus, burning, or dyspareunia) or clinical history (previous infection) obtained from the medical survey with the professional gynecologist. Absence of *Candida* cells in more than two microscopic fields and/or a low number of *Candida* spp. result on wet smears observation and culture growth was considered as normal *Candida* colonization rather than VVC (Marot-Leblond et al., 2009).

DNA Extraction of Vaginal Swabs

The Cobas® 4800 system (Roche Molecular Systems Inc., Pleasanton, CA) was used to extract the DNA of vaginal brushes, according to the manufacturer's instructions. DNA was quantified with a Nanovue spectrophotometer (GE Healthcare Life Science). DNA was eluted at 20 ng/μl with molecular grade water and stored at −20°C until the Polymerase Chain Reaction (PCR) analysis was performed. The quality of DNA was evaluated

TABLE 1 | Parameters used for the diagnosis of vaginal infections.

Infection	Symptoms	Discharge	Odor	Diagnosis	References
Vulvovaginal candidiasis	Pruritus	Thick, white to yellow	Absent	Microscopic examination (Gram-stained smears and Wet mount preps), medical survey and growth culture	Carr et al., 1998; Marot-Leblond et al., 2009
Aerobic vaginitis	Inflammation	Yellow	Foul, rotten	Microscopic examination (Gram-stained smears and Wet mount preps) and medical survey	Donders et al., 2002; Donders et al., 2005
Bacterial vaginosis	Irritation, 50% asymptomatic	Thin, white to gray, homogeneous	Fishy	Microscopic examination (Gram-stained smears and Wet mount preps) and medical survey	Carr et al., 1998; Nugent et al., 1991

TABLE 2 | PCR primers used in this study.

Set	Name	Sequence (5′-3′)	Target	T (°C) of annealing	Size of fragment	Target gene	Specificity %	Validation	References
1	Primer E1	ATCAAGTACAGTTAGTCTT	*Enterococcus faecalis*	54°C	941 bp	*ddl*	100.0%	Increase of the annealing temperature at 54°C	DTU National Food Institute, 2014
	Primer E2	ACGATTCAAAGCTAACTG							
2	adk F	ATTCTGCTTGGCGCTCCGGG	*Escherichia coli*	57°C	583 bp	*adk*	49.0% 98.0%	Increase of the annealing temperature at 57°C	Sepehri et al., 2009
	adk R	CCGTCAACTTTCGCGTATTT							
3	Gard154-Fw	CTCTTGGAAACGGGTGGTAA	*Gardnerella* spp.	60°C	301 bp	*16S* rRNA	100.0%	N/d	Henriques et al., 2012
	Gard154-Rv	TTGCTCCCAATCAAAAGCGGT							
4	LactoF	TGGAAACAGRTGCTAATACCG	*Lactobacillus* spp.	62°C	233 bp	*16S* rRNA	47.1% 66.7%	N/d	Henriques et al., 2012
	LactoR	GTCCATTGTGGAAGATTCCC							
5	Mobil-577F	GCTCGTAGGTGGTTCGTCGC	*Mobiluncus mulieris*	62°C	449 bp	*16S* rRNA	100.0%	N/d	Fredricks et al., 2007
	M.mulie-1026R	CCACACCATCTCTGGCATG							

N/d – Non-determined.

by measuring the concentration of phenolic compounds or the presence of salts (260/230) and protein contaminants (260/280).

Identification of the Major Bacterial Species by PCR

From 217 vaginal samples previously diagnosed by classical criteria through microscopy analysis, 97 were selected for molecular characterization by PCR in a Bio-Rad Thermocycler (Bio-Rad, Hercules, CA). Samples with scores between 0 and 1 of Nugent criteria were selected as healthy microbiota, while samples with scores between 9 and 10 of Nugent criteria (BV) and diagnosed as representing aggravated AV (see section Microbiological Classification of Vaginal Infections) were used as dysbiotic microbiota. Thus, sixty samples with healthy microbiota (38 pregnant and 22 non-pregnant women), 23 samples with AV (14 pregnant and 9 non-pregnant women), and 14 samples with BV microbiota (6 pregnant and 8 non-pregnant women) were included. All samples were analyzed with a total of five primer pairs, targeting two anaerobes (*Gardnerella* species, and *M. mulieris*), two aerobes (*E. coli* and *E. faecalis*) and for the genus *Lactobacillus*. Single-template PCR assays were performed for each primer set. The sequence, amplicon size, target gene, and temperature of annealing for each primer pair are described in **Table 2**.

For PCR, 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), 1X 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 ∼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 (**Table 2**) 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 duplicate or 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.

Quantification of *Lactobacillus* sp. by Quantitative Real-Time PCR (qPCR)

To create positive controls and standard quantification solutions with a well-known *Lactobacillus* sp. concentration (CFU/mL), a sample of known concentration (also known as a calibrator) (e.g., number of CFU per mL) was obtained through a validated calibration curve (CFU/OD) (Begot et al., 1996). This calibrator was serially diluted tenfold and used to construct a standard curve for qPCR assays. Accordingly, *Lactobacillus gasseri* strain JCM1131 was cultured during 24 h in Mann Rogosa Sharp Agar at 37°C under microaerophilic conditions (Begot et al., 1996; Mytilinaios et al., 2012; Machado et al., 2013). The calibrator concentration was previously proved by media growth culture counting as previously described (Naghili et al., 2013). The DNA extraction was performed from the highest CFU/ml concentration, and serial dilutions from 1E9 to 1E0 CFU/mL were used as qPCR standards. The DNA extraction of this solution with the highest concentration was performed under the same procedure already described in section DNA Extraction of Vaginal Swabs. In each qPCR assay, two random controls were also used as blind samples in triplicate.

Each reaction was performed with GoTaq® Master Mix qPCR (Promega, Madison, WI, USA) in a final volume of 20 μl, 0.5 μM of each primer (LactoF-TGGAAACAGRTGCTAATACCG and LactoR-GTCCATTGTGGAAGATTCCC) and 2 μl of DNA template. Each qPCR assay was performed in a quantitative real-time PCR Thermocycler (Bio-Rad CA, USA) under the following conditions: initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, extension at 72°C for 1 min, and a final extension for 5 min at 72°C. Each qPCR assay was followed by this melt curve analysis, allowing amplicon validation and identification of false positives through its profile and the specific temperature of melting (Tm). Each sample was analyzed by triplicate, and qPCR assays were realized in different days. Negative target controls and no template controls were included in all plates.

Primers used for these qPCR assays were previously described to amplify *Lactobacillu*s spp. through classical PCR, but not for quantitative real-time PCR. Therefore assay metrics were determined by testing their performance across the limit of quantification (LoQ) and limit of detection (LoD), as well as linearity as previously described (Price et al., 2012). The optimized assay exhibited the LoQ and LoD to be 1E2 CFU per ml, while the range of linearity of the assay was from 1E9 to 1E3 CFU/mL. The load of lactobacilli in each sample was determined by running six or five standard dilutions (1E9-1E3 CFU/mL), both in duplicate or triplicate on each qPCR assay.

For the quantification of *Lactobacillus* spp., 83 samples were selected for qPCR analysis from the initial subset of 97 vaginal samples previously characterized by PCR assays to molecular characterization of the main bacteria (see section Identification of the Major Bacterial Species by PCR), i.e., 60 from healthy microbiota samples and 23 from AV samples.

Statistical Analysis

Statistically significant differences in *Lactobacillus* spp. quantity among women with healthy and dysbiotic microbiota were evaluated using Kruskal Wallis one-way ANOVA and Mann Whitney tests. In addition, the same statistical analysis was carried out among pregnant and non-pregnant women. Finally, multivariable analysis was performed for sociodemographic and behavioral factors by using Minitab 2017 (Version 17, Minitab, State College, PA).

RESULTS

Population Study

The sociodemographic characteristics for 217 women were included in the statistical analysis and presented in **Table 3**. Half of the women in the study were pregnant (51.2%) and approximately half (47.4%) were non-pregnant. They were between 21 and 30 years of age. Only 11 women (5.1%) identified themselves as White, Afro-Ecuadorian, or Indigenous women. So, the majority of the women in our study set (94.9%) were categorized as "Half-blood," being of Hispanic ethnicity mixed with another background ethnicity (Caucasian, African, or Indigenous women). When performing an overall statistical analysis of age, the results do not show a significant relationship between age and the probability of having a specific diagnosis. Hence, there is no statistical evidence to determine that a woman's age is directly related to a specific vaginal disruption or having a healthy microbiota. From all sociodemographic factors analyzed, only the occupation category had a statistical significance over the diagnostic classification of vaginal infection with a *P*-value of 0.003 through the Chi-square test (see **Table 3**). Similarly, the use of contraceptive methods, having different sexual partners, vaginal douches, or cigarette smoking did not show any relation to the development of any vaginal infection type during the study (see **Table 4**).

Diagnosis of Vaginal Infections

The vaginal samples were evaluated in the Microbiology Institute of USFQ, according to microbiological criteria of Nugent et al. (1991) to identify healthy microbiota, BV, and intermediate microbiota (Nugent et al., 1991); Schröders classification under criteria of Donders et al. (2005) to characterize AV (Donders et al., 2005), and the criteria of Marot-Leblond et al. (2009) to determine VVC (Marot-Leblond et al., 2009). As shown in **Table 3**, 112 (52.0%) vaginal samples were classified as healthy microbiota, 16 (7.0%) were identified as intermediate microbiota, and 89 (41.0%) were diagnosed as dysbiotic (41.0%), which includes single cases of BV, AV, and VVC but also co-infections. The presence of a unique type of vaginal infection was identified in 85 vaginal samples (39.2%), whereby AV was the most prevalent infection with 26.7% of the vaginal samples, followed by BV (8.3%) and 4.1% with VVC. Furthermore, four vaginal samples were diagnosed with co-infections (1.8%), more

TABLE 3 | Sociodemographic among women in this study with healthy vaginal microbiota, intermediate vaginal microbiota, and vaginal infections (bacterial vaginosis, aerobic vaginitis, candidiasis, and co-infections).

	Healthy microbiota *N* (%)	Intermediate microbiota *N* (%)	Candidiasis *N* (%)	Bacterial vaginosis *N* (%)	Aerobic vaginitis *N* (%)	Co-infections *N* (%)	Total *N*	*P* (X^2)
Focus group								
Non-pregnant	52 (49.1)	7 (6.6)	4 (3.8)	12 (11.3)	28 (26.4)	3 (2.8)	106	0.566 (3.9)
Pregnant	60 (54.1)	9 (8.1)	5 (4.5)	6 (5.4)	30 (27.0)	1 (0.9)	111	
Age								
≤ 20	38 (47.5)	4 (5.0)	5 (6.3)	10 (12.5)	21 (26.3)	2 (2.5)	80	0.799 (92.7)
21–25	26 (41.9)	6 (9.7)	1 (1.6)	4 (6.5)	24 (38.7)	1 (1.6)	62	
26–30	24 (58.5)	3 (7.3)	3 (7.3)	2 (4.9)	9 (21.9)	0 (0.0)	41	
31–40	24 (70.6)	3 (8.8)	0 (0.0)	2 (5.9)	4 (11.8)	1 (2.9)	34	
Global Incidence	112 (52.0)	16 (7.0)	9 (4.0)	18 (8.0)	58 (27.0)	4 (2.0)	217	0.308 (22.6)
Ethnicity								
Afro Ecuadorian	1 (25.0)	1 (25.0)	0 (0.0)	0 (0.0)	2 (50.0)	0 (0.0)	4	0.737 (11.2)
Half-blood	107 (51.9)	15 (7.3)	9 (4.4)	18 (8.7)	53 (25.7)	4 (1.9)	206	
Indigenous	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	2	
White	4 (80.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	5	
Occupation								
Housewife	31 (46.3)	1 (1.5)	1 (1.5)	11 (16.4)	22 (32.8)	1 (1.5)	67	0.003 (26.7)
Student	39 (46.4)	6 (7.1)	5 (6.0)	5 (6.0)	26 (31.0)	3 (3.6)	84	
Employee	42 (63.6)	9 (13.6)	3 (4.5)	2 (3.0)	10 (15.2)	0 (0.0)	66	
Civil Status								
Married	24 (63.2)	3 (7.9)	0 (0.0)	2 (5.3)	8 (21.1)	1 (2.6)	38	0.245 (18.4)
Divorced	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2	
Single	58 (49.6)	6 (5.1)	6 (5.1)	7 (6.0)	37 (31.6)	3 (2.6)	117	
Free Union	29 (48.3)	6 (10.0)	3 (5.0)	9 (15.0)	13 (21.7)	0 (0.0)	60	
Education Level								
None	2 (66.7)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3	0.916 (24.2)
Basic (High-school students)	13 (41.9)	2 (6.5)	0 (0.0)	4 (12.9)	10 (32.3)	2 (6.5)	31	
Bachelor (Undergraduate students)	52 (47.7)	10 (9.2)	7 (6.4)	9 (8.3)	30 (27.5)	1 (0.9)	109	
Superior (Bachelor graduates)	30 (55.6)	3 (5.6)	1 (1.9)	5 (9.3)	14 (25.9)	1 (1.9)	54	
Higher Degree Research (HDR) candidates (Master and Doctor's degree students)	15 (75.0)	0 (0.0)	1 (5.0)	0 (0.0)	4 (20.0)	0 (0.0)	20	

N number of women who responded in the survey within each category; % assigned percentage for each classification within each category. P (X2) p-value through the Chi-square test show any relation among each sociodemographic factor and the possibility of having a vaginal disruption or healthy microbiota.

precisely two of them with AV and BV, one with AV and VVC, and one with BV and VVC. None of the co-infection samples was further evaluated during qPCR analysis.

Prevalence of Vaginal Infections Among Pregnant and Non-pregnant Women

Each focus group (pregnant and non-pregnant women) was analyzed to identify any relation between vaginal infection and pregnancy (see **Table 5**). Although pregnant and non-pregnant women have similar prevalence values in the healthy microbiota, most cases of BV and co-infection were found in non-pregnant women with 67% (12/18) and 75% (3/4) of the cases, respectively, as shown in **Table 5**. However, these differences were not significant.

Presence of Opportunistic Species and *Lactobacillus* spp. in Vaginal Microbiota

The presence of *G. vaginalis* and *M. mulieris* (as BV biomarkers), *E. coli* and *E. faecalis* (as AV biomarkers), and *Lactobacillus* spp. (as healthy biomarker) were analyzed by PCR assays from the selected 97 samples (see section Identification of the Major Bacterial Species by PCR). As previously mentioned, almost a half of population set was chosen by classical criteria through microscopy analysis (data not shown), more exactly, healthy microbiota samples with 0–1 and BV samples with 9–10 according to Nugent criteria (Nugent et al., 1991), and the most aggravated AV samples (Donders, 1999).

The results still evidenced the presence of *Lactobacillus* spp. in both types of vaginal dysbiosis, although their presence decreased to 21% in BV ($P = 0.006$) and 13% in AV (P

TABLE 4 | Behavioral variables among women in this study with healthy vaginal microbiota, intermediate vaginal microbiota, and vaginal infections (bacterial vaginosis, aerobic vaginitis, candidiasis, and co-infections).

	Healthy microbiota *N* (%)	Intermediate microbiota *N* (%)	Candidiasis *N* (%)	Bacterial vaginosis *N* (%)	Aerobic vaginitis *N* (%)	Co-infections *N* (%)	Total *N*	*P* (X^2)
Has sexual partner								
No	16 (45.7)	3 (8.6)	3 (8.6)	2 (5.7)	10 (28.6)	1 (2.9)	35	0.707 (3.0)
Yes	96 (52.7)	13 (7.1)	6 (3.3)	16 (8.8)	48 (26.4)	3 (1.6)	182	
Different sexual partners								
No	54 (58.1)	4 (4.3)	4 (4.3)	7 (7.5)	24 (25.8)	0 (0.0)	93	0.205 (13.3)
Yes	52 (49.1)	10 (9.4)	5 (4.7)	7 (6.6)	29 (27.4)	3 (2.8)	106	
Do not answer	6 (33.3)	2 (11.1)	0 (0.0)	4 (22.2)	5 (27.8)	1 (5.6)	18	
Uses birth control method								)
No	45 (46.9)	1 (25.0)	6 (6.3)	7 (7.3)	29 (30.2)	1 (1.0)	96	0.877 (5.2)
Yes	65 (55.1)	15 (7.3)	3 (2.5)	11 (9.3)	28 (23.7)	3 (2.5)	118	
Do not answer	2 (66.7)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	3	
Smokes								
No	105 (51.5)	14 (6.9)	8 (3.9)	18 (8.8)	55 (27.0)	4 (2.0)	204	0.683 (3.1)
Yes	7 (53.8)	2 (15.4)	1 (7.7)	0 (0.0)	3 (23.1)	0 (0.0)	13	
Vaginal douching								
No	31 (57.4)	3 (5.6)	1 (1.9)	6 (11.1)	12 (22.2)	1 (1.9)	54	0.881 (5.2)
Yes	79 (49.7)	12 (7.5)	8 (5.0)	12 (7.5)	45 (28.3)	3 (1.9)	159	
Do not answer	2 (50.0)	1 (25.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	4	

N number of women who responded in the survey within each category; % assigned percentage for each classification within each category. P (X2) P-value through the Chi-square test.

TABLE 5 | Contingency table of vaginal samples between Focus Group and the diagnosis of vaginal infections, healthy and intermediate vaginal microbiota.

		Diagnostic						
Group		**Aerobic vaginitis**	**Bacterial vaginosis**	**Candidiasis**	**Co-infection**	**Healthy**	**Intermediate**	**Total**
Non-Pregnant	Number	28	12	4	3	52	7	106
	(% within the column)	(48.3)	(66.7)	(44.4)	(75.0)	(46.4)	(43.8)	(48.8)
Pregnant	Number	30	6	5	1	60	9	111
	(% within the column)	(51.7)	(33.3)	(55.6)	(25.0)	(53.6)	(56.3)	(51.2)

*Number of women who responded in the survey within each category; % assigned percentage for each classification within each category. No statistically significant differences were found between pregnant and non-pregnant groups among vaginal infections, healthy and intermediate microbiota (P-value = 0.566[3.888]; see **Table 3**).*

= 0.019) when compared to healthy microbiota samples (see **Figure S1**). Regarding the presence of *Gardnerella* species, it was present less frequently in healthy microbiota (37%) while in BV and AV prevalence was 71% ($P = 0.001$) and 78% ($P = 0.033$), respectively. On the other hand, *M. mulieris* and *E. coli* were found in BV at 79% and 36%, respectively; while being detected in low frequency in healthy microbiota and AV cases, as shown in **Figure S1**. The presence of *M. mulieris* was low in frequency on AV and normal microbiota, when compared to BV cases. However, presence of *M. mulieris* was statistically different among healthy microbiota against BV ($P < 0.001$) and AV cases ($P = 0.002$), being less recurrent in healthy samples. Finally, *E. coli* did not show statistical differences among healthy microbiota and BV cases ($P = 0.062$). Also, it is important to mention that *E. faecalis* was found to be absent in the population set of the present study.

Among pregnant and non-pregnant women with healthy microbiota, we found that prevalence of *Lactobacillus* spp. was similar, as shown in **Figure 1**. On the other hand, pregnant women evidenced higher presence of *Gardnerella* species (39%), and *M. mulieris* (16%) when compared to non-pregnant women (32% *Gardnerella* spp., and 9% of *M. mulieris*).

In the presence of vaginal dysbiosis and infection, no statistically significant differences were found around opportunistic species between pregnant and non-pregnant women, as shown in **Figure 1**. However, *Lactobacillus* spp. showed statistically significant differences between pregnant and non-pregnant women in both BV and AV cases, as shown in **Figure 1**. In the case of BV, a higher prevalence of *Lactobacillus* spp. is shown in pregnant women (100%) compared to non-pregnant women (63%; $P = 0.028$). No significant statistically differences were found on the frequency of *Gardnerella* spp. and *M. mulieris* in these samples. In opposition, a drop of

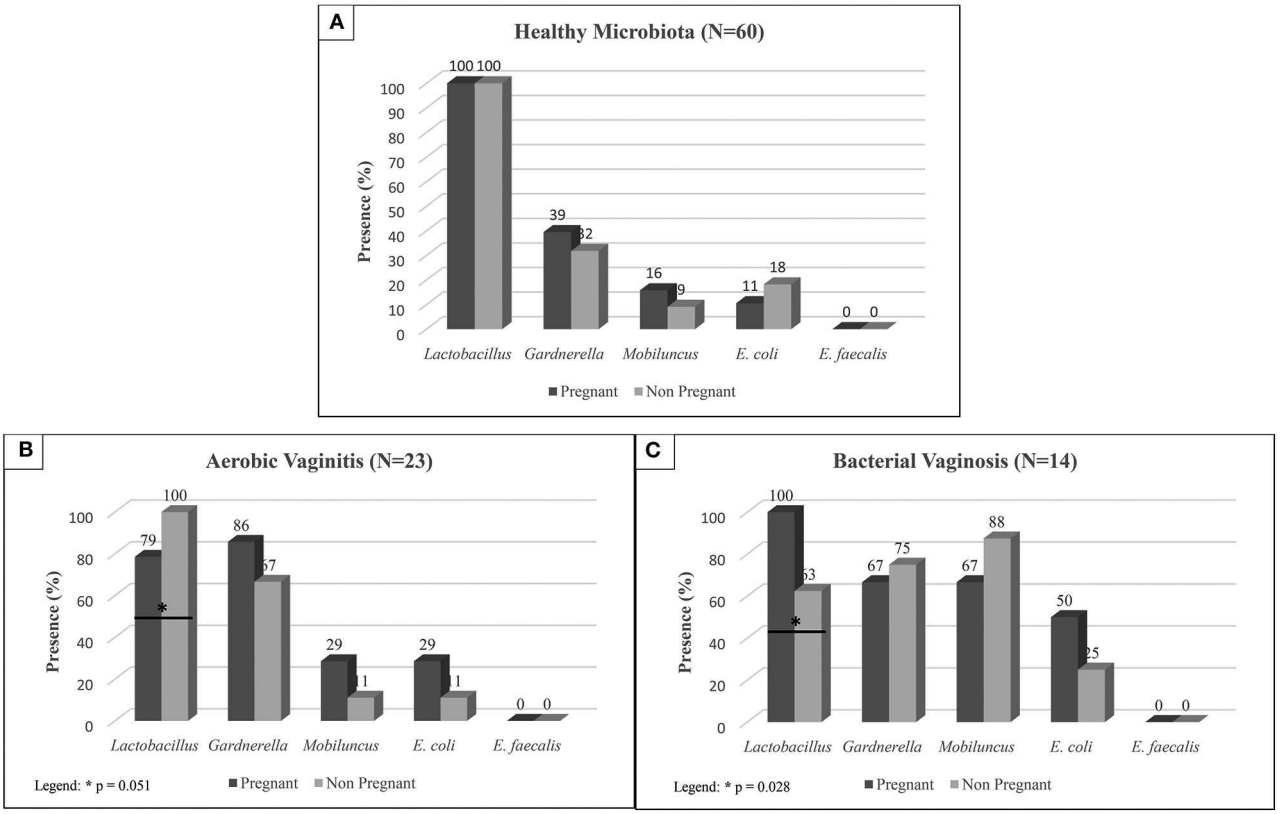

FIGURE 1 | Prevalence of each bacterium in pregnant and non-pregnant women diagnosed as: **(A)** Healthy Microbiota, **(B)** Aerobic Vaginitis, and **(C)** Bacterial Vaginosis according to the microbiological diagnosis. Statistically significant differences were evaluated by Chi-square tests.

Lactobacillus spp. prevalence is shown in pregnant women with AV (79%; $P = 0.051$) when compared to non-pregnant women (100%).

Lactobacilli Quantification by Quantitative Real-Time PCR (qPCR)

Due to the small number of samples with BV, we restricted comparison of the lactobacilli quantification to healthy (60) vs. AV (23) cases.

Due to the low number of data, a non-parametrical statistical analysis was performed by means of a Mann-Whitney. Significant differences were shown between healthy and AV groups ($P < 0.001$; see **Figure 2**), whereby *Lactobacillus* spp. varied between 1E6 and 1E7 CFU/ml in healthy microbiota decreased to between 1E3 and 1E5 CFU/ml in AV cases. This was confirmed by Kruskal–Wallis one-way ANOVA testing ($P < 0.001$; see **Figure 2**).

Mann-Whitney testing indicated no statistically significant differences between pregnant and non-pregnant women with healthy vaginal microbiota and with AV ($P = 0.330$ and $P = 0.637$), as shown in **Figure 2**. However, the analysis showed statistically significant differences ($P < 0.001$) when comparing pregnant women with healthy microbiota against AV. Likewise, we found slight differences ($P = 0.006$) when comparing non-pregnant women with healthy microbiota against AV. Finally, it is worth noting that the same significance levels were also observed between healthy pregnant women against AV non-pregnant women ($P < 0.001$) and between healthy non-pregnant women against AV pregnant women ($P = 0.0041$). These preliminary results showed similar ranges of lactobacilli load in pregnant and non-pregnant women from each group set (AV and healthy vaginal microbiota).

DISCUSSION

Sociodemographic and Behavioral Variables Among Women

This study evaluated a possible relationship between vaginal infection, vaginal dysbiosis and sociodemographic or behavioral variables among pregnant and non-pregnant women. A disruption of the vaginal microbiota usually occurs when any cause promotes a diminution in lactobacilli levels, leading to other microorganisms' augmentation (primary or opportunistic pathogens). These causes of imbalance can be due to several intrinsic and extrinsic factors (Bolton et al., 2008; Borges et al., 2014). As intrinsic factors, the vaginal microbiota of women is driven mainly by hormonal changes during their reproductive

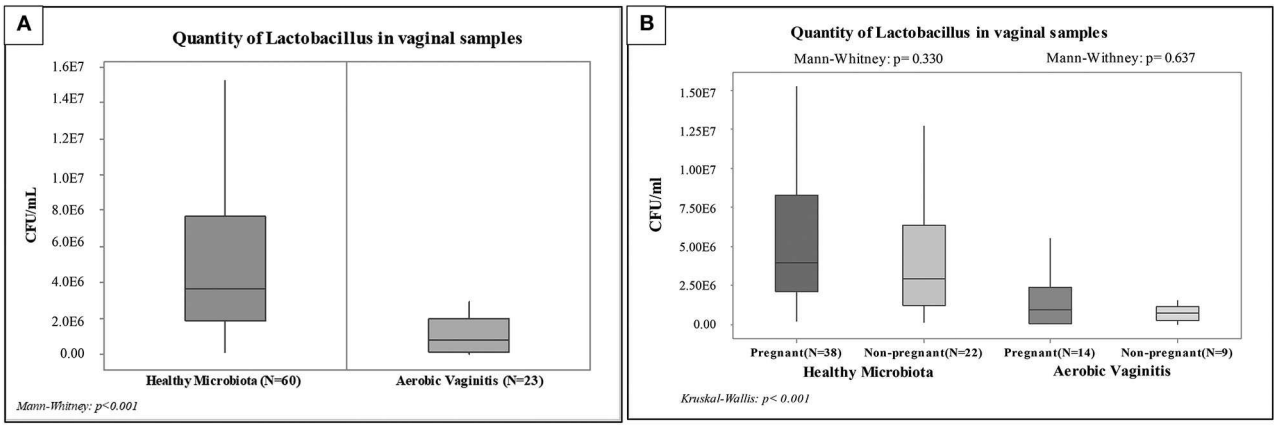

FIGURE 2 | Box plot of the quantification by qPCR of *Lactobacillus* spp. among vaginal samples: **(A)** Non-parametric. Statistical analysis among the overall groups (Healthy Microbiota and Aerobic Vaginitis), **(B)** Non-parametric. Statistical analysis among pregnant and non-pregnant women of each overall group.

life (Farage et al., 2010). These intrinsic factors were distinctively different in the two focus groups (pregnant and non-pregnant women). However, no statistically significant differences regarding composition of vaginal microbiota were detectable (see **Table 3**). Likewise, in this study, there was no statistically significant relationship among any extrinsic factor (behavioral variables) obtained in the questionnaire by multivariate analysis (see **Table 4**). This differs from other studies that established statistically significant association with some of the extrinsic factors analyzed by this study, such as the number of sexual partners (Schwebke et al., 1999), and ethnicity (Zhou et al., 2004). Others could not establish associations with the use of contraception, lubricant or spermicide, as well as personal hygiene habits (Keane et al., 1997). Similarly, others did not find any effect of oral contraceptives on the vaginal microbiota of 36 women (Eschenbach et al., 2000). As such, several studies reported contradicting results regarding sociodemographic and behavioral variables, making conclusive comparisons difficult to achieve.

Prevalence and Types of Vaginal Infection and Vaginal Dysbiosis

In our study set, 52% of women were characterized by a healthy vaginal microbiota, 7% were diagnosed with intermediate vaginal microbiota and 41% with some vaginal infection or vaginal dysbiosis (BV). Similar results were reported in the United Kingdom (Keane et al., 1997), identifying 48% of female participants with healthy vaginal microbiota and 19% with an abnormal microbiota. Similarly, Gondo et al. (2011) reported that 47.5% of the women showed infection in a study enrolling 245 Brazilian women (Gondo et al., 2011). Bacterial vaginosis (BV) is usually reported as the most prevalent vaginal infection around the world (Nelson et al., 2009; Ling et al., 2013; Machado et al., 2013), followed by vulvovaginal candidiasis (VVC) (Ringdahl, 2006). Another condition, aerobic vaginitis (AV), has been recently characterized by Donders and colleagues in 1999, and has been shown to play an important role for vaginal health (Donders, 1999; Datcu et al., 2014; Donders et al., 2017). Furthermore, Donders (2007) showed that this type of vaginal infection could easily be confused with an intermediate microbiota or even bacterial vaginosis (Donders, 2007), which may be a major reason why reliable data on the prevalence of AV in the general population are not very abundant (Donders et al., 2017).

AV can also be associated with the increased risk of preterm pre-labor rupture of membranes, chorioamnionitis, and preterm delivery (Donders et al., 2017). Contrary to previous studies (Schwebke et al., 1996; Cauci et al., 2002; Donders et al., 2005; Vieira-Baptista et al., 2017), in the present study AV was the most prevalent vaginal infection with a similar percentage of AV among pregnant (51.7%) and non-pregnant (48.3%) women. Again, the latter is in contradiction with other studies that reported low AV prevalence among pregnant women. Although Donders et al. (2009) postulated that AV was not common in pregnancy, a more recently publication by Donders et al. (2017) reported that AV could easily be confused with an intermediate microbiota and bacterial vaginosis and so reliable data on the prevalence of AV could be available in few amounts. In 2013, Jahic and colleagues diagnosed AV in 51% of the enrolled female participants, where *E. coli* and *E. faecalis* were the most prevalent bacteria (Jahic et al., 2013). In agreement, Fan et al. (2013) reported the same main bacteria and *S. epidermidis* in their AV cases (Fan et al., 2013).

In non-pregnant women, several studies reported a prevalence of AV between 5 and 10.5% in symptomatic women (Bologno et al., 2011; Marconi et al., 2012; Donders et al., 2017), whereby the most frequently identified bacteria were *E. coli* (4–23%) (Tansarli et al., 2013), *Staphylococcus* (around 27%), *Streptococcus* (0.7–58.7%) and *Enterococcus* spp. (0.3–2.4%) (Von Gruenigen et al., 2000; Iavazzo et al., 2008; Tansarli et al., 2013). These previous studies could partially explain the absence of *E. faecalis* in our study due to the low rate of detection. Finally, *E. coli* prevalence in pregnant (28.57%) and non-pregnant (11.11%), as established in our study, were within the range described by Tansarli et al. (2013) and in agreement with postulations made by Donders (2007).

Presence of Opportunistic Pathogens in Healthy Microbiota

The vaginal microbiota complexity in healthy and dysbiosis samples had already been described by several authors in women with AV and BV (Tempera and Furneri, 2010; Zozaya-Hinchliffe et al., 2010; Rumyantseva et al., 2016). Similar to Zozaya-Hinchliffe et al. (2010), we believed that the PCR characterization of the major bacterial species by PCR and the development of qPCR assays would be facilitated by first working with specimens whose microbiota would be most likely to differ significantly (Zozaya-Hinchliffe et al., 2010). So, we only selected vaginal samples from sixty women with normal vaginal microbiota who had Nugent scores of 0 and 1, twenty tree women with aggravated AV diagnosis (see section Microbiological Classification of Vaginal Infections), and fourteen women with BV who had Nugent scores of 9 and 10. These 97 women were selected to identify the major bacterial species by PCR, and then healthy and AV women (83 samples) were evaluated by qPCR (see section Amount of *Lactobacillus* spp. Among Healthy Women and Women With Vaginal Infections). However, this selection of samples could be considered a limitation of the present study.

The presence of *Gardnerella* species in a low number in the vaginal microbiota is not an indicator of BV (De Backer et al., 2007; de Vos et al., 2012; Mendling, 2016), being considered as part of the healthy vaginal microbiota. Meanwhile, several studies have shown that there are almost four different groups of *Gardnerella* species (A, B, C, and D), previously all considered as *Gardnerella vaginalis* (Vaneechoutte et al., 2019), which not all of them are related to the development of BV (Santiago et al., 2011; Hardy et al., 2017; Hill et al., 2019). In 2019, Vaneechoutte and colleagues amended several species of *G. vaginalis*, through Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), and described then as *Gardnerella leopoldii*, *Gardnerella piotii* and *Gardnerella swidsinskii*, Therefore, not all *Gardnerella* species detected in several studies constituted *Gardnerella vaginalis* and could explain virulence differences between *Gardnerella* species (Iavazzo et al., 2008; Leite et al., 2010; Muzny and Schwebke, 2013). Since these species could not be delineated using full-length 16S rRNA gene sequences, Hill and colleagues applied partial chaperonin 60 (cpn60) sequences to resolve these four group species (Hill et al., 2019). Both studies showed that *G. swidsinskii* and *G. leopoldii* constituted group A, *G. piotii* corresponded to group B, *G. vaginalis* belonged to group C, and finally, group D was the most diverse subgroup with several *Gardnerella* sp. (such as strains 101, 1500E, 6119V5, and 00703Dmash). However, this last group will require an analysis of additional isolates to establish a species differentiation (Hill et al., 2019; Vaneechoutte et al., 2019). Nonetheless, an abundance of *G. vaginalis* and *G. swidsinskii* was associated with vaginal symptoms of abnormal odor and discharge in their study set (Hill et al., 2019). This heterogeneity and diversity within the genus *Gardnerella* may distinguish clades and how these features may impact BV development (Castro et al., 2020).So, future studies should isolate all *Gardnerella* species of the vaginal samples and further analysis could allow the qPCR methodology to quantify different species of *Gardnerella*.

Amount of *Lactobacillus* spp. Among Healthy Women and Women With Vaginal Infections

As previously mentioned in results, statistically significant differences were found among the amount of *Lactobacillus* spp. between healthy and AV women ($P < 0.001$). Moreover, lactobacilli load among healthy women was established between 1E6 and 1E7 CFU/ml; meanwhile, the amount of *Lactobacillus* spp. in altered microbiota (AV) was defined between 1E3 and 1E5 CFU/ml. These results are comparable to previous studies with BV (Sha et al., 2005; De Backer et al., 2007; Ling et al., 2011). However, it is important to mention that the specificity of the lactobacilli primers (LactoF: 47.1%%; LactoR: 66.7%) was a limitation of the present study.

Amount of *Lactobacillus* spp. Among Pregnant and Non-pregnant Women

Furthermore, the results of the present study showed that both healthy and AV pregnant women have a higher concentration of *Lactobacillus* spp. when compared to non-pregnant women of the same categories. These results agree with Walther-António et al. (2014). These authors reported that lactobacilli augmentation during pregnancy and preterm birth help to prevent vaginal infection and counteract higher immune tolerance (Walther-António et al., 2014; Kim et al., 2017). However, there were no statistically significant differences between the amount of *Lactobacillus* spp. of pregnant and non-pregnant women per category. Although the present study is a preliminary analysis of lactobacilli load between pregnant and non-pregnant women, these results point to the possibility to use the same lactobacilli load range to evaluate AV and healthy vaginal microbiota (whether pregnant or non-pregnant) and thus to avoid future vaginal infection establishment in women by monitoring lactobacilli load through qPCR.

These results could corroborate with several studies, which postulated an increment of lactobacilli load in pregnant women (Aagaard et al., 2012; Walther-António et al., 2014). However, there are some major limitations of this study: (1) with 97 participants in PCR assays and 83 participants in qPCR assays, small numbers of particular cases were retained in each subgroup, (2) in PCR assays not all possible species of aerobic bacteria could be targeted in AV samples and, (3) in qPCR assays, normalized concentrations of lactobacilli were realized through low specificity primers for *Lactobacillus* spp. Therefore, future studies must optimize lactobacilli quantification, also quantify certain *Lactobacillus* species and other aerobic bacteria among pregnant and non-pregnant women. Previous studies showed that the presence of different *Lactobacillus* species is a major determinant to the stability of the vaginal microbial community in pregnancy (Verstraelen et al., 2009; Ling et al., 2010). Furthermore, Verstraelen and colleagues demonstrated *L. crispatus* ability to promote and stabilize the normal microbiota while *L. gasseri* and *L. iners* predisposed to some extent to the occurrence of abnormal microbiota (Verstraelen et al., 2009).

Future studies should be realized with a bigger and more diverse population set as well as quantification of specific *Lactobacillus* species (such as *L. crispatus*, *L. gasseri*, and *L. iners*) as postulated by others authors (Verstraelen et al., 2009; Walther-António et al., 2014; Vaneechoutte, 2017a).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Universidad San Francisco de Quito (USFQ) and the Ministry of Health of Ecuador (Protocol code: 2016-140M by MSP-SDM-10-2013-2019-O review board). The volunteers were recruited to our study set, after having read and signed the informed consent or, in the case of the underage volunteers, from their parents or legal representatives.

AUTHOR CONTRIBUTIONS

AM, GV, and DP-H were responsible for modeling and experimental design. Samples collection oversaw doctors of Gynecology and Obstetrics Service of Carlos Andrade Marin Hospital (HCAM), Gynecological-Obstetric Hospital Isidro Ayora (HGOIA), and Center for Teaching Health Cipriana Dueñas. The DNA extraction was performed at Hospital Carlos Andrade Marín by CC-B. Molecular characterization was conducted by DP-H in the Microbiology Institute at USFQ. Biostatistics analysis was developed by VB and DP-H at USFQ. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00303/full#supplementary-material

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Lateral Gene Transfer Shapes Diversity of *Gardnerella* spp.

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Gardnerella spp. are pathognomonic for bacterial vaginosis, which increases the risk of preterm birth and the transmission of sexually transmitted infections. *Gardnerella* spp. are genetically diverse, comprising what have recently been defined as distinct species with differing functional capacities. Disease associations with *Gardnerella* spp. are not straightforward: patients with BV are usually infected with multiple species, and *Gardnerella* spp. are also found in the vaginal microbiome of healthy women. Genome comparisons of *Gardnerella* spp. show evidence of lateral gene transfer (LGT), but patterns of LGT have not been characterized in detail. Here we sought to define the role of LGT in shaping the genetic structure of *Gardnerella* spp. We analyzed whole genome sequencing data for 106 *Gardnerella* strains and used these data for pan genome analysis and to characterize LGT in the core and accessory genomes, over recent and remote timescales. In our diverse sample of *Gardnerella* strains, we found that both the core and accessory genomes are clearly differentiated in accordance with newly defined species designations. We identified putative competence and pilus assembly genes across most species; we also found them to be differentiated between species. Competence machinery has diverged in parallel with the core genome, with selection against deleterious mutations as a predominant influence on their evolution. By contrast, the virulence factor vaginolysin, which encodes a toxin, appears to be readily exchanged among species. We identified five distinct prophage clusters in *Gardnerella* genomes, two of which appear to be exchanged between *Gardnerella* species. Differences among species are apparent in their patterns of LGT, including their exchange with diverse gene pools. Despite frequent LGT and co-localization in the same niche, our results show that *Gardnerella* spp. are clearly genetically differentiated and yet capable of exchanging specific genetic material. This likely reflects complex interactions within bacterial communities associated with the vaginal microbiome. Our results provide insight into how such interactions evolve and are maintained, allowing these multi-species communities to colonize and invade human tissues and adapt to antibiotics and other stressors.

Keywords: *Gardnerella* spp., recombination, evolution, bacterial vaginosis, lateral gene transfer

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INTRODUCTION

Gardnerella spp. are Gram-variable, facultative anaerobes found in the vaginal microbiome of healthy women (Schellenberg et al., 2017). However, *Gardnerella* spp. are also associated with vaginal dysbiosis and bacterial vaginosis (BV), which is characterized by symptoms such as abnormal vaginal discharge, malodor, and pain (Hilbert et al., 2017). Additionally, BV can increase the risk of preterm birth and transmission of sexually transmitted diseases, including HIV (Hilbert et al., 2017).

Gardnerella spp. were previously considered a single species (i.e., *Gardnerella vaginalis*), but more recent research proposes that what was previously designated *G. vaginalis* in fact comprises several distinct species (Vaneechoutte et al., 2019). Biochemical tests (Piot et al., 1984) and phylogenetic methods (Ahmed et al., 2012; Cornejo et al., 2018) have delineated at least four distinct clades/species. While *Gardnerella* spp. are known to be associated with BV, clear consensus is lacking for the clades or combinations of clades that are most consequential for disease (Janulaitiene et al., 2017; Hill et al., 2019). Women with BV are often infected with strains from two or more clades (Hilbert et al., 2017). Recently, a study used average nucleotide identity and digital DNA-DNA hybridization to update the description of *G. vaginalis*, describe 3 new species, and 9 different genomospecies within "*Gardnerella* spp." (Vaneechoutte et al., 2019). Researchers have tried to identify which groups of isolates are more pathogenic than others; however, this complex relationship remains to be fully defined (Harwich et al., 2010; Balashov et al., 2014; Janulaitiene et al., 2017; Hill et al., 2019). Our analyses build on previous work that used comparative genomics approaches to predict potential functional differentiation of this multi-species community (Cornejo et al., 2018). Here we investigate how these functional differences have evolved and are maintained in *Gardnerella* spp. populations.

We previously found evidence suggesting lateral gene transfer (LGT) is frequent among *Gardnerella* spp. (Devault et al., 2017). We and others found that LGT appeared to be structured by species, and that despite abundant intergenomic recombination, the species have maintained their genetic distinctiveness (Ahmed et al., 2012). Here we sought to characterize LGT in detail in a larger sample and to investigate whether patterns of recombination differ between species. The mechanism(s) of recombination in *Gardnerella* spp. are not known, but previous investigators found four predicted competence genes in a sample of three isolates (Yeoman et al., 2010). Additionally, prophage genes have been identified in *Gardnerella* spp. (Malki et al., 2016). As part of our investigation of LGT in *Gardnerella* spp. we further sought to identify and characterize genes with a potential role in shaping LGT, including competence genes, phage, and restriction modification systems, in a larger and more diverse sample of isolates. Elucidating how DNA is exchanged in bacterial populations helps to illuminate how clinically relevant traits, such as virulence and antibiotic resistance, evolve in these complex communities.

TABLE 1 | Number of genes identified in the pangenomes of 106 *Gardnerella* isolates.

Program		Frequency of isolates	Number of genes	Amino acid % identity
PIRATE	Core genes	99–100	608	50–100
	Soft core	95–99	195	50–100
	Total genes	0–100	4,653	50–100
Roary	Core genes	99–100	343	>75
	Soft core	95–99	275	>75
	Total genes	0–100	6,055	>75

Amino acid percent identity used with Roary was inferred from the output of PIRATE, which uses a range of amino acid percent identity thresholds to calculate the pangenome.

MATERIALS AND METHODS

Data Set

We obtained whole genome sequencing reads or *de novo* assemblies (when reads were unavailable) for 97 isolates of *Gardnerella* spp. from NCBI. We additionally sequenced nine clinical *Gardnerella* spp. isolates. The accessions for the nine newly sequenced isolates are listed in **Table S1**. Accession numbers and available clinical data for all 106 isolates are listed in **Table S2**.

Bacterial Growth and Isolation

Strains were streaked on human blood bilayer with Tween (HBT) agar (Bd Diagnostic Systems) and incubated at 37°C in 5% CO_2 for 3–4 days. Growth was subcultured in 6 mls of brain heart infusion (BHI) broth (Teknova) + 10% fetal bovine serum (FBS) + 5% Fildes enrichment + 1 μg/mL amphotericin for 48 h at 37°C in 5% CO_2.

DNA Extraction

We used the gBac Mini gDNA Bacteria Kit (IBI, Lot No JM14117) for DNA extraction of 9 clinical isolates with the following modifications: the entire 6 ml culture was centrifuged for 5 min at 5,000 g to pellet cells and incubated with lysozyme for 1 h.

Library Preparation and Sequencing

For the 9 new clinical isolates, libraries were prepared using a modified Nextera protocol as described by Baym et al. (2015) with a reconditioning PCR to minimize chimeras with fresh primers and polymerase for an additional 5 cycles and a bead based size selection (650 b). Libraries were sequenced on an Illumina HiSeq 2500 (paired-end, 150 bp).

De novo Assembly and Annotation

We used the iMetAMOS (Koren et al., 2014) pipeline to compare *de novo* assemblies from SPAdes (Bankevich et al., 2012), MaSurCA (Zimin et al., 2013), and Velvet (Zerbino and Birney, 2008). KmerGenie (Chikhi and Medvedev, 2014) was used to select kmer sizes for assembly. Quality of reads and assemblies were assessed using FastQC (Andrews, 2010), QUAST (Gurevich et al., 2013), REAPR (Hunt et al., 2013), LAP (Ghodsi et al., 2013), ALE (Clark et al., 2013), FreeBayes (Garrison and Marth, 2012),

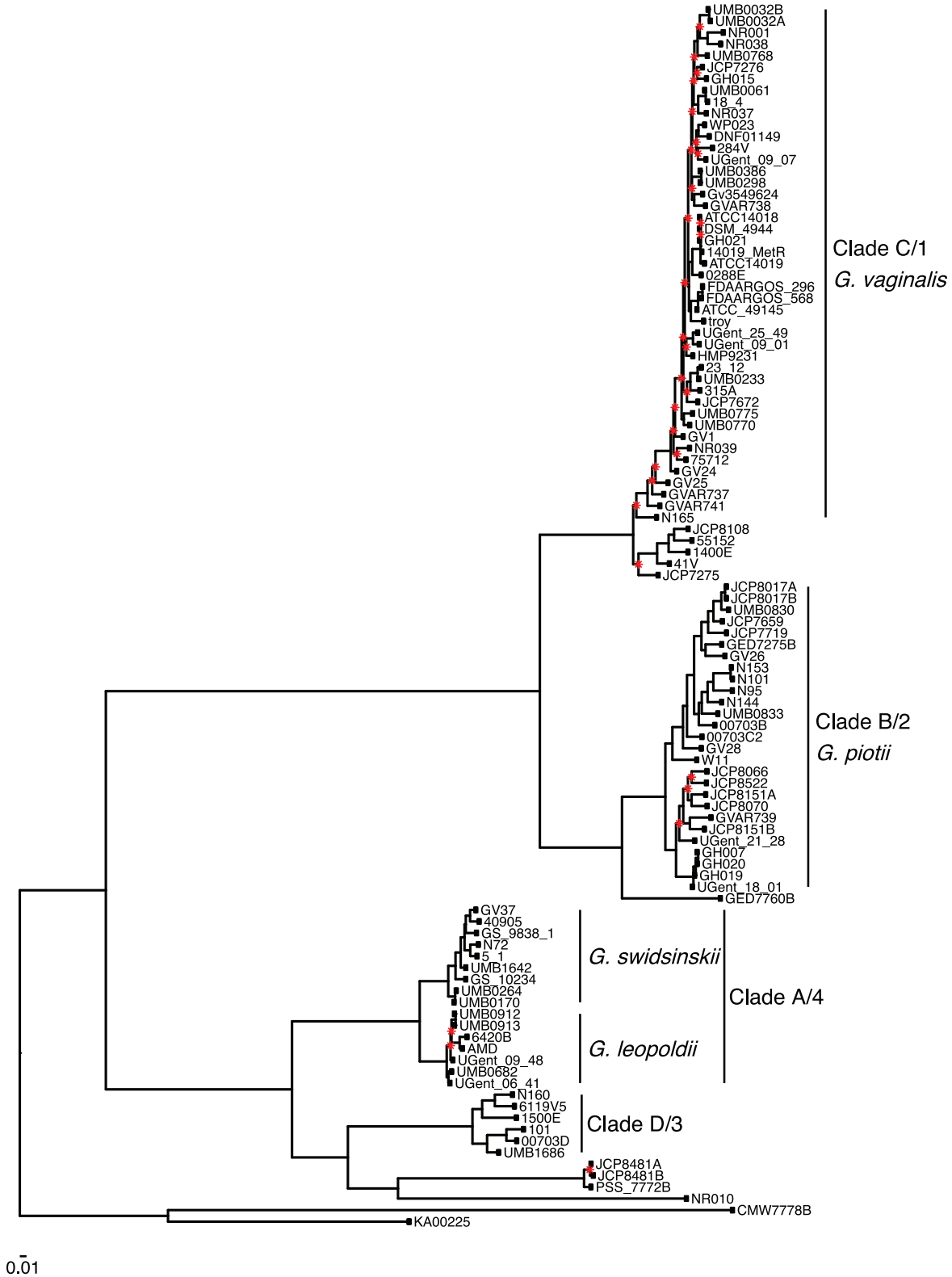

FIGURE 1 | *Gardnerella* core genome maximum likelihood phylogeny supports distinct species/clade structure. We inferred a maximum likelihood phylogeny from a core genome alignment of 106 *Gardnerella* isolates. Species/clade labels reflect classification schemes from Ahmed et al. (2012) and Hill et al. (2019). Newly named species indicated (Vaneechoutte et al., 2019). The phylogeny is midpoint rooted, and nodes with bootstrap values lower than 70 shown in red. Branch lengths are scaled by the number of substitutions per site.

and CGAL (Rahman and Pachter, 2013), and contamination was detected with Kraken (Wood and Salzberg, 2014). *De novo* assemblies were annotated with Prokka (Seemann, 2014).

Core Genome Identification and Alignment

We used PIRATE (Bayliss et al., 2019) to identify the core and pan genomes for newly sequenced and publicly available *Gardnerella* spp. genomes (**Table 1**). Using PIRATE, we clustered orthologous gene families using an amino acid identity threshold ranging from 50 to 100% to obtain a clearer understanding of the breadth of diversity across the pangenomes of *Gardnerella* spp. We used the pangenome information from PIRATE and concatenated core genome alignments of single copy genes at 100% frequency for the entire dataset as well as individual species. In addition, we used Roary (Page et al., 2015) to identify core and pan genomes at an amino acid percent identity threshold of >75%. This allowed us to compare gene homologs at a consistent threshold across all genes.

Recombination Detection

To identify recombination events between the major clades/species of *Gardnerella* spp., we used FastGEAR (Mostowy et al., 2017) on a concatenated core genome aligned with MAFFT (Katoh and Standley, 2014). Briefly, FastGEAR uses a Hidden Markov Model approach to cluster isolates into lineages, detect ancestral and recent recombination, and measure the statistical strength of the recombination events. We used Gubbins (Croucher et al., 2015) to identify recombination events within the core genome alignments of clade 1 (*Gardnerella vaginalis*) and clade 2 (*Gardnerella piotii*). Briefly, Gubbins identifies recombination by using spatial scanning statistics to identify loci with elevated single nucleotide polymorphism (SNP) densities. To account for differences in sample size in *G. vaginalis* and *G. piotii*, we subsampled *G. vaginalis* to the size of *G. piotii* and used Gubbins to identify recombination in the subsampled dataset. We calculated the proportion of sites affected by recombination per isolate within *G. vaginalis* and *G. piotii* and compared the means using a Mann-Whitney-Wilcoxon test (Mann and Whitney, 1947).

Codon Usage

We calculated codon adaptation index (CAI) for the core genes of *G. vaginalis* and *G. piotii* using the EMBOSS CUSP program (Rice, 2000). We compared the means of CAI values using a Student's *t*-test.

Phylogenetic Network Inference

Using the concatenated core genome alignment constructed from PIRATE output, we inferred a phylogenetic network using SplitsTree 4 (Huson and Bryant, 2006) of all *Gardnerella* isolates, *G. vaginalis*, and *G. piotii* isolates.

Maximum Likelihood Phylogenetic Inference

We performed maximum likelihood phylogenetic inference on the concatenated alignment of core genes using RAxML v 8.2.3 (Stamatakis, 2014) with the GTR model of nucleotide substitution and gamma distribution of rate variation. Twenty trees were estimated for the alignment, and the tree with the maximum likelihood was chosen. We performed bootstrapping using the autoMR convergence criteria.

Competence Machinery and Vaginolysin Identification

We systematically identified competence genes, tad pilus assembly homologs, and vaginolysin using PIRATE and Roary gene homolog output, as well as manually using the Prokka (Seemann, 2014) annotations of the *Gardnerella* isolates. We

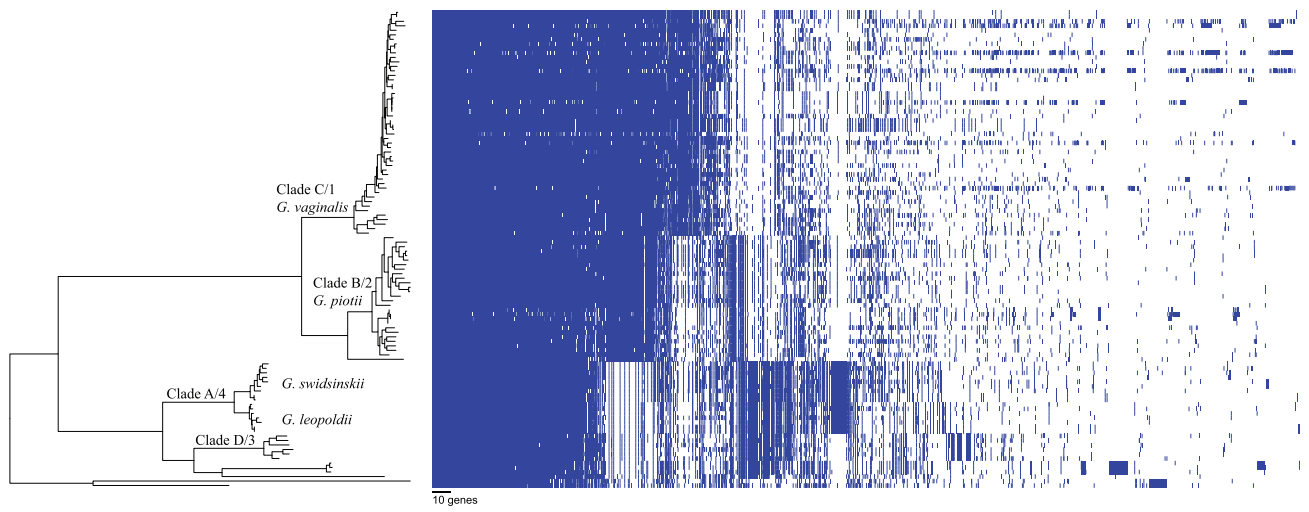

FIGURE 2 | Accessory genome of *Gardnerella* is structured by species/clade. Gene homologs identified using Roary with 75% amino acid threshold are plotted (excluding singleton genes). Order of species in the phylogenetic tree (left) corresponds to species order in **Figure 1**. Accessory content differs among *Gardnerella* spp. species, which is consistent with barriers to LGT between species.

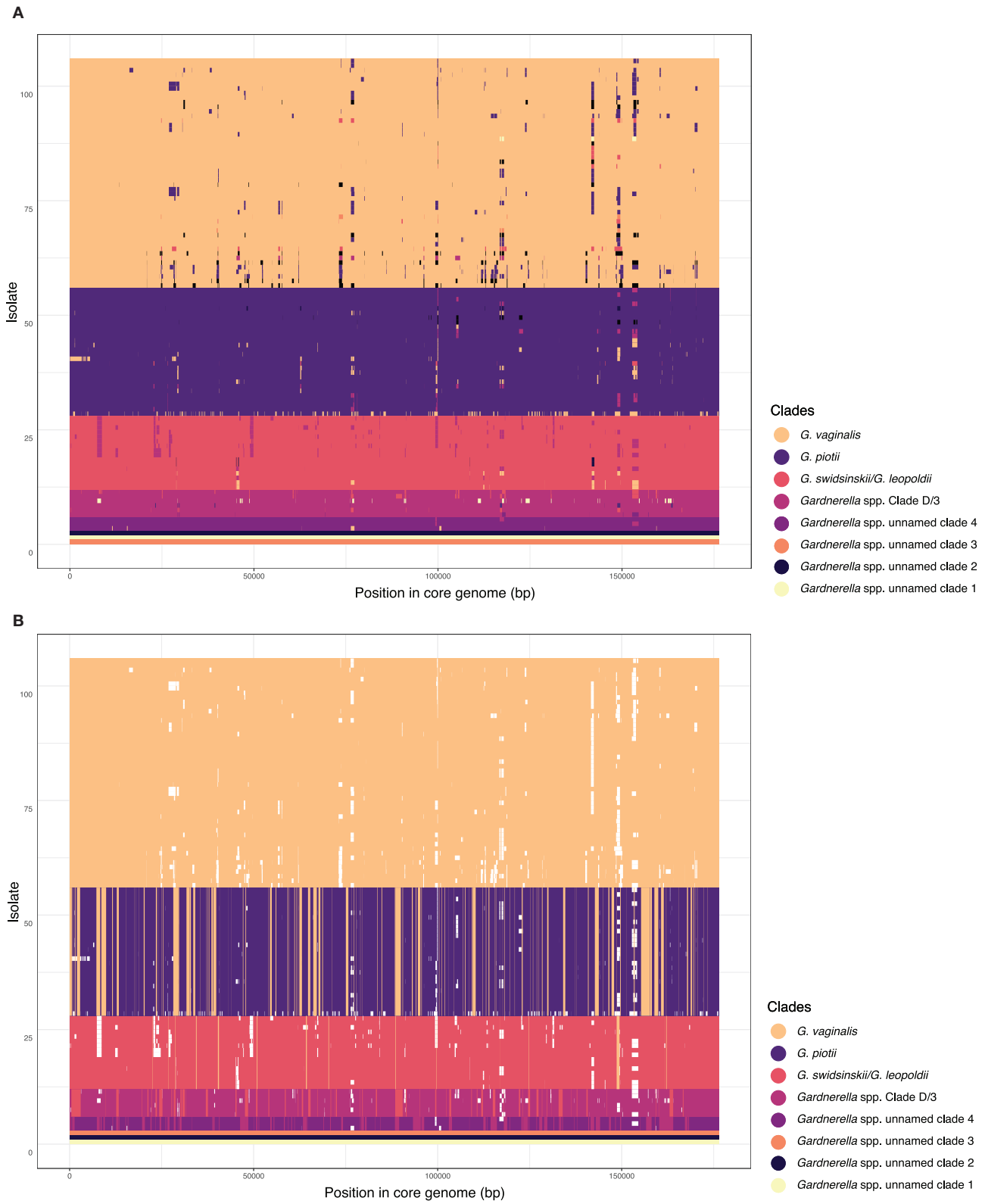

FIGURE 3 | Recombination occurs most often between closely related species/clades. FastGEAR inference of recombination from a core genome alignment. Briefly, FastGEAR uses a Hidden Markov Model approach to cluster isolates into lineages, detect ancestral and recent recombination, and measure the statistical strength of

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FIGURE 3 | the recombination events. FastGEAR identified 8 clades in the dataset, consistent with **Figure 1** and published delineations (Ahmed et al., 2012; Schellenberg et al., 2017; Vaneechoutte et al., 2019). Isolates are ordered according to core genome phylogeny and colored according to each of 8 clades identified by FastGEAR. Each horizontal line refers to an isolate's core genome with colors representing the inferred origin of that region. The clades/species colors are labeled in the legend. **(A)** Recent recombinant tracts identified with FastGEAR. Overall there are few recombination events between clades/species, which appear to be more common between *G. vaginalis* and *G. piotii*, than other combinations. **(B)** Ancestral recombination shows a similar pattern of species structured LGT. White fragments correspond to recent recombination events (shown in **A**) and are masked when inferring ancestral recombination. Recent recombination inferred with a Bayesian factor (BF) > 1 and ancestral recombination with BF > 10 shown.

included homologs of *cinA, recA, comEA, comEC*, *cpaB, cpaF, tadB, tadC, tadE,* and *tadG* in our selection analyses (Tomich et al., 2007; Yeoman et al., 2010). We aligned each gene with PRANK and constructed individual maximum likelihood gene trees using FastTree (Price et al., 2010). We used the aBSREL method implemented in Hyphy (Smith et al., 2015) to test for selection along branches in the phylogenies of competence genes. To investigate the effects of LGT and selection on the diversity of the competence machinery, vaginolysin, and the core genome, we compared these genomic regions in the two species that are well-sampled: *G. vaginalis* and *G. piotii*. We calculated dN/dS using the yn00 implementation (Yang and Nielsen, 2000) in PAML (Yang, 1997).

Prophage Identification

We used ProphET (Reis-Cunha et al., 2019) to detect prophage in our collection of genomes. Briefly, ProphET performs a similarity search of annotated proteins from bacterial genomes against a database of known phage proteins to identify prophage within bacterial genomes. ProphET discards regions with a low density of phage-associated genes. Next, we blasted the known prophage regions against a custom nucleotide database of the *de novo* assembled contigs from all 106 *Gardnerella* spp. isolates to identify additional phage. To assess whether we missed prophage that were split between contigs, we blasted the known prophage against the custom database and filtered the results to identify hits found within 50 bp of a contig end and plotted the sequence length distribution of these hits. After identifying prophage regions, we calculated pairwise mash (Ondov et al., 2016) distances, which is based on shared k-mer (sequences of length k) content between prophage nucleotide sequences. Using these pairwise distances, we performed multidimensional scaling (MDS) to identify clusters of similar prophage. We also created a presence/absence matrix for each prophage cluster in our data set. To compare genetic content of each prophage cluster, we annotated the nucleotide sequences of all prophage using Prokka (Seemann, 2014), and cross referenced these results with the Clusters of Orthologous Groups (COG) database (Tatusov et al., 2000).

CRISPR/*cas* Identification

We identified CRISPR/*cas* genes in genome annotations produced by Prokka. Additionally, we used the PIRATE output to identify homologous CRISPR/*cas* genes. To look for an association between the presence of CRISPR/*cas* and particular prophage cluster we used Fisher's Exact Test (Fisher, 1934) with Bonferroni correction (Bonferroni, 1935). We also performed a Mann-Whitney-Wilcoxon test (Mann and Whitney, 1947) to test for an association between the presence of CRISPR/*cas* and genome assembly length.

Restriction Modification Identification

We used the Prokka annotations and PIRATE output to identify homologous clusters of genes associated with restriction modification (RM) systems. We created a presence/absence matrix for each RM associated gene in our data set.

Pangenome Diversity Analyses

We calculated pangenome accumulation and rarefaction curves of *G. vaginalis* and *G. piotii* isolates. Additionally, we calculated the gene frequency of the accessory genomes of both species. Using Egglib (De Mita and Siol, 2012), we calculated average π per accessory gene within and between *G. vaginalis* and *G. piotii* isolates using a subset of genes found at intermediate frequencies (1–99%) in both species. We performed a Kruskal-Wallis test in R to determine differences in average gene π values by group (Kruskal and Wallis, 1952). We then performed pairwise Mann-Whitney-Wilcoxon tests in R with Bonferroni correction to identify which distribution pairs were significantly different (Bonferroni, 1935; Mann and Whitney, 1947).

RESULTS

Pangenome and Phylogenetic Analysis of *Gardnerella* spp.

Pan genome analysis of 106 clinical isolates of *Gardnerella* spp. identified 4,653 genes in the pan-genome. Six hundred and eight of these formed the "strict core" genome (i.e., found in 100% of isolates) whereas an additional 195 genes were found in 95% of the sample (**Table 1**).

For initial categorization of genes in this diverse sample, we used an amino acid identity threshold of 50% or greater, implemented in PIRATE (Bayliss et al., 2019), to identify 4,653 gene families. We found an average of 75% amino acid percent identity among genes found in at least 95% of the isolates. Based on this finding, we performed additional pangenome analysis using Roary with an amino acid percent identity threshold of 75% to identify core and accessory genes (**Table 1**). At this more restrictive threshold, we found 6,055 total gene families, 343 of which were found in at least 99% of isolates.

We found that *G. vaginalis, G. piotii, G. swidsinskii, G. leopoldii,* clade 3/D, and four unnamed additional clades were clearly differentiated in their core genomes, as shown by long branches separating them on a core genome phylogeny (**Figure 1**; **Figure S1**). The same pattern held in the accessory genome, where *Gardnerella* spp. could be clearly distinguished on the basis of accessory gene content (**Figure 2**).

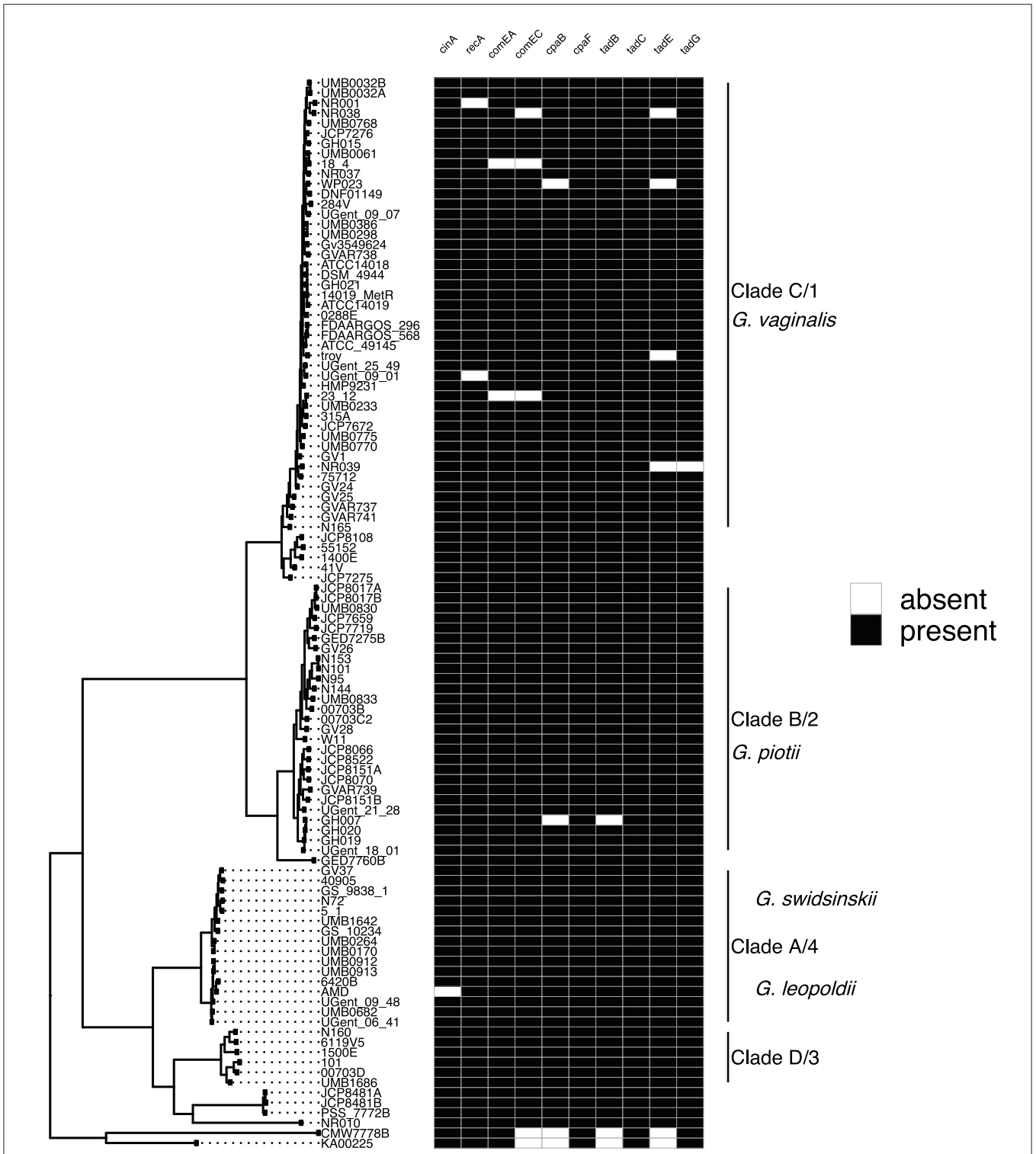

FIGURE 4 | Competence gene homologs are ubiquitous across *Gardnerella*. Presence absence matrix of gene homologs likely to be related to competence **(right)** and maximum likelihood phylogenetic tree **(left)**. Most of the genes are found among all clades/species, indicating competence related machinery is conserved among *Gardnerella* spp. Gene homologs were identified using both Roary and PIRATE. Additionally, we looked in the annotations to identify homologs missed using Roary or PIRATE alone.

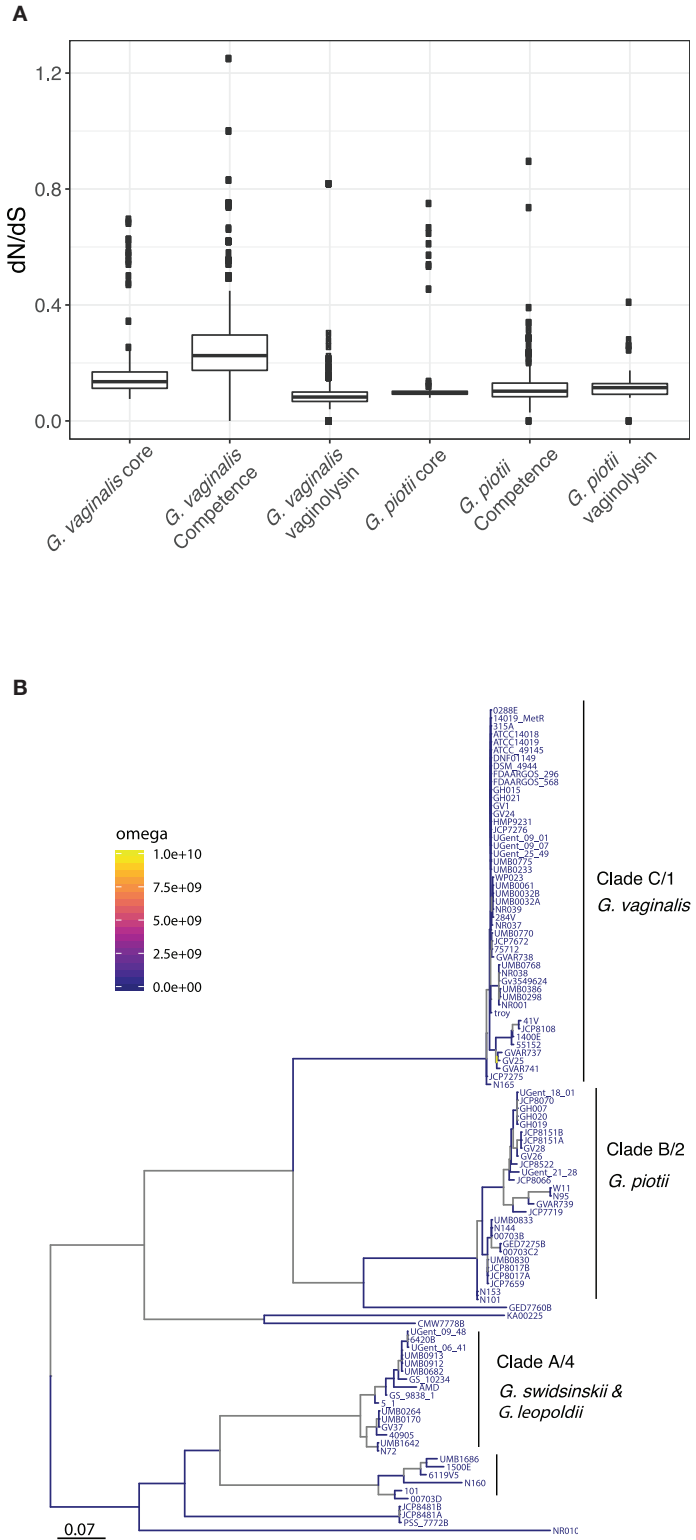

FIGURE 5 | Purifying selection is the primary force shaping diversity in *Gardnerella* core, competence, and vaginolysin genes. **(A)** Diversity of core genomes, competence genes, and vaginolysin within *G. vaginalis* and *G. piotii*. Distributions shown are of dN/dS across core genomes, concatenations of competence genes,

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FIGURE 5 | and vaginolyisn. The box spans the interquartile range, the median is represented by the middle line, and the whiskers extend to ±1.5 times the interquartile range. Data beyond the end of the whiskers are outlying points and plotted individually. All genes appear to be under purifying selection (dN/dS <1). **(B)** Proportion of sites under positive selection along branches of the *comEA* maximum likelihood phylogeny. Using the aBSREL test in HyPhy, we identified branches with significant evidence ($p < 0.05$) of selection. Branch specific omega values show little evidence of positive selection, suggesting purifying selection.

Lateral Gene Transfer in the *Gardnerella* Core Genome

Our analyses of lateral gene transfer indicated that this species structure is maintained by barriers to recombination. We used FastGEAR to identify sub-groups within the sample and infer patterns of core genome recombination in the recent and remote past. FastGEAR analyses defined eight clades, consistent with previously published genetic classifications. Few recombination events were inferred between these eight clades (**Figure 3A**). Interestingly, between-clade recombination appeared to have occurred more commonly in the remote past (**Figure 3B**). More recombination events were inferred between closely related clades/species (i.e., between *G. vaginalis* and *G. piotii*) than distantly related clades/species (e.g., *G. vaginalis* and clade D/3).

Mechanisms of LGT

To identify potential mechanisms of LGT in *Gardnerella,* we first systematically searched for and identified a collection of competence related genes. A previous study identified four competence genes (*cinA, recA, comEA, comEC*) in a sample of three *Gardnerella* isolates (Yeoman et al., 2010). We expanded this finding to our collection of 106 *Gardnerella* isolates and identified 6 additional genes (*cpaB, cpaF, tadB, tadC, tadE, tadG*) involved in tad pilus assembly (Tomich et al., 2007). We found competence homologs to be encoded by most isolates (**Figure 4**). These genes were highly differentiated among species (e.g., **Figure S2**), mirroring patterns of diversity in the core genome. This suggests that competence genes are not commonly exchanged across species.

It is possible that patterns of diversity in competence genes reflect functional differentiation that could contribute to reproductive isolation among *Gardnerella* spp. To test this hypothesis, we performed selection analyses: in the event that divergence was driven by functional differentiation we expect to find evidence of positive selection reflected in excess non-synonymous (coding) variation separating species-specific versions of these genes. Pairwise values of dN/dS are consistent with purifying selection (i.e., selection against deleterious mutations) as the predominant influence on both core genes and competence homologs (**Figure 5A**). Estimates of branch specific omega values also indicate that the competence genes are generally evolving under purifying selection (**Figure 5B**; **Figure S3**). Thus, patterns of diversity in competence machinery do not suggest that their differentiation has been driven by selection for advantageous mutations/ functional differentiation.

Patterns of Diversity at a Virulence Locus

Vaginolysin is a pore-forming toxin and acute virulence factor (Gelber et al., 2008) previously identified as a core gene with evidence of between-clade recombination in a sample of 17 isolates (Ahmed et al., 2012). Here we sought to further investigate evolution of this locus: genes that mediate host pathogen interactions are often characterized by high levels of diversity reflecting selection for advantageous mutations (Andrews and Gojobori, 2004; Kennemann et al., 2011; Osório et al., 2013) and we were interested in dynamics of this well-characterized virulence factor in *Gardnerella* spp. By contrast with Ahmed et al., we did not find vaginolysin to be part of the core genome in this larger sample of isolates. Using a >80% amino acid similarity threshold in the PIRATE gene homolog output, we found vaginolysin to be present in 83% of *Gardnerella* isolates. A presence/absence matrix for this gene implies that vaginolysin has been gained or lost a limited number of times during evolution of our sample (**Figure S4**). The vaginolysin gene tree is not consistent with the core phylogeny, indicating that while it is infrequently lost or gained, it appears to be readily exchanged between *Gardnerella* spp. (**Figures S4, S5**). Interestingly, we found that vaginolysin appears to be evolving under strong constraint, similar to the core genome and competence machinery in *G. vaginalis* (**Figure 5A**). Taken together, these results suggest that the vaginolysin toxin performs an important function in *Gardnerella* spp., but bacteria occasionally adapt to loss of this function.

Prophage

To further explore mechanisms of LGT, we used ProphET (Reis-Cunha et al., 2019) to identify 130 prophage within our set of 106 genomes. We found *Gardnerella* spp. to encode between zero and four prophage per genome (median 1). Using mash distances (Ondov et al., 2016), we identified 5 clusters of prophage, with at least one prophage found in 70% of *Gardnerella* isolates. Prophage clusters 1 and 2 are found across *Gardnerella* spp. (**Figure 6**), while prophage clusters 3 and 4 are restricted to *G. vaginalis* and *G. piotii,* with the exception of one cluster 4 prophage found in a *G. swidsinskii* isolate. Cluster 5 is the smallest group, found in 2 isolates from an unnamed Gardnerella spp. clade (**Figure 6**). Phages in clusters 2 and 3 form well-differentiated sub-clusters that correspond with host species designations (**Figures 7B,C**), whereas cluster 1 and 4 phages from different hosts co-mingle (**Figures 7A,D**). This suggests there are barriers to between-species transfer of phage clusters 2 and 3 whereas clusters 1 and 4 are readily transferred among diverse bacterial hosts. We found the genetic content of prophage to vary between clusters (**Figure S6**); however, the majority of the genes were uncharacterized hypothetical genes (82%). To assess if we missed prophage split over contigs, we blasted all prophage against a custom database of all *de novo* assembled contigs and filtered the results to find hits within 50 bp of the end of a contig. We plotted the sequence length distribution of these blast results

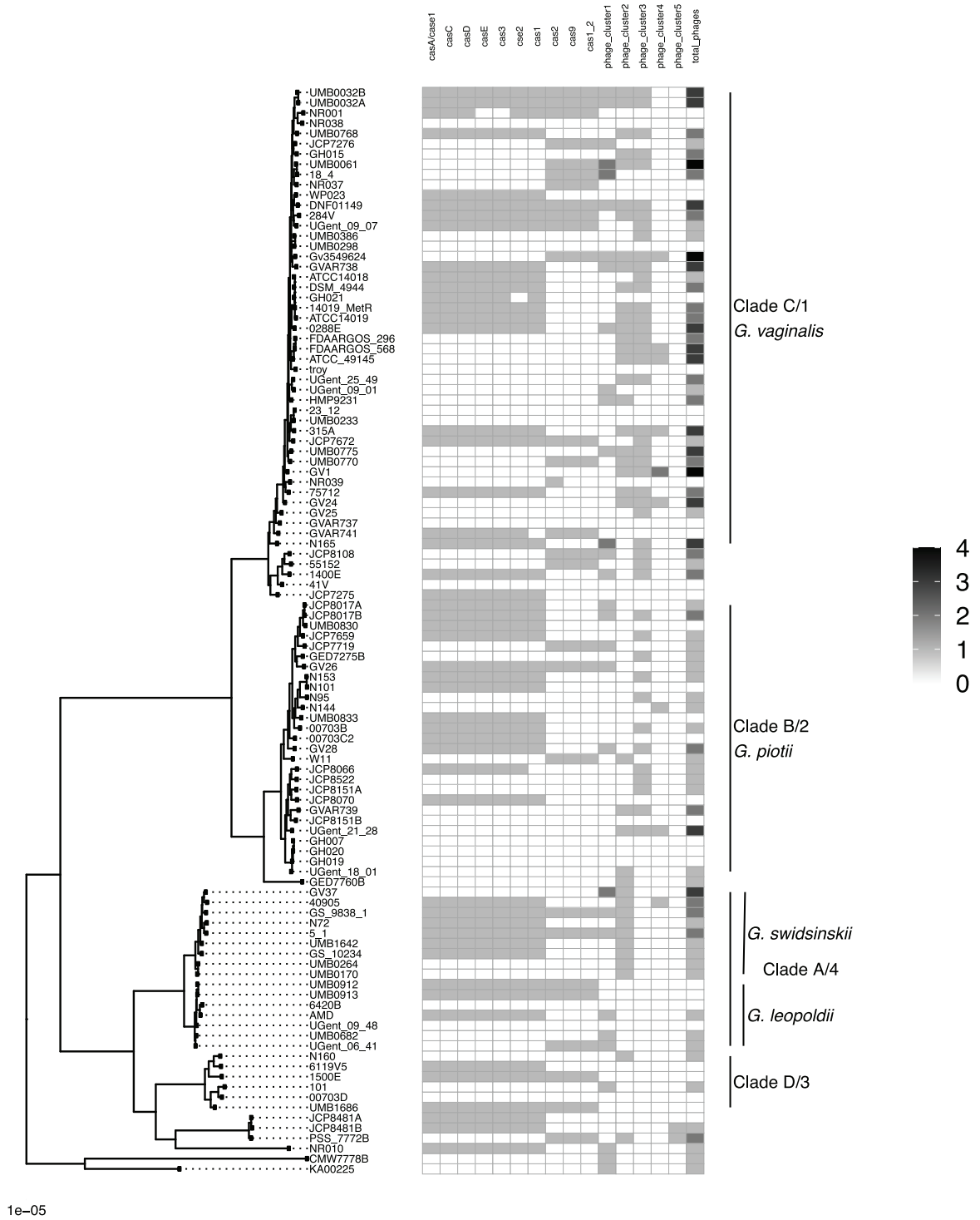

FIGURE 6 | CRISPR/*cas* and prophage are not ubiquitous across *Gardnerella* clades/species. Presence absence matrix of CRISPR/*cas* genes and phage clusters. CRISPR/*cas* genes were identified using PIRATE output and Prokka genome annotations. We identified prophage regions using ProphET and calculated pairwise mash distances of the nucleotide sequences to define prophage clusters. Prophage clusters 1 and 2 are found across *Gardnerella* spp., while prophage clusters 3 and 4 are restricted to *G. vaginalis* and *G. piotii,* with the exception of one cluster 4 prophage found in *G. swidsinskii*. We identified prophage clusters in 70% of *Gardnerella* isolates.

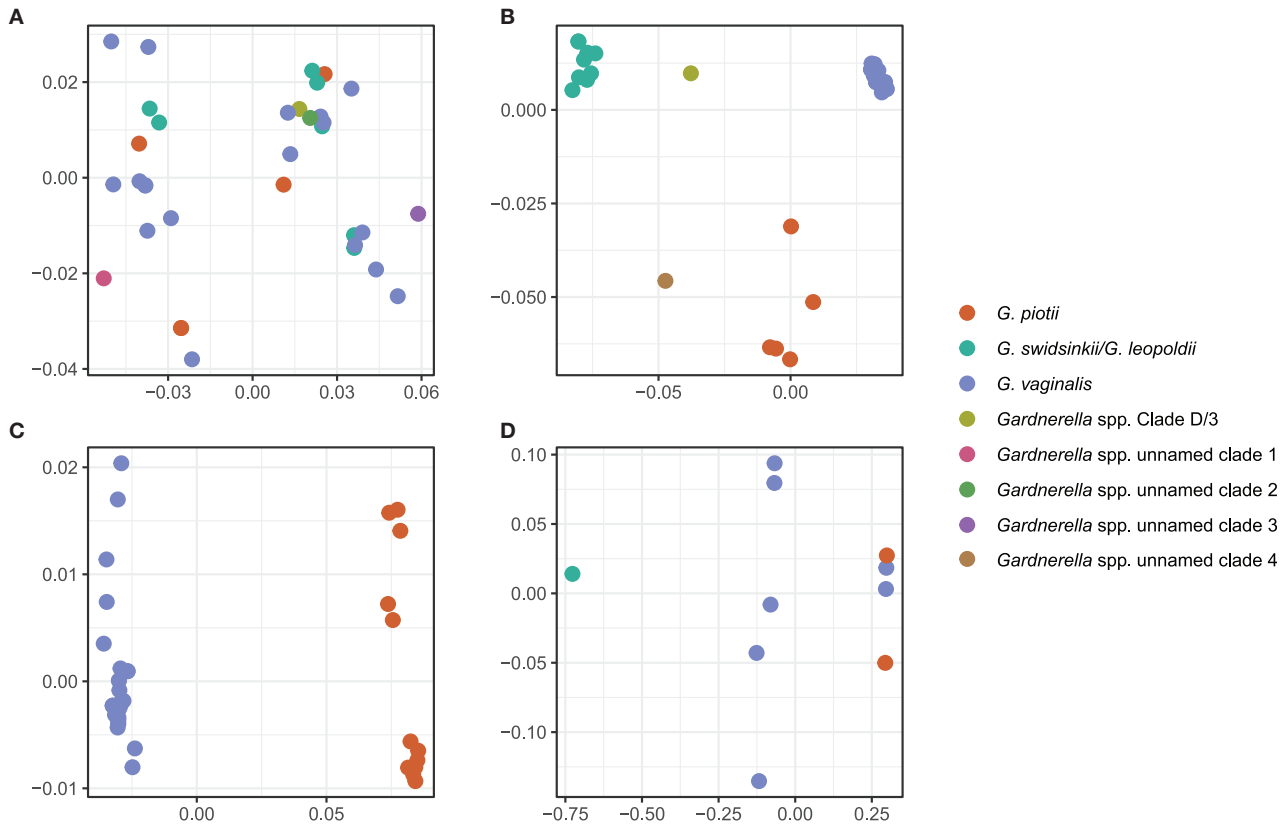

FIGURE 7 | Prophage clusters have distinct clade/species restrictions and patterns of transfer between clades/species. We used multi-dimensional scaling (MDS) of pairwise mash distances of prophage nucleotide sequences identified using ProphET to visualize prophage clustering across clades/species. Prophage clusters 2 and 3 cluster based on the core genome species designations **(B,C)**, and prophage clusters 1 and 4 do not cluster according to core genome species designations **(A,D)**, suggesting that the prophage have been transferred across clades. We did not include an MDS plot of cluster 5, as it is found in only 2 isolates.

and found the majority were very small in length, which suggests they are unlikely to be identified prophage (**Figure S7**).

CRISPR/*cas*

CRISPR/*cas* protect bacterial genomes from foreign DNA, including phage, and thus could potentially play a role in maintaining genetic barriers among *Gardnerella* spp. Presence/absence matrix of CRISPR/*cas* loci in our sample is shown in **Figure 6**. Patterns of carriage were variable, with some strains carrying up to 10 distinct loci and others without any loci identified. Certain loci tended to co-occur. Loss of CRISPR/*cas* can lead to proliferation of mobile genetic elements (Hullahalli et al., 2018), in which case we might expect genomes lacking CRISPR/*cas* to accumulate mobile genetic elements and increase in size. To test this hypothesis, we compared the presence of CRISPR/*cas* to the total length of *de novo* assembled contigs. We found that CRISPR/*cas* is not associated with increased genome length (Mann-Whitney-Wilcoxon test, $W = 1{,}058$, $p = 0.08$) (**Figure S8**).

Restriction Modification

Restriction modification (RM) systems can also protect bacterial genomes by cleaving foreign DNA (Tock and Dryden, 2005), and thus may shape barriers to lateral gene transfer in *Gardnerella* spp. To identify genes involved in restriction modification (RM) systems, we used PIRATE homologous gene family output and Prokka genome annotations. We found a wide diversity of RM genes found at varying frequencies across the *Gardnerella* spp. phylogeny, and *Gardnerella* spp. are not defined by the presence/absence of particular RM gene families (**Figure S9**).

Codon Usage in *G. vaginalis* and *G. piotii*

Differences in codon usage (Tuller et al., 2011) are another potential mechanism driving differentiation and genetic isolation of *Gardnerella* species. To investigate this hypothesis, we focused on *G. vaginalis* and *G. piotii*, sister species in the *Gardnerella* phylogeny that are common and well-sampled (50 *G. vaginalis*, 28 *G. piotii*). We found codon usage to be similar in *G. vaginalis* and *G. piotii* isolates and thus it does not appear to be an explanation for the reproductive isolation of these species (*t*-test, $p > 0.99$) (**Figure S10**).

Lateral Gene Transfer in Core Genomes of *G. vaginalis* and *G. piotii*

To further characterize patterns of recombination in *G. vaginalis* and *G. piotii*, we identified recombinant tracts in core genomes

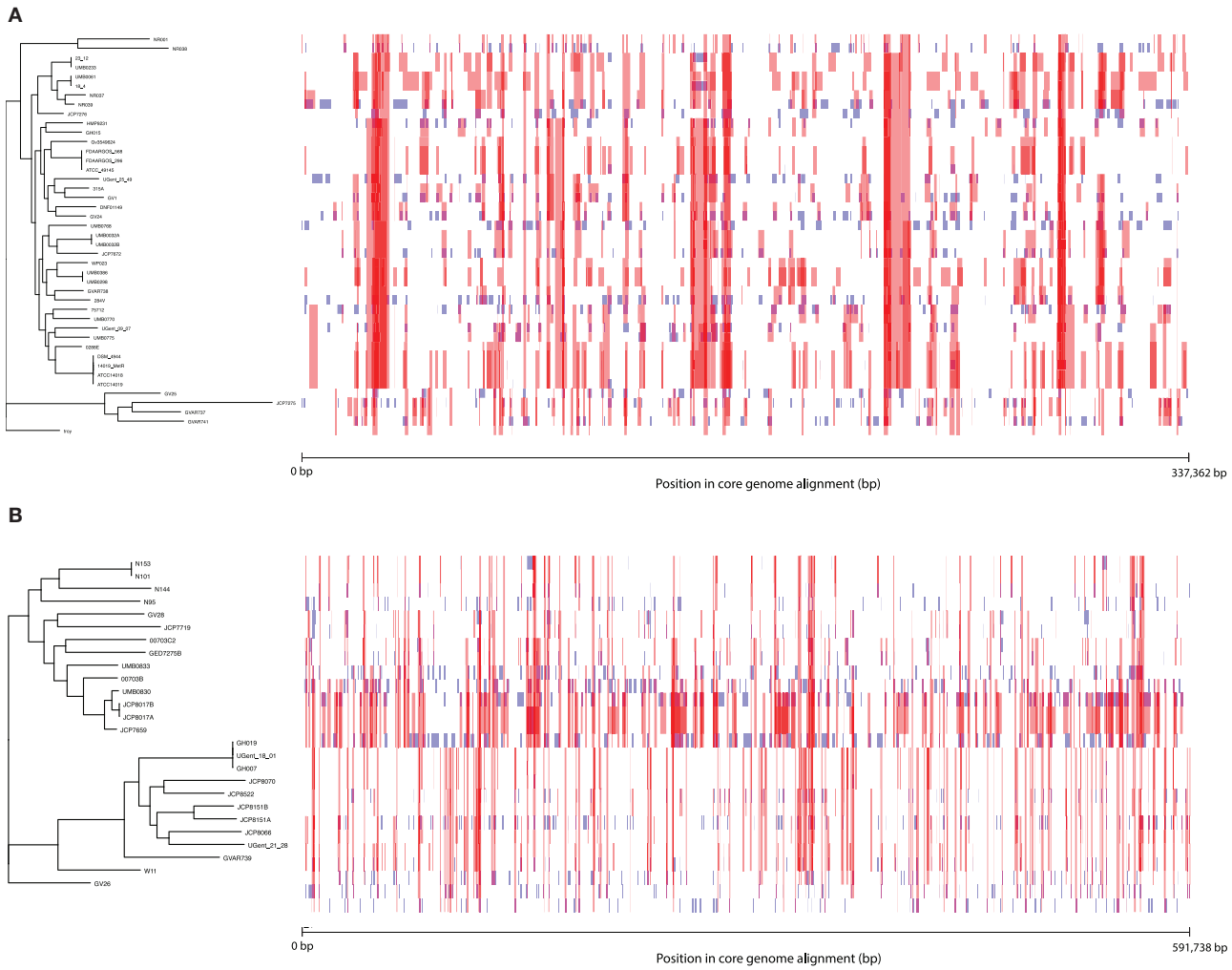

FIGURE 8 | Within species recombination is frequent in *Gardnerella* core genomes. Recombination in the core genomes of *G. vaginalis* **(A)** and *G. piotii* **(B)**. Each row corresponds to the core genome of an isolate in the phylogenetic tree to the left. Blue segments represent laterally transferred fragments unique to an individual isolate. Red segments indicate laterally transferred fragments that are shared across multiple isolates. The proportion of the alignment affected by recombination (both red and blue fragments) is 0.94 for *G. vaginalis* and 0.92 for *G. piotii*.

with Gubbins. LGT appears qualitatively more frequent in *G. vaginalis* than in *G. piotii* (**Figure 8**). The proportion of the total alignment affected by recombination is similar in the two species (0.94 for *G. vaginalis* and 0.92 for *G. piotii*), in an uncorrected analysis. To account for differences in sample size in *G. vaginalis* and *G. piotii* isolates, we sub-sampled the number of *G. vaginalis* isolates to the number of *G. piotii* isolates and used Gubbins to identify recombination in the subsampled dataset. The mean proportion of each isolate's core genome affected by recombination is greater for *G. vaginalis* (26.1%) than for *G. piotii* (23.1%) (Mann-Whitney-Wilcoxon test, $W = 473$, $p = 0.0138$) (**Figure 9**). The *G. vaginalis* network contains more reticulations than *G. piotii*, which also supports overall higher rates of recombination in *G. vaginalis* (**Figure S11**).

Patterns of Lateral Gene Transfer in Accessory Genomes of *G. vaginalis* and *G. piotii*

Inter-species barriers to gene exchange were evident in comparisons of the accessory genomes of *G. vaginalis* and *G. piotii*. Accessory gene frequencies *in G. vaginalis* and *G. piotii* isolates indicated that most accessory genes were unique to an individual species, with few genes found at similar frequencies across species (**Figure 10**). If accessory genes were freely exchanged between species, we would expect their frequencies to equilibrate.

To further characterize lateral gene transfer in the accessory genome, we quantified pairwise diversity (π) for accessory genes common to *G. vaginalis* and *G. piotii*. We performed

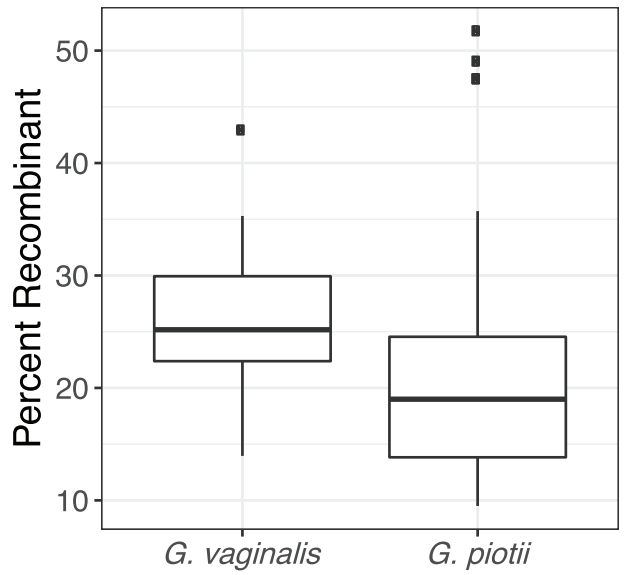

FIGURE 9 | *G. vaginalis* core genomes are more recombinant than *G. piotii*. Boxplots show proportion of each isolate's core genome affected by recombination, as estimated with Gubbins. The box spans the interquartile range, the median is represented by the middle line, and the whiskers extend to ±1.5 times the interquartile range. Data beyond the end of the whiskers are outlying points and plotted individually. To account for differences in sample size of *G. vaginalis* and *G. piotii* isolates, we subsampled *G. vaginalis* isolates to the number of *G. piotii* isolates and used Gubbins to identify recombination in the subsampled dataset. The mean of affected core genomes in the subsampled *G. vaginalis* his higher than that of *G. piotii* (Mann-Whitney-Wilcoxon test, W = 473, $p = 0.0138$).

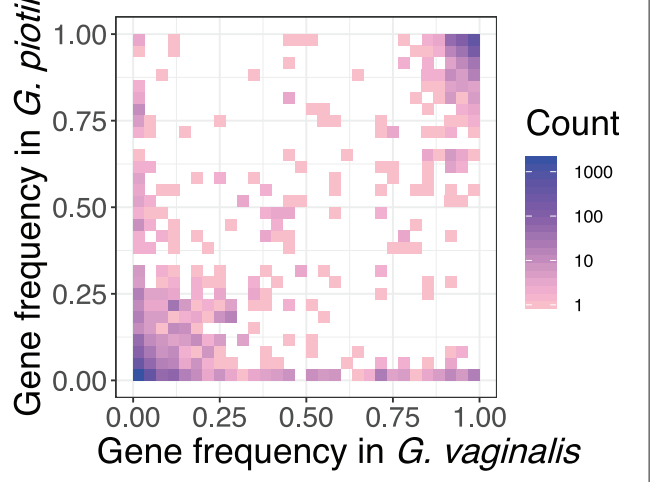

FIGURE 10 | Accessory genes are not maintained at similar frequencies in *G. vaginalis* and *G. piotii*. Heat map of pangenome gene frequencies in *G. vaginalis* and *G. piotii*. Accessory genes are not maintained at similar frequencies in the two species suggesting that selection pressures for the shared accessory genes are not the same across species. For example, some genes are maintained at high frequencies in one species, but low in the other. Number of genes are colored on a log scale.

a Kruskal-Wallis test and found there was a statistically significant difference between average gene π values by group (H = 214.7, p < 2.2e-16). We then performed pairwise Mann-Whitney-Wilcoxon tests with Bonferroni correction and found the distributions of average gene π values of *G. vaginalis* (W = 373,928, p < 3.32e-13) and *G. piotii* isolates (W = 399,762, p < 3.37e-13) were lower than between species (**Figure 11**). In species that regularly exchange accessory gene content, we expect to see similar levels of diversity in between- and within-species comparisons. However, we observed lower diversity within species, indicating that accessory gene variants are transferred more frequently within species than between them.

Differences in the amount of recombination between *G. vaginalis* and *G. piotii* appear to affect not only the core genome, but also the accessory genome. *G. vaginalis* has a larger pangenome than *G. piotii*, consistent with acquisition of novel gene content from diverse sources (**Figure 12**).

DISCUSSION

Structure of *Gardnerella* spp. Pangenome

Gardnerella spp. comprise diverse bacteria with a distinct genetic structure (Ahmed et al., 2012). This diversity has been delineated both phylogenetically and metabolically (Ahmed et al., 2012; Cornejo et al., 2018; Vaneechoutte et al., 2019). Using WGS data from 106 *Gardnerella* isolates, we identified eight major clades from a core genome alignment that are consistent with published genomic analyses (Ahmed et al., 2012; Devault et al., 2017; Schellenberg et al., 2017; Cornejo et al., 2018; Vaneechoutte et al., 2019). Our data primarily consist of isolates from *G. vaginalis* and *G. piotii*, which are found most commonly in clinical samples with higher Nugent scores (Janulaitiene et al., 2017).

The eight major clades in our sample were differentiated with respect to both allelic variation in their core genomes (**Figure 1**) and gene content in their accessory genomes (**Figure 2**). Prior research found evidence of frequent homologous recombination within clades/species (Ahmed et al., 2012) and functional differentiation of the accessory genome (Cornejo et al., 2018). Our results extend these observations with the observation that allelic variants in the core genome appear to be more readily exchanged within species than between them (**Figures 3**, **8**). In addition, we found evidence of barriers to recombination in the accessory genome, as shared accessory genes are more similar within species than between species (**Figure 11**). Accessory genes are not maintained at similar frequencies across species, suggesting that selection pressures for the shared accessory genes are not the same across species (**Figure 10**).

Mechanisms and Barriers to LGT in *Gardnerella*

Previous studies identified competence genes in a handful of *Gardnerella* isolates (Yeoman et al., 2010). We systematically examined our sample for previously identified competence genes as well as genes known to encode tad pili (Tomich et al., 2007;

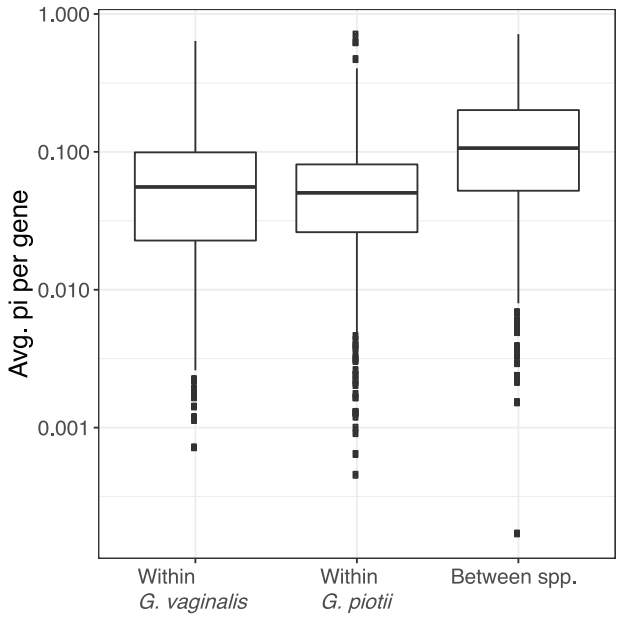

FIGURE 11 | Accessory gene flow occurs more often within *Gardnerella* species than between species. The distributions of nucleotide diversity (π) per shared accessory gene within and between *G. vaginalis* and *G. piotii*, were calculated and log transformed. The box spans the interquartile range, the median is represented by the middle line, and the whiskers extend to ± 1.5 times the interquartile range. Data beyond the end of the whiskers are outlying points and plotted individually. Average gene π values differ significantly by group (Kruskal-Wallis test, $H = 214.7$, $p < 2.2e\text{-}16$). The distributions of average gene π values of *G. vaginalis* (Mann-Whitney-Wilcoxon, $W = 373{,}928$, $p < 3.322e\text{-}13$) and *G. piotii* (Mann-Whitney-Wilcoxon, $W = 399{,}762$, $p < 3.37e\text{-}13$) are lower than between species. In species that regularly exchange accessory gene content, we might expect to see similar levels of diversity in shared accessory gene content. However, we observed lower diversity within species, consistent accessory gene flow occurring more often within *G. vaginalis* and *G. piotii* than between them.

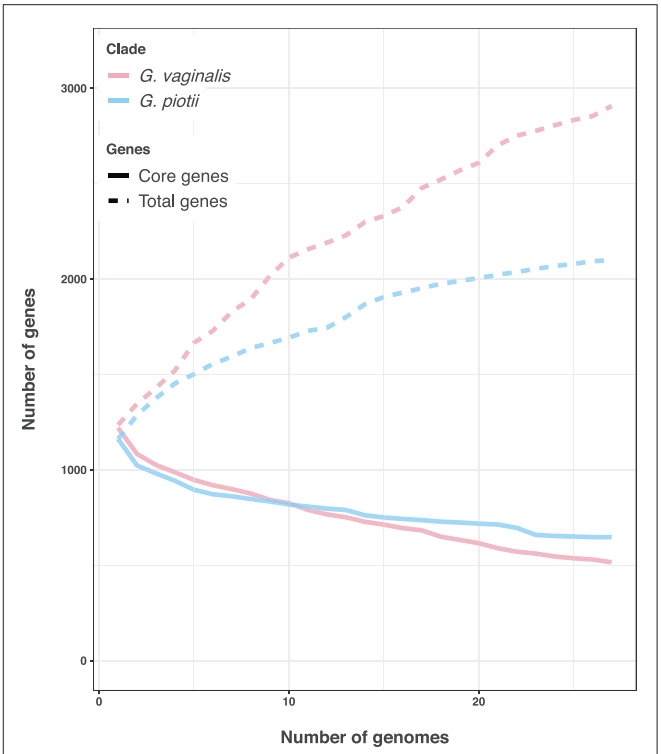

FIGURE 12 | *G. vaginalis* has a larger accessory genome than *G. piotii.* Rarefaction curves of core and total gene content. *G. vaginalis* isolates were subsampled to the number of *G. piotii* isolates, and both species were iteratively sampled 100 times. The median value is shown. *G. vaginalis* has a larger accessory genome than *G. piotii,* consistent with acquisition of novel gene content from a diverse pool.

Yeoman et al., 2010). We found these genes to be encoded by most isolates from the eight clades/species in our sample (**Figure 4**). The competence machinery, was however, highly genetically differentiated among clades/species (**Figure S2**). This raises the possibility of functional differentiation among competence genes, which could contribute to genetic isolation of clades/species (Porse et al., 2018). We investigated this hypothesis by computing the ratio of non-synonymous to synonymous variation in pairwise comparisons and across phylogenies of competence genes (**Figure 5**; **Figure S3**). We did not find any evidence of functional divergence among competence genes. Our results instead suggest that competence genes are under similar evolutionary pressures as the core genome, with both evolving under purifying selection (**Figure 5**; **Figure S3**).

A previous study identified predicted prophage genes in *Gardnerella* spp. (Malki et al., 2016). This does not necessarily indicate the presence of prophage, particularly if phage orthologs are not found within a cluster of phage associated genes. We used ProphET (Reis-Cunha et al., 2019) to identify prophage clusters, which we found in 70% of isolates (**Figure 6**). One limitation of this method is that prophage found in poorly assembled regions or across multiple contigs in the assembly may remain unidentified thus the true carriage frequency of prophage could be higher than our estimate. However, we blasted the prophage regions against the *de novo* assembled contigs and found that hits along the end of contigs are very small in length, suggesting they are not unidentified prophage (**Figure S7**).

CRISPR/*cas* are adaptive immune systems that can protect bacterial genomes from mobile genetic elements, such as phage, and thus could potentially shape and reinforce the genetic barriers in *Gardnerella* spp. Loss of CRISPR has been shown to enable the proliferation of mobile elements in *Enterococcus* (Pleckaityte et al., 2012; Hullahalli et al., 2018). We did not find an association between the presence of CRISPR/*cas* genes and the absence of any prophage clusters, suggesting these interactions may not be straightforward (**Figure 6**). Restriction modification (RM) is another mechanism by which foreign DNA is cleaved and thus prevented from integrating into bacterial genomes (Tock and Dryden, 2005). We found a wide diversity of RM genes at varying frequencies among *Gardnerella* spp. (**Figure S9**). The role they play in shaping and maintaining LGT barriers is unknown.

Differences in codon usage are another possible barrier to recombination among species (Tuller et al., 2011). To investigate this possibility, we compared codon usage between the two most well-sampled species. We did not, however, find evidence of differential codon usage among *G. vaginalis* and *G. piotii* isolates (**Figure S10**). It's intriguing that codon usage appears to be harmonized amongst these highly genetically differentiated species. By comparison, a study of a single relatively clonal species (*S. aureus*) found evidence of differences in codon usage among ecotypes (Richardson et al., 2018). We and others have found evidence that the pore-forming toxin vaginolysin is freely exchanged among *Gardnerella* spp. (**Figure S5**) (Ahmed et al., 2012). Harmonization of codon usage could facilitate exchange of genes like vaginolysin that are critical to diverse *Gardnerella* spp. We found evidence that vaginolysin genes are evolving under strong constraint (**Figure 5A**), which supports the idea that the toxin is important to the fitness of diverse *Gardnerella* spp.

Our results indicate that clades/species of *Gardnerella* spp. are reproductively isolated despite being found in the same niche. This differentiation does not appear to be driven by functional differentiation of competence genes, nor by differences in patterns of codon usage. The likelihood of LGT events and compatibility of transferred genes with the recipient genome increases between closely related genomes (Popa and Dagan, 2011). It is probable that general patterns of differentiation in the core and accessory genomes of *Gardnerella* spp. have a role maintaining species separation (Porse et al., 2018). It is intriguing that between-species LGT appears to have been more common in the remote past (**Figure 3B**), when the species may have shared more sequence similarity. Other potential mediators of reproductive isolation among species may be restriction-modification systems and CRISPR/*cas* systems, both of which can target degradation of foreign DNA, and thus shape patterns of LGT (Tock and Dryden, 2005; Marraffini and Sontheimer, 2008; Dupuis et al., 2013; Hullahalli et al., 2018). We did not find evidence of interactions between CRISPR/*cas* loci and prophage (**Figure 6**); suggesting interactions among these elements are complex in *Gardnerella* spp.

Patterns of Within and Between Species LGT in *G. vaginalis* and *G. piotii*

We found patterns of LGT to vary among *Gardnerella* spp. Specifically, we found evidence of distinct patterns of within-species recombination in comparisons of *G. vaginalis* and *G. piotii*. *G. vaginalis* appears to engage more frequently in LGT (**Figure 8**; **Figure S11**) as a larger proportion of each isolate's core genome is estimated to have been affected by recombination (**Figure 9**). Additionally, *G. vaginalis* has a larger pangenome (**Figure 12**), consistent with higher levels of gene importation in the accessory genome. These findings raise the possibility that *G. vaginalis* exchanges DNA with more diverse partners than does *G. piotii*, potentially with other non-*Gardnerella* members of the complex polymicrobial BV biofilm.

We do not know the underlying mechanism for differences in LGT between *G. vaginalis* and *G. piotii*. Variation in rates of transduction as well as restriction-modification systems could play a role. A previous study found *G. vaginalis* to be enriched for "phage-associated protein" and three genes involved in a type I RM system (Cornejo et al., 2018). We observed a wide diversity of RM systems, with multiple type I variants found within each species (**Figure S9**). Thus, even if RM systems explain the differences in LGT patterns within species, it is not a one to one relationship between species and RM systems. Teasing apart those interactions will require more research.

In conclusion, *Gardnerella* spp. are genetically distinct in both their core and accessory genomes. We found evidence of more within species LGT in the core and accessory genomes, suggesting active maintenance of reproductive barriers despite similar patterns of codon usage. The putative competence machinery is genetically differentiated between clades/species; however, we found no evidence of functional divergence/positive selection driving clade/species separation. We identified a larger pangenome in *G. vaginalis* than in *G. piotii* as well as more LGT in the core genome, suggesting *G. vaginalis* engages more frequently in LGT with more diverse partners. Taken together, our results demonstrate that co-localized bacterial populations can maintain a complex genetic structure in which genetic exchange appears to be restricted to specific sub-populations with exceptions for individual genes (e.g., vaginolysin). The forces maintaining this structure are yet to be fully elucidated but likely include patterns of sequence similarity and possibly phage, CRISPR/*cas*, RM systems and interactions among them. Defining evolutionary interactions in bacterial populations helps to illuminate how clinically important traits such as antibiotic resistance and virulence emerge and are maintained in these complex communities.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are freely available at NCBI, PRJNA602880.

AUTHOR CONTRIBUTIONS

CP and TM conceived of the study. CP developed the study design, with input from the other authors. LB and TM performed the analyses. LB drafted the manuscript with input from CP. All authors provided critical feedback and contributed to manuscript revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00293/full#supplementary-material

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The Vaginal Microbiota Among Adolescent Girls in Tanzania Around the Time of Sexual Debut

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The aetiology of bacterial vaginosis (BV) is not well-understood, and prevalence appears to be higher among women living in sub-Saharan Africa. A recent conceptual model implicates three main bacteria (*Gardnerella vaginalis; Atopobium vaginae;* and *Prevotella bivia*), sexual activity, sialidase activity, and biofilm formation in the pathogenesis of BV. We describe the vaginal microbiota, presence of the putative sialidase A gene of *G. vaginalis,* and biofilm among 386 adolescent girls aged 17 and 18 years in a cross-sectional study in Mwanza, Tanzania around the time of expected sexual debut. Vaginal swabs were collected and tested by quantitative polymerase chain reaction (qPCR) for five *Lactobacillus* species, *G. vaginalis, A. vaginae, P. bivia*, the sialidase A gene of *G. vaginalis*, and by fluorescence *in situ* hybridisation (FISH) for evidence of *G. vaginalis* and *A. vaginae* biofilm. We conducted a risk factor analysis of *G. vaginalis, A. vaginae* and *P. bivia*, and explored the associations between biofilm, the presence of the sialidase A gene, and non-optimal vaginal microbiota (Nugent 4–7). *L. crispatus* and *L. iners* were detected in 69 and 82% of girls, respectively. The prevalence of *L. crispatus* was higher than previously reported in earlier studies among East and Southern African women. *G. vaginalis, A. vaginae, P. bivia* were independently associated with reported penile-vaginal sex. Samples with all three BV-associated bacteria made up the highest proportion of samples with Nugent-BV compared to samples with each bacterium alone or together in pairs. Of the 238 girls with *G. vaginalis*, 63% had the sialidase A gene detected, though there was no difference by reported sexual activity ($p = 0.197$). Of the 191 girls with results for sialidase A gene and FISH, there was strong evidence for an increased presence of sialidase A gene among those with evidence of a biofilm ($p < 0.001$). There was a strong association between biofilm and non-optimal microbiota (aOR67.00; 95% CI 26.72–190.53). These results support several of the steps outlined in the conceptual model, although the role of sexual activity is less clear. We recommend longitudinal studies to better understand changes in vaginal microbiota and biofilm formation around the time of sexual debut.

Keywords: bacterial vaginosis, vagina microbiota, Africa, biofilm, *Lactobacillus crispatus*, *Gardnerella vaginalis*, sialidase, adolescence

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INTRODUCTION

Vaginal microbiota plays an important protective role in the female reproductive tract. Optimal vaginal microbiota is dominated by lactic acid producing bacteria (*Lactobacillus spp*) which maintains a low pH in the vaginal niche. Bacterial vaginosis (BV), an example of non-optimal vaginal microbiota, is characterised by the loss of protective *Lactobacillus spp*. (*L. crispatus,* as well as *L. gasseri, L. jensenii*, and *L. vaginalis)* and a high relative abundance or load of facultative and/or obligate anaerobes, resulting in the breakdown of the protective mucin layer and inflammation (McKinnon et al., 2019). BV is associated with adverse urogenital and reproductive health outcomes including an increased risk of HIV acquisition (Low et al., 2011; Buvé et al., 2014; Eastment and Mcclelland, 2018). While BV and BV-associated bacteria have been well-described, it is not well-understood how the high abundance of BV-associated bacteria is established and maintained, and how BV develops and resolves (van de Wijgert et al., 2014). Sexual activity has been strongly associated with BV; however, it is not clear whether it is a sexually transmitted or sexually enhanced condition (Fethers et al., 2008; Verstraelen et al., 2010). Describing the vaginal microbiota around the time of sexual debut may offer important insights for understanding the pathogenesis of BV.

Lactobacillus spp.-dominated vaginal microbiota is associated with health; however, not all *Lactobacilllus spp*. are equally protective. *L. crispatus,* as well as *L. gasseri, L. jensenii*, and *L. vaginalis,* have been associated with health; while *L. iners* is often found among women with BV (van de Wijgert et al., 2014). A recent prospective study among South African adolescent girls showed that *L. crispatus*-dominated vaginal microbiota, not *L. iners*, was associated with a decreased risk of acquiring HIV (Gosmann et al., 2017). In addition, this study reported that only 10% of women had microbiota dominated by *L. crispatus*, while 32% were dominated by *L. iners*. This is in contrast to a study conducted among Caucasian women in high income countries 45% of whom had *L. crispatus*-dominated vaginal communities (Ravel et al., 2011). Several studies have reported that Black African and African-American women compared to Caucasian or Asian women are less likely to carry *L. crispatus*, and more likely to carry *L. iners* (van de Wijgert et al., 2014). It has been hypothesised that such differences in the vaginal microbiota may partly explain differences in prevalence of BV between different populations with the highest prevalences found in women in sub-Saharan Africa (Kenyon et al., 2013; Buvé et al., 2014).

In 2019, Muzny et al. presented a conceptual model that implicated three main bacteria and their interactions in the pathogenesis of BV: *Gardnerella vaginalis; Atopobium vaginae;* and *Prevotella bivia*. The model postulated that that virulent strains of *G. vaginalis* are acquired by sexual transmission which adhere to the host epithelium, displace lactobacilli and create a biofilm (Muzny et al., 2019), a structured community of bacteria in a self-produced extracellular matrix which sequesters bacteria making it difficult to treat (Hardy et al., 2017a). Recently it has been shown that different strains of *G. vaginalis* may explain differences in virulence (Vaneechoutte et al., 2019). For example, some, but not all, strains can produce sialidase, which facilitate the destruction of the protective mucin layer on the vaginal epithelium (Lopes Dos Santos Santiago et al., 2011). After this first step, the Muzny model proposes that the synergistic effect of *P. bivia* and *G. vaginalis* enhances growth of both bacteria which both produce sialidase and loss of the mucin layer of the vaginal epithelium. Next, the loss of the mucin layer leads to increased adherence of other BV-associated bacteria, including *A. vaginae*. *A. vaginae* has been shown to elicit a stronger immune response than *G. vaginalis*, and may also have higher resistance to BV treatments such as metronidazole.

We recently published a paper showing a BV prevalence of 25% among girls attending secondary school in Tanzania. In this study the BV prevalence among girls who reported no penile-vaginal sex was 19% compared to 33% in girls reporting having had penile-vaginal sex (Francis et al., 2018). These data strongly suggest that penile-vaginal sex increases the risk for BV. In this paper, we describe the vaginal microbiota from the same study, including the results of quantitative PCR tests for *L. crispatus*, *L. gasseri, L. jensenii*, *L. iners, L. vaginalis, G. vaginalis, A. vaginae* and *P. bivia*; putative sialidase A gene of *G. vaginalis* in specimens containing *G. vaginalis*; and BV-associated biofilm dominated by *G. vaginalis* and *A. vaginae* by fluorescence *in situ* hybridisation (FISH). We investigate factors associated with the presence of *G. vaginalis*, *A. vaginae* and *P. bivia*, and the association between biofilm, putative sialidase gene of *G. vaginalis* and non-optimal microbiota.

MATERIALS AND METHODS

The enrolment of the study population and the study procedures have been described in detail elsewhere (Francis et al., 2018). In brief, all government secondary schools in Mwanza city, north-western Tanzania, were mapped and 26 schools with more than 25 girls aged 17 and 18 years were identified and asked to collaborate in the study. The parents of all girls aged 17 and 18 years in forms 1–3 were informed about the study and asked for their informed consent for their daughter to participate in the study if she was <18 years old. The girls were asked for their assent/consent. Assenting/consenting girls were invited to a research clinic where they were interviewed and samples of urine, blood and vaginal fluid were taken for testing for sexually transmitted and reproductive tract infections (STIs/RTIs) and characterisation of the vaginal microbiota. Non-pregnant girls were taught how to self-collect vaginal swabs. They were asked to collect five sequential swabs in the presence of a nurse who provided assistance if needed.

Laboratory Tests

Laboratory testing was performed according to standard operating procedures. Urine samples were tested for pregnancy using the QuickVue+ Test (QUIDEL, USA). Serum samples were used to test for IgG antibodies for HSV-2 by ELISA (Kalon Biological Ltd., UK). Syphilis was determined by the Immutrep Rapid Plasma Reagin test (Omega Diagnostics, Scotland) and the Treponema pallidum particle agglutination assay (SERODIA, Fujirebio Inc., Japan).

All blood samples were screened with the Determine HIV 1/2 rapid test (Alere, Japan). Reactive samples were tested with the Uni-GoldTM HIV rapid test (Trinity Biotech, Ireland). If both tests were reactive, the final result was deemed positive. If the Uni-Gold test was not reactive, the sample was tested with the HIV ½ Stat-Pak test (Chembio, USA). The final result was considered positive if the Stat-Pak result was reactive.

Two flocked swabs (Copan, USA) were eluted in 1.2 mL of diluted phosphate buffered saline (dPBS) (pH 7.4–1:9, PBS:saline), pooled and aliquots were prepared. One aliquot was used to test for *Neisseria gonorrhoeae, Chlamydia trachomatis* and *Mycoplasma genitalium* by in-house PCR (Chen et al., 2008; Hopkins et al., 2010). A second aliquot was used to test for human papillomavirus (HPV) using the Roche Linear Array HPV Genotyping Test (Roche, USA), which detects 37 HPV high and low risk genotypes. A third aliquot was used to assess the presence and concentrations of *L. crispatus*, *L. gasseri, L. jensenii*, *L. iners, L. vaginalis, G. vaginalis, A. vaginae* using a qPCR as previously described (Jespers et al., 2012), with the exception of DNA extraction where another method, the QIAmp DNA mini kit, was applied. A fourth aliquot was used for the determination of the bacterial load of *P. bivia* and the detection of the putative sialidase A gene of *G. vaginalis* in specimens containing *G. vaginalis*. DNA was extracted using the Abbott m2000sp automated extraction platform (Abbott Laboratories, USA) according to the manufacturer's instructions for the plasma custom program and incorporating an extra lysis step (Crucitti et al., 2018). For the quantification of *P. bivia*, an in-house qPCR assay was performed targeting the mucin-desulfating sulfatase (*mdsC*) gene. The 25 μL PCR mixture consisted of 12.5 μL Sybr Green master mix (Qiagen, Germany), 0.5 μL of 10 μM of each primer (forward primer: PBsulF 5'ACGTTTGGGCAAAGCTCCTTGTCT3', reverse primer: PBsulR 5'GCGTGTACGCCAGTTGCAAGA3), 6.5 μL Rnase free water, and 5 μL of DNA extract (Lopes dos Santos Santiago et al., 2012). The amplification comprised an initial heating of 95°C for 5 min followed by 45 cycles of 95°C for 5 s and 65°C for 10 s. For samples containing *G. vaginalis*, a qPCR for the putative *G. vaginalis* sialidase A gene was performed as previously described (Hardy et al., 2017b). All qPCRs were run in duplicate and the organism concentrations were expressed as genome equivalents per ml (geq/ml) and $\log_{10}$-transformed.

A cotton-tipped vaginal swab was used to prepare two slides. The first slide was Gram stained and examined for vaginal yeast and for BV using the Nugent score for diagnosing BV (Nugent et al., 1991). A Nugent score of 0–3 indicated normal microbiota, 4–6 indicated intermediate microbiota, and 7–10 indicated BV. A Superfrost Plus® slide (Menzel-Gläser, Germany) was heat fixated and stored at room temperature until shipment to the Institute of Tropical Medicine in Antwerp, Belgium (ITM) where it was re-fixated with a Carnoy solution (6:3:1, ethanol:chloroform:glacial acetic acid) for at least 12 h. For all participants who had *G. vaginalis* detected by qPCR, the slide was examined using Peptide Nucleic Acid (PNA)-FISH employing species-specific probes for *A. vaginae* (AtoITM1) and *G. vaginalis* (Gard162), and the broad-range BacUni-1 probe. Procedures and definitions of observations were applied as described elsewhere (Hardy et al., 2015). The Superfrost Plus® slide was not available for the first few weeks of the study; therefore, not all participants have results for the FISH examination. The same swab was inoculated in an InPouch™ TV culture device (BioMed Diagnostics, USA), incubated at 37°C and read every other day for the presence of motile trichomonads for 5 days or until positive.

All laboratory tests were carried out at the Mwanza Intervention Trials Unit at the National Institute of Medical Research (NIMR/MITU) laboratory in Mwanza with the exception of the qPCR on *P. bivia*, the putative sialidase A gene tests on *G. vaginalis* and the visualization of *G. vaginalis* and *A. vaginae* by FISH which were carried out at ITM. The primers and probes at NIMR were ordered from Eurogentec S.A. (Belgium) and PCRs were run on the QIAGEN Rotorgene Q. The primers and probes used at ITM were synthesized by Integrated DNA Technologies (IDT), Illinois, USA, and amplifications were performed using the Corbett Life Science Rotor-Gene™ 6000 (Qiagen, The Netherlands). In order to assess the variability in the results of the qPCR a random selection of 100 stored samples were re-tested at ITM. Initial qPCR results for *L. crispatus* carried out at NIMR/MITU were higher than expected; therefore, we repeated *L. crispatus* qPCR at ITM and compared results. There was less than a log difference between the geometric means between the NIMR/MITU and ITM results (8.72 vs. 7.76, respectively); therefore, we used the NIMR/MITU results in the analysis described below.

Data Management and Statistical Analysis

Pen and paper questionnaire data and laboratory worksheets were double entered into OpenClinica LLC (Akaza Research, USA). The data were analysed using R version 3.6.3.

Two parameters were analysed for each bacterial species: percentage of girls in which the bacterial species was detected and, if the species was present, the concentration of the bacteria expressed as geq per ml. The presence of the different bacterial species in girls who reported no penile-vaginal sex was compared to that in girls reporting having had penile-vaginal sex using chi-squared tests, and for those in whom the species was present, the mean $\log_{10}$ concentrations were compared using *t*-tests. In order to allow for the effect of STIs/RTIs on the vaginal microbiota, the same analyses were repeated excluding the girls with STIs/RTIs (vaginal yeast, HSV-2 infection, gonorrhoea, chlamydial infection, *M. genitalium* infection, HPV, trichomoniasis, syphilis, and HIV). Lastly, the presence and concentrations of the different species were compared in girls in the different categories of Nugent score (1–3, 4–6, 7–10), stratified by reported penile-vaginal sex.

A hierarchical approach was used for the risk factor analysis for each of the BV-associated bacteria: *G. vaginalis; A. vaginae;* and *P. bivia.* We first estimated the crude and independent effects of the socio-demographic characteristics on the presence of the bacteria (level 1). Socio-demographic variables included age, socioeconomic status (SES) and if the participant lived with a parent. Socioeconomic status (SES) was estimated using an indicator based on the type of possessions owned by the head of the household. The independent effects were estimated using

multivariable logistic regression adjusted for any other socio-demographic variables with a p-value of <0.10. This procedure was repeated for the analysis of behavioural risk factors (level 2), except that the multivariable model at this level was adjusted for not only other behavioural factors with adjusted associations with $p < 0.10$, but also the socio-demographic characteristics that were found to be independently associated with BV from the first stage of the analysis. Behavioural factors that were explored in all girls included menstrual hygiene management, intravaginal cleansing, direction of cleaning after defecation, sexual touching with hands, receptive oral sex and life-time number of sexual partners for penile-vaginal sex. In girls who reported that they had had penile-vaginal sex the same behavioural variables were explored as well as condom use with last partner and age of first sexual partner. *P*-values were obtained with likelihood ratio tests.

Samples tested by FISH were categorised into five groups based on the presence and absence of dispersed and/or adherent *G. vaginalis* and *A. vaginae*: no *G. vaginalis* or *A. vaginae* visualised; dispersed *G. vaginalis* only, no *A. vaginae*; dispersed *G. vaginalis* and *A. vaginae;* adherent *G. vaginalis* only, no *A. vaginae*; and a combination of *G. vaginalis* and *A. vaginae* dispersed and adherent. The latter category included all samples with both *G. vaginalis* and *A. vaginae*, and with at least one bacterium showing adherence. We considered the last two categories with adherent bacteria to be indicative of biofilm. We compared these categories by Nugent score and reported penile-vaginal sex using the Chi squared test for trend.

Samples in which *G.vaginalis* was found were characterised by the presence of the sialidase A gene using two parameters: percentage of girls in which the sialidase A gene was present and, if the sialidase A gene was present, the concentration of the sialidase A gene expressed as geq per ml. The presence of the sialidase A gene and the mean $\log_{10}$-transformed concentrations were compared by Nugent score, reported penile-vaginal sex and results of the FISH, again using chi-squared tests and t-tests, respectively.

The associations between the presence of the sialidase A gene and biofilm, and between biofilm and non-optimal vaginal microbiota were explored. We combined Nugent score 4–10 to make vaginal microbiota status binary and defined the categories 0–3 as optimal microbiota and 4–10 as non-optimal microbiota. Likewise, we combined the FISH categories to make a binary variable: the first three categories as described above were defined as non-adherent bacteria, and the last two categories were defined as adherent bacteria indicative of biofilm. Crude odds ratios were calculated for the association between detection of the sialidase A gene and microbiota, and then adjusted for the FISH results. Likewise, the crude OR was calculated for the association between FISH results and microbiota, and then adjusted for detection of the sialidase A gene.

Ethics Approval

The Institutional Review Board of the Institute of Tropical Medicine in Antwerp (867/13), the Ethics Committee of the University Teaching Hospital in Antwerp (13/14/147), the Lake Zone Institutional Review Board in Mwanza (MR/53/100/86) and the National Ethics Committee of the NIMR Coordinating Committee in Dar es Salaam (NIMR/HQ/R.8a/Vol.IX/1544) approved the study protocol. Permission to conduct the study was obtained from the Mwanza City Education Department and from Nyamagana and Ilemela Districts Education Authorities.

RESULTS

Study Population

A total of 401 girls were enrolled in the study, of whom 2 girls were outside the age range of 17–18 years and excluded from the analyses. Results for STI testing, Nugent score and qPCR testing were available for 386 girls (**Table 1**). Of these girls, 216 (56%) were aged 17 years, 58 (15%) reported intravaginal cleansing, 9 (2%) reported receptive oral sex, and 163 (42%) reported that they had ever had penile-vaginal sex. Of the girls who reported having penile-vaginal sex, 69 (42%) reported having always used a condom with their last partner, and 64 (39%) reported that the difference in ages between the girls and her first sexual partner was 3 years or more.

The overall prevalence of BV (Nugent score 7–10) was 25%: 33% among the girls who reported penile-vaginal sex; and 19% among the girls who reported no penile-vaginal sex. Intermediate microbiota (Nugent score 4–6) was found in 7.5% of the girls: 17% among the girls who reported penile-vaginal sex; and 3% among the girls who reported no penile-vaginal sex. Five percent of the girls had a candida infection. Overall, 2% of girls had gonorrhoea, 2% had chlamydia, 4% had trichomoniasis, and 2% had *M. genitalium* infection. None of the girls was diagnosed with active syphilis and 2% had IgG antibodies against HSV-2. HIV infection was detected in three girls (1%). The most common STI/RTI was any HPV (high or low risk) infection, with 125 (32%) girls infected. A total of 19 (9%) of the 223 participants who reported no penile-vaginal sex, tested positive for HSV-2, chlamydia, gonorrhoea, *M. genitalium* ($n = 12$) or Y-chromosome ($n = 7$).

Prevalence of Bacterial Species

Table 2 presents the proportion of girls in which each of the bacterial species was found as well as the mean $\log_{10}$ concentrations of the species if present. In the 223 girls who reported that they had never had penile-vaginal sex, the most commonly found bacterial species were *L. iners* (80%), *L. crispatus* (75%) and *L. vaginalis* (74%), followed by *L. jensenii* (55%). Among the 163 girls who reported that they had penile-vaginal sex, the prevalence of *L. crispatus* (60%), *L. vaginalis* (55%) and *L. jenseniii* (40%) was lower ($p < 0.01$). The BV associated bacteria *A. vaginae*, *G. vaginalis* and *P. bivia* were found in the girls who did not report penile-vaginal sex though in lower proportions than among the girls who reported sexual activity ($p < 0.01$). There was no association between the prevalence of *L. iners* ($p = 0.20$) and of *L. gasseri* ($p = 0.64$) and reported penile-vaginal sex. A similar pattern was seen after excluding the girls with STIs/RTIs (**Supplementary Table 1**).

The prevalence of *L. crispatus*, *L. jensenii* and of *L. vaginalis* decreased with increasing Nugent score (all $p < 0.001$), whereas the prevalence of *L. iners* increased with increasing Nugent score ($p = 0.008$) (**Figure 1** and **Supplementary Table 2**). The

TABLE 1 | Socio-demographic characteristics, reported sexual history and hygiene management and reproductive tract infections among adolescent schoolgirls in Mwanza city, Tanzania ($N = 386$).

Total	386	100%
Age (years)		
17	215	56%
18	171	44%
Born in		
Mwanza region	290	75%
Other region	96	25%
Lives with		
Mother (+/– father/other person)	245	63%
Father (+/– other person, but not mother)	24	6%
Does not live with mother or father	117	30%
Number of people in household		
1–5	134	35%
6–7	130	34%
8 or more	122	32%
SES indicator (possessions)		
Car	24	6%
TV, but no car	165	43%
Cell phone, no car or TV	183	47%
None of the above	14	4%
Nights outside home, last 3 months		
None	340	88%
One or more	46	12%
Menstrual hygiene management		
Reusable cloth	150	39%
Underpants	372	96%
Sanitary pads	282	73%
Tampons or toilet paper	3	1%
Intravaginal cleansing		
No cleansing	328	85%
Plain water	34	9%
Soap	22	6%
Cloth, cotton wool, detergents	2	1%
Method of cleaning after defecation		
Water only	342	89%
Toilet paper	31	8%
Other	13	3%
Direction of cleaning after defecation		
Front to back	294	76%
Back to front	92	24%
Ever touched a penis with her hands	22	6%
Man/boy ever touched her vagina with hands	35	9%
Ever had penis in her mouth	3	1%
Ever had receptive oral sex	9	2%
Ever has a penis rub against her genitals	9	2%
Ever had anal sex[a]	2	1%
Life-time sexual (penile-vaginal sex) partners		
None	223	58%
One	123	32%
Two	31	8%
Three or more	9	2%
Age of first sexual partner (years older than the girl at the time of sexual debut)[b]		
< 1 year older	16	10%
1-2 years older	31	19%
2-3 years older	30	18%
>3 years older	64	39%
Don't know/no answer	22	13%
Condom use with current/latest partner[b]		
Never	67	41%
Some of the time	24	15%
Always	69	42%
Don't know/no answer	3	2%
Bacterial vaginosis (Nugent 7–10)	95	25%
Intermediate microbiota (Nugent 4–6)	29	7%
Vaginal yeast	21	5%
Chlamydia trachomatis	9	2%
Neisseria gonorrhoeae	8	2%
Trichomonas vaginalis	17	4%
Mycoplasma genitalium	9	2%
Active syphilis	0	0%
Human papillomavirus–any genotype	125	32%
Herpes simplex virus-2	9	2%
HIV[c]	3	1%

[a]Missing data for one participant. [b]Restricted to participants who reported having at least one sexual partner. [c]Missing data for two participant.

prevalence of *L. gasseri* was low in girls with Nugent score 0–3 (25%) and even lower in girls with Nugent score 7–10 (8%, $p = 0.004$). The prevalence of *A. vaginae*, *G. vaginalis* and *P. bivia* increased with increasing Nugent score (all $p < 0.001$). Furthermore, the mean $\log_{10}$ concentrations of the *Lactobacillus spp.*, if present, were lower in girls with BV compared with girls with Nugent score 0-3, except for *L. iners*. The mean $\log_{10}$ concentrations of *A. vaginae*, *G. vaginalis* and *P. bivia* were higher in girls with BV than in girls with Nugent score 0-3 (all $p < 0.001$).

The composition of BV-associated bacterial species for each category of Nugent score is shown in **Supplementary Table 3**. Samples with *G. vaginalis*, *A. vaginae* and *P. bivia* only (single pathogens) did not substantially contribute to the total Nugent-BV cases (0-1% of BV cases). Neither did samples with both *A. vaginae* and *P. bivia* (2%) nor samples with both *G. vaginalis* and *P. bivia* (2%). Samples with both *G. vaginalis* and *A. vaginae* contributed to 34% of the Nugent-BV cases, and samples with all three BV associated bacteria contributed 58% of the Nugent-BV cases. None of the three BV-associated bacteria were detected in two samples with Nugent score 7-10, but in one of these samples *L. iners* was detected.

Factors Associated With the Presence of BV-Associated Bacteria

For each of the three BV-associated bacteria there was strong evidence of an association with penile-vaginal sex

TABLE 2 | Presence of Lactobacillus spp and BV-associated microbiota among secondary school girls in Mwanza, Tanzania ($N = 386$).

		All participants $N = 386$	No reported penile-vaginal sex $N = 223$	Reported penile-vaginal sex $N = 163$	*p*-value
L. crispatus	Present[a] *N* %	266 (69)	168 (75)	98 (60)	0.001
	Mean conc[b]	8.3	8.6	7.8	< 0.001
L. iners	Present *N* %	318 (82)	179 (80)	139 (85)	0.20
	Mean conc	7.7	7.6	8.0	< 0.001
L. jensenii	Present *N* %	188 (49)	123 (55)	65 (40)	0.003
	Mean conc	6.6	6.7	6.5	0.26
L. vaginalis	Present *N* %	255 (66)	166 (74)	89 (55)	< 0.001
	Mean conc	5.8	5.8	5.8	0.44
L. gasseri	Present *N* %	85 (22)	51 (23)	34 (21)	0.64
	Mean conc	5.9	5.9	5.9	0.94
A. vaginae	Present *N* %	168 (44)	76 (34)	92 (56)	< 0.001
	Mean conc	6.9	6.8	6.9	0.63
G. vaginalis	Present *N* %	239 (62)	116 (52)	123 (75)	< 0.001
	Mean conc	6.6	6.5	6.8	0.06
P. bivia	Present *N* %	197 (51)	98 (44)	99 (61)	0.001
	Mean conc	4.8	4.7	4.9	0.18

[a] *Proportion of samples with a concentration of bacteria. P-values were obtained with Chi square for the association presence of each bacteria and reported penile-vaginal sex.* [b] *Mean log_{10} concentration (geq/ml), if species is present. P-value was obtained by t-test for the association between mean log_{10} concentration and reported penile-vaginal sex.*

(**Supplementary Tables 4A, 5A, 6A**). In addition, as SES increased, there was a decreased odds of the presence of *G. vaginalis* (**Supplementary Table 4A**). Among the girls who reported that they had penile-vaginal sex an association was found between presence of *A. vaginae* and older age of the first sexual partner (**Supplementary Table 5B**); and between presence of *P. bivia* and life-time number of sexual partners (**Supplementary Table 6B**).

Results of Fluorescence *in situ* Hybridisation (FISH) for *Gardnerella vaginalis* and *Atopobium vaginae*

Of the 239 samples in which *G. vaginalis* was present, 191 (80%) had FISH results. Of these, 63 (33%) had no *G. vaginalis* or *A. vaginae* visualised, 17 (9%) had dispersed *G. vaginalis* only, 5 (3%) had dispersed *G. vaginalis* and *A. vaginae*, 19 (10%) had adherent *G. vaginalis* only (no adherent or dispersed *A. vaginae*), and 87 (46%) had a combination of dispersed or adherent *G. vaginalis* and *A. vaginae*. The latter category included 65 with both adherent *G. vaginalis* and *A. vaginae*, 20 with adherent *G. vaginalis* and dispersed *A. vaginae*, and 2 with dispersed *G. vaginalis* and adherent *A. vaginae*. There was 1 sample with *A. vaginae* dispersed only (no *G. vaginalis*); this sample was included in the category of dispersed *G. vaginalis* and *A. vaginae*. A Nugent score of 0–3 was more frequent among girls with no *G. vaginalis* or *A. vaginae* visualised compared to other categories. Conversely, a Nugent score of 7–10 was more frequent among girls with a combination of adherent and dispersed *G. vaginalis* and *A. vaginae* compared to other categories (χ^2: $p < 0.001$; **Figure 2**). There was no difference in the FISH results by reported sexual activity (χ^2: $p < 0.211$; **Table 3**).

Results of the Detection of Sialidase A Gene Among Samples Positive for *G. vaginalis*

Among the 239 girls with *G. vaginalis*, 236 had data for the presence of the sialidase A gene. Of these, 151 (63%) had *G. vaginalis* with the sialidase A gene. There was a positive correlation between the presence of sialidase A gene and high Nugent score; among the girls with *G. vaginalis,* Nugent score of 7–10 was more frequent among girls with sialidase A gene present compared to girls without sialidase A gene present (χ^2: $p < 0.001$; **Figure 3**).

Girls who reported not having had penile-vaginal sex had a similar prevalence of sialidase A gene compared to girls reporting penile-vaginal sex with at least one partner (45 vs. 55%, respectively, $p = 0.197$, data not shown).

Association Between Presence of the Sialidase A Gene, Biofilm and Non-optimal Microbiota

Of the 191 samples with *G. vaginalis* for which both FISH and sialidase A gene results were available, samples with adherent bacteria had higher proportions of sialidase positive *G. vaginalis* (χ^2: $p < 0.001$; **Table 4**).

Table 5 presents the association between presence of the sialidase A gene and non-optimal vaginal microbiota. The crude OR was 6.14 (95% CI 3.15–12.55), but after adjusting for FISH results, the OR was 2.29 (95% CI 0.77–6.65). The crude association between the FISH results and non-optimal vaginal microbiota was an OR of 83.12 (95% CI 33.74–232.60), and after adjusting for sialidase A gene, the OR was 67.00 (95% CI 26.72–190.53).

DISCUSSION

Few studies have examined the effect of sexual debut on the vaginal microbiota of young women. To our knowledge, this is the first paper to describe the vaginal microbiota, including vaginal biofilm among girls or young women in sub-Saharan Africa around the time of expected sexual debut. In this cross-sectional study, reported penile-vaginal sexual intercourse was associated with quantifiable changes in vaginal microbiota characterised by decreases in *Lactobacillus spp.* and increases in BV-associated bacteria. The prevalence of *L. crispatus* was higher than reported in previous studies among women in sub-Saharan Africa (Jespers et al., 2012). In our study population, samples with all three BV-associated bacteria present (*G. vaginalis, A vaginae* and *P. bivia*) made up the highest proportion of samples with Nugent-BV compared to samples with each bacterium alone or

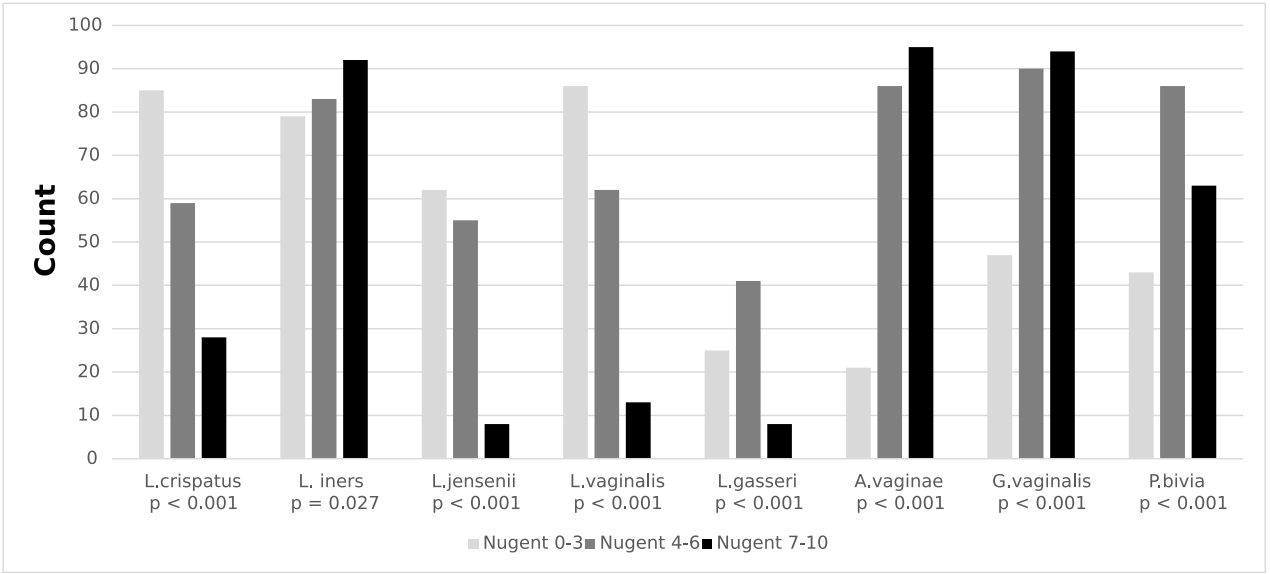

FIGURE 1 | Presence of *Lactobacillus spp* and BV-associated microbiota by Nugent score among secondary school girls in Mwanza, Tanzania ($N = 385$).

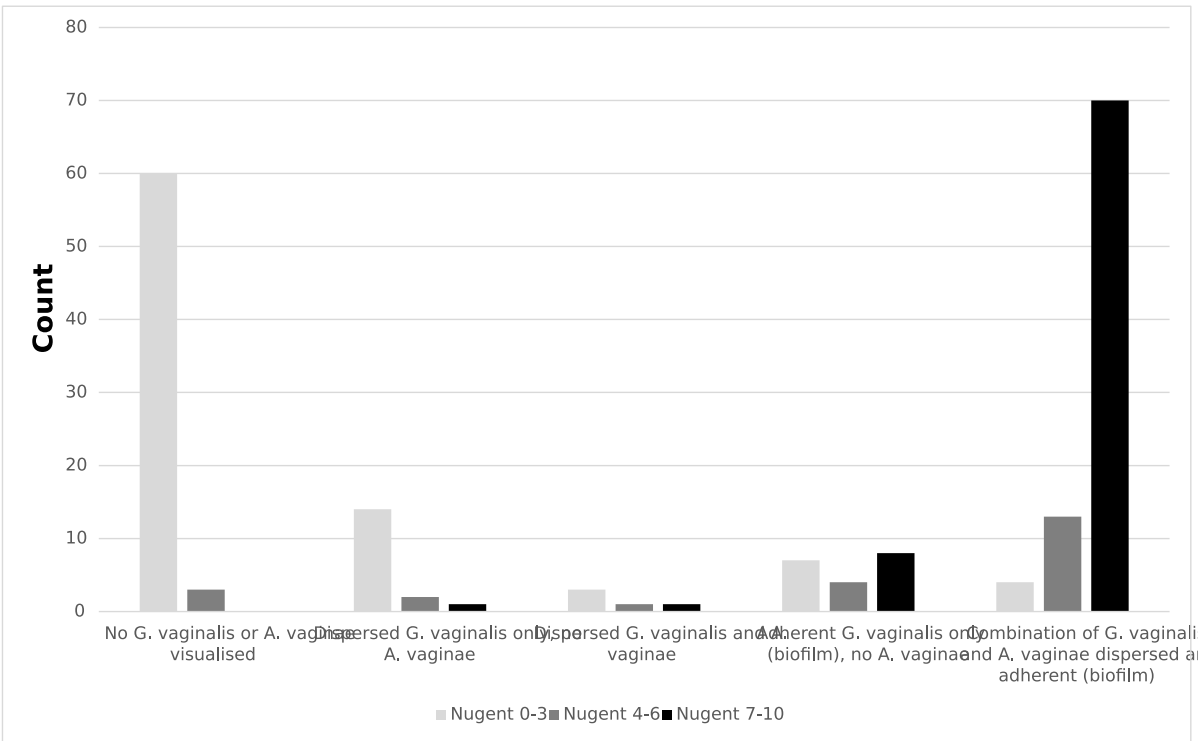

FIGURE 2 | Results of fluorescence *in situ* hybridisation (FISH) for *Gardnerella vaginalis* and *Atopobium vaginae* by Nugent score among secondary school girls in Mwanza, Tanzania ($N = 191$; $p < 0.001$).

together in pairs, supporting the conceptual model presented by Muzny et al. (2019). In addition, non-*G. vaginalis* biofilm was rare, further supporting the hypothesis that colonisation with a virulent strain of *G. vaginalis* is a necessary first step in the pathogenesis in BV (Muzny et al., 2019). Among those with detectable *G. vaginalis*, two-thirds had sialidase A positive *G. vaginalis*, and presence of this gene was higher among women with adherent bacteria indicative of biofilm supporting *in vitro*

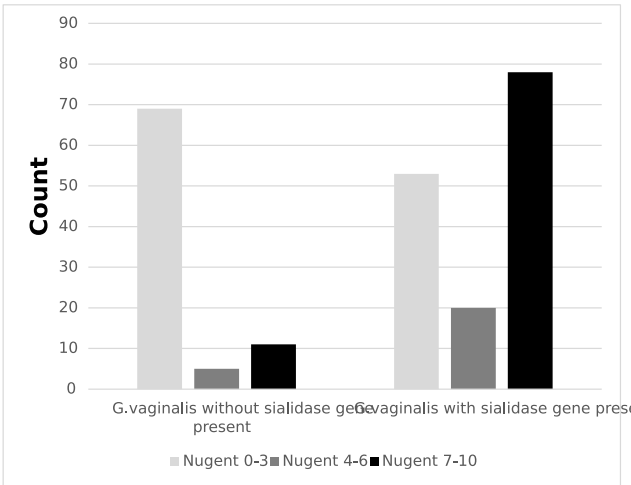

FIGURE 3 | Frequencies of detection of *Gardnerella vaginalis* with or without putative sialidase A gene by Nugent score among secondary school girls in Mwanza, Tanzania ($N = 236$; $p < 0.001$).

TABLE 3 | Results of fluorescence *in situ* hybridisation (FISH) for *Gardnerella vaginalis* and *Atopobium vaginae* by sexual activity among secondary school girls in Mwanza, Tanzania ($N = 191$).

	Reported no penile-vaginal sex *N* = 90 (100%)	**Reported penile-vaginal sex with at least one partner *N* = 101 (100%)**
No *G. vaginalis* or *A. vaginae* visualised	36 (40%)	27 (27%)
Dispersed *G. vaginalis* only, no *A. vaginae*	6 (7%)	11 (11%)
Dispersed *G. vaginalis* and *A. vaginae*	3 (3%)	2 (2%)
Adherent *G. vaginalis* only (biofilm), no *A. vaginae*	6 (7%)	13 (13%)
Combination of *G. vaginalis* and *A. vaginae* dispersed and adherent (biofilm)	39 (43%)	48 (47%)

$\chi^2(df) = 5.85(4)$, $p = 0.211$.

evidence that sialidase A gene is an important virulence factor for *G. vaginalis* biofilm formation.

The high prevalence of *L. crispatus* among girls in our study is in contrast to other studies from sub-Saharan Africa and may reflect vaginal microbiota around the time of sexual debut. In a study investigating the vaginal microbiota among women from different risk groups in East and Southern Africa, the prevalence of *L. crispatus* was 38% among sexually active adolescents in Kenya compared to 60% in our study (Jespers et al., 2015). Several studies conducted in the United States and in Europe have compared the vaginal microbiota in women of different ethnic backgrounds, showing that a microbiota dominated by *L. crispatus* was less frequent in women of African ancestry than in Caucasian women, and that the microbiota of women of African ancestry was more often dominated by *L. iners* or bacterial species other than lactobacilli (Zhou et al., 2007; Ravel et al., 2011; Fettweis et al., 2014; Borgdorff et al., 2017). Yet, our study shows a similar prevalence of *L. crispatus* among girls who report not yet having penile-vaginal sex in Tanzania to sexually naïve Caucasian girls attending school in Belgium (69% in Tanzania and 68% in Belgium) (Jespers et al., 2016). These results are hypothesis generating, and longitudinal studies are needed to investigate if the *L. crispatus* prevalence diminishes with increased numbers of partners, uncircumcised partners or the initiation of intravaginal practices, which are more common in more sexually experienced women in the region (Gray et al., 2009; Allen et al., 2010).

Our data provide evidence for the role of penile-vaginal sex in the presence of *G. vaginalis, A. vaginae* and *P. bivia*: girls who reported having had at least one sexual partner had a higher prevalence and bacterial load, as well as strong independent associations between reported penile-vaginal sex and each bacterium. However, there was also evidence that some girls had prevalent *G. vaginalis*, *A. vaginalis* and *P. bivia* before reported sexual debut. This is consistent with longitudinal studies among adolescent girls in the US, Australia and in Belgium: *G. vaginalis* and *A. vaginae* were detected among some girls with no reported sexual experience, and the initiation of penile-vaginal sex was associated with increased presence of these BV-associated bacteria (Fethers et al., 2012; Mitchell et al., 2012; Jespers et al., 2016). An Australian study also found that engaging in unprotected penile-vaginal sex was associated with having multiple strains of *G. vaginalis,* including more virulent strains, such as *G. vaginalis* clade 4 which has been shown to develop a biofilm, produce the toxin vaginolysin, and express sialidase activity. In our study, we saw no difference in the FISH results or sialidase A positive *G. vaginalis* between girls reporting sexual activity vs. girls not. However, all these results must be considered in the context of underreporting of sexual activity. We found that 9% of girls who did not report penile-vaginal sex had a laboratory confirmed STI or tested positive on the Y-chromosome. Underreporting of sexual behaviour among adolescents has been well-documented internationally especially

TABLE 4 | Results of fluorescence *in situ* hybridisation (FISH) for *Gardnerella vaginalis* and *Atopobium vaginae* by presence of the sialidase A gene among 191 samples that have *G. vaginalis* present.

	***G.vaginalis*; no sialidase gene (*N* = 60)**		***G.vaginalis*; sialidase gene present (*N* = 131)**	
	N Present (%)	**Mean conc if present**	**N Present (%)**	**Mean conc if present**
No *G. vaginalis* or *A. vaginae* visualised	35 (58)	6.0	28 (21)	6.1
Dispersed *G. vaginalis* only, no *A. vaginae*	9 (15)	6.7	8 (6)	7.3
Dispersed *G. vaginalis* and *A. vaginae*	1 (2)	9.3	4 (3)	6.7
Adherent *G. vaginalis* only (biofilm), no *A. vaginae*	3 (5)	6.3	16 (12)	7.4
Combination of *G. vaginalis* and *A. vaginae* dispersed and adherent (biofilm)	12 (20)	7.2	75 (57)	7.6

$\chi^2(df) = 53.69(4)$, $p < 0.001$.

TABLE 5 | Association between the presence of the sialidase A gene and results from fluorescence *in situ* hybridisation (FISH) with non-optimal vaginal microbiota (Nugent score 4–10) (*N* = 191).

	N	**Non-optimal microbiota Pos (%)**	**Odds ratio (95% CI)**	***p*-value**	**Adjusted odds ratio (95% CI)[a]**	***p*-value[b]**
Sialidase A gene						
Absent	60	15 (25%)	1	<0.001	1	0.134
Present	131	88 (67%)	6.14 (3.15–12.55)		2.29 (0.77–6.65)	
Fluorescence *in situ* hybridisation (FISH) for *Gardnerella vaginalis* and *Atopobium vaginae*						
Non-adherent	85	8 (9%)	1	<0.001	1	<0.001
Adherent (biofilm)	106	95 (90%)	83.12 (33.74–232.60)		67.00 (26.72–190.53)	

[a] *Variables were adjusted by the other variables in the model.* [b] *P-values obtained with likelihood ratio tests.*

during face-to-face interviews (Langhaug et al., 2010), and fears of stigmatization, school expulsion and punishment may be even more relevant in the setting where this study was conducted (Houlihan et al., 2014).

Our FISH results are similar to other reports that show few or no samples with biofilm in the absence of *G. vaginalis* (Hardy et al., 2016). *In vitro* studies have shown that *G. vaginalis* is able to adhere to epithelial cells and displace *L. crispatus*, while other BV-associated bacteria, such as *A. vaginae* and *P. bivia* were outcompeted by protective lactobacilli (Rosca et al., 2019). Once a *G. vaginalis* biofilm is established, BV-associated bacteria can colonise and form synergistic interactions, by stimulating growth and upregulating key virulence factors such as genes encoding for sialidase. Indeed, our study shows a higher proportion of Nugent-BV among samples with both *G. vaginalis* and *A. vaginae* compared to biofilm with *G. vaginalis* alone, also seen in a previous study conducted in Rwanda (Hardy et al., 2016).

There was a strong association between the samples with adherent bacteria indicative of biofilm and non-optimal microbiota. This was expected as "clue cells," one of the criteria for BV diagnosis with Amsel's criteria, represent adherent bacteria. Importantly, this association was not substantially attenuated after adjustment for presence of the sialidase A gene. However, the association between sialidase A gene and non-optimal microbiota was substantially attenuated by adjusting for the FISH results, suggesting that biofilm lies on the causal pathway between sialidase A gene and non-optimal microbiota (**Figure 4**). In other words, much of the effect of sialidase production on non-optimal microbiota occurs through biofilm formation. This is supported by biological plausibility: sialidase destroys the protective mucus layer on the vaginal epithelium facilitating adhesion, successfully bypassing protective lactobacilli and providing a matrix in which secondary BV-associated bacteria can adhere and sequester. This is in alignment with earlier findings that showed an increased probability of having BV (diagnosed by Nugent scoring) when high concentration of *G. vaginalis* and the putative *G. vaginalis* sialidase A gene are present (Hardy et al., 2017b). The current study adds to the *in vivo* evidence of the importance of sialidase in a different population.

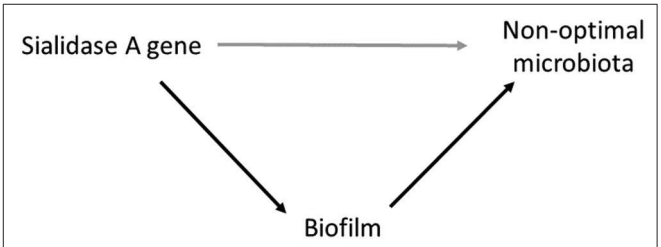

FIGURE 4 | Conceptual model illustrating the relationship between Sialidase A gene, non-optimal microbiota (Nugent 4–10) and biofilm in which biofilm lies on the causal pathway between the Sialidase A gene and non-optimal microbiota.

Our study has several limitations which call for caution in the interpretation of the results. Firstly, as noted above, there is evidence of underreporting of sexual activity. Furthermore, in the multivariable analyses of *G. vaginalis*, *A. vaginae and P. bivia*, we did not adjust each bacterium by the other two; therefore, it is possible that the associations with sexual activity is confounded by associations between the bacteria. To better answer the question of sexual acquisition of BV-associated bacteria, longitudinal studies are needed with validated behaviour measures and biomarkers for sexual activity. Secondly, we only tested a limited number of BV-associated bacteria by qPCR, and may have omitted a range of bacteria associated with enhanced vulnerability to HIV and other STIs (Eastment and Mcclelland, 2018). Our study did not test for sub-groups, clades or strains within *G. vaginosis* which may explain the difference in virulence (Vaneechoutte et al., 2019). Our study was also limited by FISH probes for *G. vaginalis* and *A. vaginae* only; future studies should investigate other species *in vivo* biofilm beyond *G. vaginalis* and *A. vaginae,* such as *P. bivia*, to better understand the interactions between vaginal microbiota. Importantly, while FISH is a useful method for identifying biofilm *in vivo* samples, the sensitivity is 66.7% (95% CI 54.5–77.1%) for *A. vaginae* 86.3% (95% CI: 77.4–92.2%) for *G. vaginalis* compared to qPCR (Hardy et al., 2015). To confirm the biofilm structure, future studies should complement the FISH analysis visualizing the bacteria constituting the biofilm with an assay detecting the extracellular matrix. Lastly, we tested samples for sialidase A gene, but did not measure sialidase production directly. While a previous study has shown that detection of sialidase A gene correlates very well with sialidase production (Lopes Dos Santos Santiago et al., 2011), another showed that some strains of *G. vaginalis* with sialidase A gene tested negative for sialidase production (Janulaitiene et al., 2018). We also did not investigate other virulence factors such as vaginolysin and others (Castro et al., 2019). A multi-omic approach would have provided a more comprehensive description of the interaction between the human host and microbiome, including identifying species/strains, genes, proteins, metabolites, and functional pathways.

In conclusion, this study among adolescent girls in Tanzania provides a snapshot of the vaginal microbiota around the time of expected sexual debut. These data challenge our understanding of *L. crispatus* prevalence among women with African ancestry, and confirms previous research showing a strong effect of sexual activity on the vaginal microbiota, although it remains unclear if this association is due to sexual transmission or sexual enhancement. This study also provides strong evidence for the role of *G. vaginalis* sialidase in biofilm formation and supports the conceptual model for the pathogenesis of BV that centres on the roles of virulent strains of *G. vaginalis*, as well as *P. bivia* and *A. vaginae*.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The parents of all girls aged 17 and 18 years in forms 1-3 were informed about the study and asked for their written informed consent for their daughter to participate in the study if she was less than 18 years old. After parental consent, girls who were less than 18 years were asked for their written informed assent. Girls who were 18 years old were asked for their written informed consent.

AUTHOR CONTRIBUTIONS

SF, AB, TC, AA, VJ, JC, KB, RH, and DW-J contributed to the conception and design of the study. DW-J, JI, CH, and AA carried out the study. TS and CH performed the statistical analysis. AB and SF wrote the first draft of the manuscript. TC and LH wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00305/full#supplementary-material

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REVIEW
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Bacterial Vaginosis: Current Diagnostic Avenues and Future Opportunities

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A healthy female genital tract harbors a microbiome dominated by lactic acid and hydrogen peroxide producing bacteria, which provide protection against infections by maintaining a low pH. Changes in the bacterial compositions of the vaginal microbiome can lead to bacterial vaginosis (BV), which is often associated with vaginal inflammation. Bacterial vaginosis increases the risk of acquiring sexually transmitted infections (STIs) like human immunodeficiency virus (HIV) and affects women's reproductive health negatively. In pregnant women, BV can lead to chorioamnionitis and adverse pregnancy outcomes, including preterm premature rupture of the membranes and preterm birth. In order to manage BV effectively, good diagnostic procedures are required. Traditionally clinical and microscopic methods have been used to diagnose BV; however, these methods require skilled staff and time and suffer from reduced sensitivity and specificity. New diagnostics, including highly sensitive and specific point-of-care (POC) tests, treatment modalities and vaccines can be developed based on the identification of biomarkers from the growing pool of vaginal microbiome and vaginal metabolome data. In this review the current and future diagnostic avenues will be discussed.

Keywords: bacterial vaginosis, female genital tract, diagnostics, vaginal microbiome, vaginal metabolome, vaginal inflammation

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INTRODUCTION

Bacterial vaginosis (BV), or vaginal dysbiosis, is one of the most common vaginal conditions associated with aberrant changes in the vaginal microbiome (VMB) (van de Wijgert et al., 2014). Bacterial vaginosis is characterized by a reduction of the resident lactic-acid producing *Lactobacillus* spp. and an overgrowth of anaerobic bacteria (Hillier et al., 1993; Fredricks et al., 2005; Ravel et al., 2013). This condition poses a major public health concern for women of reproductive age, for their offspring and for their partners as BV is associated with adverse reproductive health outcomes such as pelvic inflammatory disease, miscarriage, preterm birth and may also lead to an increased risk of human immunodeficiency virus (HIV) acquisition and transmission (Hay et al., 1994; Ness et al., 2005; Sha et al., 2005a; Atashili et al., 2008; van de Wijgert et al., 2008).

Bacterial vaginosis can be diagnosed clinically by using the Amsel's criteria (Amsel et al., 1983) and in the laboratory using the Nugent scoring system (Nugent et al., 1991). Amsel's criteria were published in 1983 for use in clinical settings and at least three of four criteria need to be met before being classified as BV positive (Amsel et al., 1983). The Nugent scoring system is a refined version

of the grading criteria introduced by Spiegel et al. (1983), where Gram-stained vaginal smears are evaluated for the presence/absence and quantity of specific bacterial morphotypes with a scoring system ranging from 0 to 10 (Nugent et al., 1991).

Although both methods have been widely used worldwide for almost three decades and are considered the "gold standard" of BV diagnosis, these methods are not free of limitations. For example, both methods are often subject to interobserver variability as the assessment of the diagnostic criteria depends on the observer's skill and experience (Klebanoff et al., 2004; Modak et al., 2011). Inaccurate BV results may lead to misdiagnosis and delays in treatment, which eventually puts women at risk for adverse reproductive health sequelae (Allsworth and Peipert, 2007; Modak et al., 2011). Misdiagnosis may also occur because of the unavailability of specific diagnostic tools in e.g., resource-limited settings and the deviation from strict diagnostic criteria by clinicians (Chavoustie et al., 2017). The complex etiology of BV contributes to inaccurate diagnosis, especially in asymptomatic carriers, and subsequently to poor treatment and clinical outcomes. The continuous development of accurate, easy-to-use point-of-care (POC) tests for BV is crucial, particularly in resource-limited settings.

The major drawbacks for the development of new diagnostic assays for BV are the lack of a uniform case definition for BV and that the etiology of BV remains poorly understood (Forsum et al., 2005; Muzny and Schwebke, 2016). In recent years, the advancement of molecular and high-throughput sequencing technologies [e.g., next-generation sequencing (NGS)] has revealed that BV is a multifactorial condition influenced by social, epidemiological, microbiological and host factors (Muzny and Schwebke, 2016; Muzny et al., 2020). Also, evidence suggests that the presence of a polymicrobial biofilm offers protection for many BV-associated bacteria against hydrogen peroxide (H_2O_2), antibiotics and host immunity (Swidsinski et al., 2005, 2008, 2015; Patterson et al., 2007) and these factors should be considered when designing a POC test. The focus of this review includes factors that complicate the diagnosis of BV, currently available diagnostic avenues and alternative approaches as potential diagnostic avenues for the diagnosis of BV.

FACTORS COMPLICATING THE DIAGNOSIS OF BV

The diagnosis of BV is complicated by the lack of consensus over what is defined as BV, the natural variation of the VMB in women of different racial backgrounds and the elusive polymicrobial etiology of BV. Several risk factors have been identified in the pathogenesis of BV, such as age, socioeconomical status, antibiotic usage, sexual behavior and ethnicity (Brumley, 2012; Singh et al., 2015; Ranjit et al., 2018). *Lactobacillus* bacteria are traditionally linked with a healthy VMB, but *L. iners* may be more pathogen than friend. These factors remain the reason that still no one method can accurately diagnose BV.

The Definition and Pathogenesis of BV

Historically, "non-specific bacterial vaginitis" was defined by Gardner and Dukes (1954) as an "infection" caused by a single etiological agent, *Haemophilus vaginalis* (now renamed as *Gardnerella vaginalis*), based on the observation that this bacterium was isolated from 92% (127/138) of women with "non-specific bacterial vaginitis." In the following year, the authors argued that the direct inoculation of *G. vaginalis* in women "free of the disease" resulted in clinical manifestations of BV and pure cultures of *G. vaginalis* could be recovered from these women (Gardner and Dukes, 1955). However, in the very same study, this argument was not true in the majority of the tested "disease-free" women (77%; 10/13), and positive cultures were not obtained from these women (Gardner and Dukes, 1955). In confirmation of these findings, studies based on molecular and culture techniques have shown that *G. vaginalis* can be found in the vaginal tract of sexually inexperienced women and of women without BV (Nugent score 0–3) (Aroutcheva et al., 2001; Fethers et al., 2012; Balashov et al., 2014; Schwebke et al., 2014a; Janulaitiene et al., 2017). Consequently, the "single etiological agent" theory was not widely accepted (Hickey and Forney, 2014).

To address the controversy of the "single etiological agent" concept, alternative definitions of BV suggested that this condition is of polymicrobial nature. There are several proposed polymicrobial hypotheses under debate, mainly to answer three questions: (i) whether BV is initiated by the establishment of an "early colonizer species" like virulent strains of *Gardnerella* spp. and *Prevotella bivia*, which create a favorable vaginal environment to facilitate the adherence and the growth of the "secondary colonizer" species like *Atopobium vaginae* or *Megasphaera* type I; (ii) whether BV is initiated by the introduction of polymicrobial communities of BV-associated bacteria; or (iii) whether the displacement of *Lactobacillus* spp. happens before the establishment of "early colonizer species" or polymicrobial BV communities (Srinivasan and Fredricks, 2008; Schwebke et al., 2014b; Muzny et al., 2018, 2019). In addition, it is also unclear whether BV is caused by *de novo* polymicrobial biofilm formation or by the transmission of polymicrobial biofilms through sexual activity (Verstraelen and Swidsinksi, 2019). While there is some evidence supporting each hypothesis, these hypotheses explaining the polymicrobial BV theory have not come to a consensus yet. In support of the first question above, a study by Pybus and Onderdonk (1997) suggested that during the onset of BV, *Gardnerella* spp. may provide a favorable environment by producing amino acids to promote the growth of other BV-associated bacteria (e.g., *P. bivia*). Muzny et al. (2018) showed that the mean relative abundance of four "key bacteria" (i.e., *P. bivia*, *Gardnerella* spp., *A. vaginae* and *Megasphaera* type I) is significantly increased zero to four days before the onset of BV, indicating their role during the initiation of BV. However, relative abundances of certain *Gardnerella* spp. are not always associated with BV as no significant association was found between relative abundances of *G. leopoldii* and Nugent scoring category by Hill et al. (2019). *Gardnerella leopoldii* is a recently-identified species within the genus *Gardnerella* and is part of 13 genomic species found within the genus through whole genome sequence analysis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis (Vaneechoutte et al., 2019). With regards to the second question above, Srinivasan

and Fredricks (2008) have hypothesized that BV is triggered when the polymicrobial communities of BV-associated bacteria are introduced into the vaginal environment, which may lead to simultaneous displacement of lactobacilli. In support of this hypothesis, there is evidence that some strains of BV-associated bacteria (*Bacteroides* spp., *Enterococcus faecium*, *G. vaginalis*, *Mobiluncus* spp., and *Peptostreptococcus* spp.) are capable of inhibiting the growth of lactobacilli (Nagy et al., 1991; Kelly et al., 2003). Furthermore, according to a study by Swidsinski et al. (2014), the polymicrobial community of BV-associated bacteria could be transmitted by means of a polymicrobial biofilm ("clue cells," characteristic vaginal epithelial cells coated with coccobacillary bacteria). This hypothesis, however, is contradicted by the studies that suggest other BV-associated bacteria are not as virulent as *Gardnerella* spp. in terms of cytotoxicity, adherence and biofilm formation (Patterson et al., 2010; Machado et al., 2013a; Alves et al., 2014).

Despite multiple molecular and genomic studies being conducted at species or microbiome level, there is no consensus on the group of bacterial species that may directly cause BV (Fethers et al., 2012; Srinivasan et al., 2012; Chen et al., 2018; Muzny et al., 2018). Srinivasan et al. (2012) performed broad-range 16S ribosomal RNA (rRNA) gene PCR and pyrosequencing to describe the composition and diversity of the VMB in women with BV, diagnosed either by Amsel's criteria or Nugent scoring. The results revealed that women with BV have a heterogeneous VMB that is not dominated by a single taxon and no species is universally present in all of BV-positive women (Srinivasan et al., 2012). It is supported by the findings of Chen et al. (2018) that significant differences in the bacterial composition of the BV microbiome were observed among BV groups defined by Amsel's criteria or Nugent scoring.

Vaginal inflammation is another controversial feature in BV. Some studies have reported that there was no statistically significant difference between any of the median concentrations of the pro-inflammatory cytokines [interleukin 6 (IL-6), IL-10, IL-12] tested for in BV-positive women vs. controls, and that high levels of prolidase and sialidase have led to the cleavage of the vaginal immunoglobulin A (IgA) and IgM (Cauci et al., 1998; Weissenbacher et al., 2010). A recent study using a mouse coinfection model also found that neither *Gardnerella* spp. or *Prevotella bivia* induced vaginal inflammation (Gilbert et al., 2019). In contrast, other studies have provided evidence that vaginal inflammation might well be present in BV and that specific BV-associated bacteria other than *Gardnerella* spp. and *Prevotella* spp. may induce proinflammatory responses (Sturm-Ramirez et al., 2000; Hedges et al., 2006; Nikolaitchouk et al., 2008; Anahtar et al., 2015; Jespers et al., 2017; Gardner et al., 2019; Gilbert et al., 2019). A few examples include a study by Sturm-Ramirez et al. (2000), in which women with BV had higher odds of having high levels of IL-1β or a tumor necrosis factor alpha (TNF-α) (adjusted odds ratio: 4.17; 95% confidence interval: 1.69–10.48; $p = 0.002$) and a study by Jespers et al. (2017), in which statistically significant increased concentrations of IL-12 were observed in women with incident BV. Similar observations were reported regarding other types of proinflammatory cytokines (IL-1α, IL-8, IL-36γ) (Pivarcsi et al., 2005; Nikolaitchouk et al., 2008; Gardner et al., 2019). Accordingly, a conceptual model was recently proposed by Muzny et al. (2020) in order to explain these observations. According to this model, epithelial cells and immune cells during a "healthy state" contribute to homeostasis by producing anti-inflammatory cytokines in response to low levels of cytokines produced by epithelial cells, whereas during early colonization by *Gardnerella* spp. and *Prevotella bivia*, sialidase is produced to induce a mucosal barrier disruption and allow the evasion of immune responses (Lewis et al., 2012; Gilbert et al., 2019; Muzny et al., 2020). Ultimately, secondary colonizers like *A. vaginae* and *Sneathia* spp. may join the polymicrobial biofilm and elicit the production of proinflammatory cytokines (Libby et al., 2008; Anahtar et al., 2015; Muzny et al., 2020). However, more research needs to be done to elucidate exact mechanisms of how the host-microbiota interactions contribute toward BV etiology.

Variation in the Vaginal Microbiome

Bacterial vaginosis has a complex pathogenesis and etiology and a disruption in the vaginal microflora is thought to be the main contributor to the altered vaginal environment and the associated clinical symptoms (Muzny and Schwebke, 2016). However, up to 50% to 75% of BV cases could be asymptomatic, complicating the diagnosis of BV and raising more questions on the etiology of this condition (Klebanoff et al., 2004; Coleman and Gaydos, 2018).

Traditionally, the vaginal environment of "healthy" women (referring to women who do not have adverse reproductive health outcomes and do not show clinical symptoms) were thought to be protected and maintained by hydrogen peroxide (H_2O_2)-producing *Lactobacillus* spp. *via* the production of H_2O_2, lactic acid and bacteriocins (Klebanoff et al., 1991; Zhou et al., 2004; O'Hanlon et al., 2011; Ravel et al., 2011). A lack or a decrease in the number of *Lactobacillus* spp. was therefore considered to indicate vaginal dysbiosis and this assertion formed the basis for the Nugent scoring system, which regards the lack of the *Lactobacillus* morphotype and the abundance of Gram-variable rods and cocci (*G. vaginalis* and *Bacteroides* spp. morphotypes) as BV (Nugent score of 7–10; Klebanoff et al., 1991; Nugent et al., 1991; Hillier et al., 1993). Indeed, a large cross-sectional study in the US by Ravel et al. (2011) showed that the VMB of 396 "healthy" women [representing four different ethnic groups (white, black, Hispanic, and Asian)] consisted of five "community state types" (CSTs), in which four CSTs were dominated by four *Lactobacillus* species each (*L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*) (72.2%; 286/396). However, women with more heterogeneous CST (dominated by strict BV-associated anaerobic bacteria such as *Prevotella* spp., *Dialister* spp., *Atopobium* spp., *Gardnerella* spp., *Megasphaera* spp., *Peptoniphilus* spp., *Sneathia* spp., *Eggerthella* spp., *Aerococcus* spp., *Finegoldia* spp., and *Mobiluncus* spp.) were asymptomatic and therefore thought to still maintain a "healthy" vaginal environment (Ravel et al., 2011). This type of VMB was also observed in several other studies (Zhou et al., 2004; Hyman et al., 2005; Fettweis et al., 2014; Gautam et al., 2015). The reason why women with this CST can still maintain "healthy" vaginal environments is yet unknown, but it is possible that anaerobic bacteria like *Atopobium* spp., *Leptotrichia* spp. and *Megasphaera* spp. may

substitute an ecological role in the absence of *Lactobacillus* spp. by e.g., producing lactic acid (Zhou et al., 2004). This substitution of an ecological role by anaerobic bacteria may partially explain why there is a high percentage of asymptomatic BV and may also contribute to the misdiagnosis of BV (Hickey et al., 2012). Therefore, with more information coming to light, the diagnosis of BV should not be focused on only the abundance of lactobacilli and other anaerobic bacteria.

The diagnosis of BV could be complicated by the fact that the vagina is a dynamic ecosystem that undergoes a natural fluctuation in the composition of the VMB throughout a woman's life, which is influenced by menstrual cycle, progesterone and estradiol levels, glycogen content in the vaginal epithelium, vaginal pH and immune responses (Brotman et al., 2010; Thoma et al., 2011; Gajer et al., 2012; Jespers et al., 2017; De Seta et al., 2019; Gliniewicz et al., 2019). At birth, it is believed that the VMB is established when the neonate is exposed to the vaginal tract of her mother during vaginal delivery or to the skin bacteria during Caesarian section (Dominguez-Bello et al., 2010). During this time, the VMB of the neonate delivered through the vaginal tract resembles that of her mother, dominated by either *Lactobacillus* spp., *Prevotella* spp. or *Sneathia* spp. (Dominguez-Bello et al., 2010). When the neonate gets older, the vaginal epithelium becomes thinner (i.e., contains a lower glycogen content) and the vaginal pH becomes neutral due to the decreased number of *Lactobacillus* spp. producing lactic acid (Farage and Maibach, 2006a). These physiological changes trigger the established VMB to undergo a compositional change from a *Lactobacillus* spp.-dominated VMB to a VMB dominated by strict anaerobes (e.g., *Bacteroides fragilis*) or enteric bacteria (e.g., *Escherichia coli*; Hammerschlag et al., 1978a,b). The vaginal pH and the number of *Lactobacillus* spp. seem to recover in adolescents and in women of reproductive age as the vaginal epithelium thickens (i.e., the glycogen level rises) under the estrogen influences (Eschenbach et al., 2000; Alvarez-Olmos et al., 2004; Farage and Maibach, 2006b). At this time (reproductive age), the VMB can undergo a natural fluctuation in composition during the menstrual cycle—some women may have a more resilient VMB while others have more extensive fluctuation over short periods of time (Gajer et al., 2012).

The role of ethnicity and race in affecting patterns of the vaginal microbiome have been considered because of the higher prevalence of BV in African-American and Mexican-American women (51.5 and 32.1%, respectively) in comparison to white, non-Hispanic women (23.2%; Alcendor, 2016). Some women (especially of African ethnicity) may also harbor a more heterogeneous VMB consisting of strict anaerobes like *Anaerococcus* spp., *Atopobium* spp., *Corynebacterium* spp., *Finegoldia* spp., *Gardnerella* spp., *Megasphaera* spp., *Prevotella* spp. and *Streptococcus* spp., along with *Lactobacillus* spp. (Zhou et al., 2007; Gajer et al., 2012; Fettweis et al., 2014). This type of VMB is similar to the VMB observed in postmenopausal women, in which the numbers of *Lactobacillus* spp. are reduced but those of strict anaerobes like *Anaerococcus* spp., *Atopobium* spp., *Finegoldia* spp., *Gardnerella* spp., *Prevotella* spp. and *Streptococcus* spp. are increased (Brotman et al., 2018; Gliniewicz et al., 2019).

The Role of *Lactobacillus* Iners in the Diagnosis of BV

The VMB is typically characterized by the dominance of a single or a few *Lactobacillus* species such as *L. crispatus, L. gasseri, L. jensenii, L. iners* and to a lesser extent species such as *L. acidophilus, L. brevis, L. delbrueckii, L. fermentum, L. mucosae, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus* and *L. vaginalis* are also VMB colonizers (Antonio et al., 1999; Pavlova et al., 2002; Tärnberg et al., 2002; Ravel et al., 2011). If characterization of the VMB is considered as part of the diagnostic criteria for BV, understanding the relevance and functional role of different *Lactobacillus* species is needed. The identification of different types of lactobacilli in the VMB may be relevant to understand the stability of the VMB and the host's susceptibility to pathogens (Spear et al., 2011). *Lactobacillus iners* has been detected in the VMB of both BV positive and BV negative women, raising questions about the role of *L. iners* in the etiology of BV (Macklaim et al., 2011; Petrova et al., 2017). While the detection of *G. vaginalis* and *A. vaginae* have received the most attention in the etiology of BV, *L. iners* has been suggested as an unusual suspect in the etiology of BV and might play a critical role in the diagnosis of BV (Vaneechoutte, 2017).

The predominance of *L. iners* in an intermediate VMB or in symptomatic and asymptomatic BV might indicate its role in homeostasis and in promoting a lactobacilli-dominated microbial community in an altered vaginal environment (Ferris et al., 2004; Jakobsson and Forsum, 2007; Lambert et al., 2013). High levels of *L. iners* in BV may also refer to its genetic composition enabling optimal adaptation and survival in altered vaginal environments (Petrova et al., 2017). For instance, functional analysis in cases of BV revealed the expression of genes for the breakdown of glycogen, mannose and maltose (Macklaim et al., 2013). Shipitsyna et al. (2013) highlighted the importance of *L. iners* after finding *L. iners* and *G. vaginalis* as the predominant species in intermediate cases of BV. Another finding in the same study was the decline in the abundance of *L. iners* in cases of BV as opposed to levels in healthy women. The depletion of the abundance of *L. iners* together with the possibility that the predominance of *L. iners* may shift an intermediate VMB further toward dysbiosis contribute to the value of *L. iners* in the diagnosis of BV (Verstraelen et al., 2009; Petrova et al., 2017).

In the study by Shipitsyna et al. (2013), the qualitative detection of *L. iners* (negative result) was determined to have a low sensitivity in the prediction of BV (7% sensitivity and 86% specificity) with also little discriminatory power between positive and negative BV cases. Microscopy techniques such as Gram-staining may also lead to false negative results due to different isolates of *L. iners* presenting with different bacterial morphologies than that previously described (De Backer et al., 2007; Petrova et al., 2017). Thus, far, the most reliable method to include *L. iners* in the diagnosis of BV would be a quantitative method, including sequencing of the 16S rRNA gene that has previously enabled a comparison of prevalence, bacterial load and relative abundance of BV associated bacteria (Shipitsyna et al., 2013). Together with the relative abundance of *L. iners,* different ethnic groups with known differences in the abundance of *L. iners* in the VMB should also be considered when BV

is suspected. Especially in cases involving Black African and African-American women where *L. iners* has been strongly associated with symptomatic and asymptomatic cases of BV (Ravel et al., 2011; Jespers et al., 2012; Srinivasan et al., 2012; Mitchell et al., 2013; Vaneechoutte, 2017).

CURRENT DIAGNOSTIC AVENUES

Bacterial vaginosis is clinically characterized by Amsel's criteria or laboratory diagnosed by the Nugent score (Bautista et al., 2017). Clinical laboratories identify changes in the vaginal environment through microscopic examination and vaginal swab culture (Hong et al., 2016). Nugent scoring involves Gram-staining of vaginal smears and has been suggested as the gold standard in the diagnosis of BV in comparison to Amsel's criteria, which is based on non-quantifiable, non-reproducible clinical symptoms only (Chawla et al., 2013; Amegashie et al., 2017; Antonucci et al., 2017; Coleman and Gaydos, 2018). The diagnosis of BV is made on quantification of the Gram-stained microorganisms, classifying these organisms based on different vaginal morphotypes as well as the identification of clue cells (part of the Amsel's criteria) which could be laborious and requiresskilled personnel (Nugent et al., 1991; Chawla et al., 2013; Antonucci et al., 2017).

Amsel's criteria involves saline microscopy and has been improved over time to include the presence of a thin watery homogenous discharge, elevated vaginal pH (>4.5), the presence of more than 20% of clue cells (vaginal epithelial cells) and a fishy odor after the addition of 10% potassium hydroxide to vaginal secretions ("whiff test") for a positive BV diagnosis (Amsel et al., 1983; Eschenbach et al., 1988; Mohammadzadeh et al., 2014). In comparison to Nugent scoring, the sensitivity and specificity for Amsel's criteria ranges from 37% to 70% and 94% to 99%, respectively and with moderate reproducibility (Schwebke et al., 1996; Sha et al., 2005b). Previous studies determined that 37% to 54% of women with an intermediate Nugent scoring had BV according to the Amsel's criteria (Taylor-Robinson et al., 2003; Bradshaw et al., 2005). A combination of Amsel's criteria and Nugent scoring may be beneficial for an accurate diagnosis of BV due to an assessment on both clinical symptoms and microbial morphology.

Another diagnostic method based on Gram-stained vaginal smears, the Ison-Hay classification criteria was described in 2002, which allows simplified grading and characterization of the vaginal microflora based on the amount of lactobacilli morphotypes compared to *Gardnerella* spp. morphotypes (Ison and Hay, 2002; Chawla et al., 2013). A vaginal microflora with a Nugent score of 4 to 6, known as an intermediate score or intermediate microflora is classified in grade II by the Ison-Hay criteria (Amegashie et al., 2017; Antonucci et al., 2017). An intermediate microflora was initially thought as a transitional step between a normal vaginal microflora and BV, or *vice versa*, but remains an uncharacterized category and is a challenge in the diagnosis of BV due to unknown clinical implications (Verhelst et al., 2005; Menard et al., 2008). Microscopy may be desired by some clinicians above other laboratory tests such as molecular assays due to a shorter turn-around time; however, the identification of different morphotypes is subjective and diagnosis may be influenced by individual skills and experience (Chawla et al., 2013; Antonucci et al., 2017). The involvement of species such as *A. vaginae, Ureaplasma* spp. and *Mycoplasma* spp., that cannot be detected by using Gram staining techniques or Nugent score, subsequently decreases the sensitivity of the Nugent score and warrants the need of a confirmatory or an additional molecular test to measure other etiological agents in the diagnosis of BV (Menard et al., 2008; Haggerty et al., 2009).

Several POC diagnostic assays exist to diagnose BV, such as saline microscopy, wet mount microscopy, chromogenic tests such as the OSOM® BVBlue® and the VGTest™ ion motility spectrometry (IMS). The VGTest™ IMS determines the levels of the malodorous biogenic amines associated with BV, whereas the OSOM® BVBlue® test detects elevated levels of the sialidase enzyme to diagnose BV (Madhivanan et al., 2014; Blankenstein et al., 2015). Rapid assays detecting the presence of proline amino peptidase in BV have also demonstrated high levels of specificity and sensitivity (Madhivanan et al., 2014). The combination of testing for vaginal pH and the whiff test is also suggested as a simple and inexpensive POC test for especially resource-limited settings (Madhivanan et al., 2009). The development of nucleic acid amplification tests (NAATs) such as the BD Affirm™ VPIII test can identify and differentiate between organisms associated with vaginitis and should be investigated to be used as a POC test (Cartwright et al., 2013).

Problem of Syndromic Management in Resource-Limited Settings

Syndromic management is based on the identification of a combination of symptoms presented during a clinical examination (Shrivastava et al., 2014). These symptoms may be easily recognized signs associated with infection and known bacterial pathogens (Shrivastava et al., 2014). A diagnosis is made by a healthcare provider within a short time, without sophisticated skills and laboratory tests (Altini, 2006). Syndromic management of sexually transmitted infections (STIs) and BV has its benefits, especially in resource-limited settings where invasive procedures and laboratory tests are not available. These benefits include the amendment of immediate treatment on the patient's first visit to a clinic, (reducing further transmission of the disorder), it's widely accessible, education is provided, and no laboratory tests are needed (World Health Organization, 2007; Shrivastava et al., 2014). The success of syndromic management relies on accurate information including the sexual history of the patient and a thorough clinical examination (National Department of Health (South Africa), 2015). Bacterial vaginosis is diagnosed based on vaginal discharge syndrome (VDS) and is treated with a stat dose of metronidazole (National Department of Health (South Africa), 2015).

Despite the advantages that have been reported with syndromic management of STIs and BV, the shortfall of this approach includes not detecting asymptomatic STIs (that may be under treatment) and a poor positive predictive value where antibiotic susceptibility testing is not available, possibly

resulting in the overuse of antibiotics (Garrett et al., 2018). In resource-limited settings, there are limited opportunities for routine surveillance, complicating the treatment and resolution of infections. Deviation from standard management guidelines by healthcare workers and the habit of relying on their own clinical judgement also contributes to insufficient treatment of vaginal infections (Leitich et al., 2003; Tann et al., 2006).

Syndromic management may lead to the misdiagnosis of BV and treatment failure and high rates of recurrencemay contribute to antibiotic resistance (Bostwick et al., 2016). A study conducted in South Africa investigating the prevalence of asymptomatic BV amongst a group of HIV unaffected pregnant women observed that 43% of BV positive (severe BV as indicated by a Nugent score of 9–10) women were asymptomatic (Joyisa et al., 2019). The high number of asymptomatic BV cases complicates syndromic management. Diagnosis and effective treatment of BV in pregnant women are warranted to avoid the development of pregnancy complications as well as the acquisition of HIV and other STIs. To diagnose and treat asymptomatic BV, low-cost but sensitive and specific diagnostic models for resource-limited countries are needed in addition to a proposed shift from syndromic to diagnostic management (Shrivastava et al., 2014; Garrett et al., 2018).

ALTERNATIVE APPROACHES AS POTENTIAL DIAGNOSTIC AVENUES FOR THE DIAGNOSIS OF BV

Rapid and accurate molecular methods targeting the nucleic acid have changed the diagnosis of BV and also provided new insight into our understanding of BV. The report that *Gardnerella vaginalis* is not just one species and does not only comprise one strain type but that several genomic species can be distinguished in the *Gardnerella* genus (Vaneechoutte et al., 2019) has emphasized how limited our understanding of bacterial vaginosis is and how the use of current and advanced technologies will shape our understanding of this condition in the future. Next generation technologies, including genomics, metabolomics, proteomics and immunomics shed light on the functional and immune processes during healthy, intermediate and BV states, that can ultimately contribute to the development of diagnostic tests, treatment and prevention strategies. The fourth industrial revolution brings about artificial intelligence and the possibility of diagnosing complex syndromes, like BV, using machine learning algorithms.

Molecular Diagnostic Methods

Given the limitations of Amsel's clinical criteria and microscopy for the diagnosis of BV, the development of molecular assays is an attractive approach for the diagnosis of BV because of the ability to identify and quantify multiple fastidious microorganisms (Adzitey et al., 2013). As described by Coleman and Gaydos (2018), an example of a molecular technique used in the diagnosis of BV is the direct probe assay (BD Affirm^{TM} VPIII probe assay) that is commercially available (Becton Dickinson, Sparks, MD) and the Bacterial Vaginosis/Vaginitis panel developed by Quest diagnostics. Direct probe assays are rapid and can detect multiple indicator organisms in a single sample but may require high concentrations of the targeted bacteria for a reliable result. The detection of only *G. vaginalis* in direct probe assays is not a specific marker for BV (thus cannot be used to diagnose BV), as this species may be present in healthy women. The specific target used in these probe assays should be verified as 13 genomic species have been identified in the genus *Gardnerella* (Vaneechoutte et al., 2019). Other clinical indicators such as Amsel's criteria could be used in conjunction with probe assays to improve diagnostic accuracy and ensure reliable diagnosis (Spiegel, 1991; Fredricks et al., 2005; Zozaya-Hinchliffe et al., 2010; Janulaitiene et al., 2017). Direct probe assays designed for the detection of *G. vaginalis* might be more specific for the diagnosis of BV in symptomatic patients than in asymptomatic patients. A sensitivity and a specificity of 90% and 97%, respectively have been reported when compared to microscopy and Nugent scoring (94% and 81%, respectively; Coleman and Gaydos, 2018). Nucleic acid amplification tests are another molecular approach that is proven to have a higher diagnostic accuracy (20% to 25%) over direct probe assays, especially in populations with varying prevalence of infective vaginitis (Cartwright et al., 2013). Commercially available NAATs as indicated in **Table 1** include the NuSwab® quantitative multiplex PCR assay, SureSwab BV DNA quantitative real-time PCR assay, BD Max vaginal panel and the BV multiplex assay with sensitivity ranging from 90.5% to 96.7% (the latter for symptomatic women) and specificity from 85.8% to 95% compared to Amsel's criteria and Nugent score as previously reported in detail by Coleman and Gaydos (2018). **Figure 1** summarizes the main diagnostic tests and/or approaches that are currently available in the diagnostic arena for BV detection and approaches that could be developed for wider use in BV diagnostics.

Nucleic acid amplification tests are dependent on the selection of specific indicator organisms; however, multiplexed NAATs allow the detection of multiple indicator organisms in a single NAAT reaction (Adzitey et al., 2013). Not only is the selection of indicator organisms, but also the inclusion of the quantitative value that would indicate an accurate BV diagnosis, a challenge. Indicator species such as *G. vaginalis* and *A. vaginae* can also be commensals of a healthy VMB (Zozaya-Hinchliffe et al., 2010; Redelinghuys et al., 2017). The value of molecular quantification in the diagnosis of BV was investigated by Menard et al. (2008) who, similar to other studies, investigated the bacterial load to determine cut-off values for both *G. vaginalis* and *A. vaginae* (Wang and Su, 2014; Redelinghuys et al., 2017). Molecular quantification also enabled the characterization of an intermediate microflora corresponding to a BV-type vaginal microflora with high levels of *G. vaginalis* and *A. vaginae* (Bradshaw et al., 2006; Menard et al., 2008). Cut-off values for *G. vaginalis* and *A. vaginae* might improve BV diagnosis using molecular techniques; however, previous attempts to determine cut-off values most specific and sensitive for the diagnosis of BV have either taken individual species into consideration or a combination of the two species because of the suggested synergism (Redelinghuys et al., 2017). Consensus has not been reached due to variables such as

TABLE 1 | Commercially-available tests for BV, with diagnostic capability and markers of detection.

Test and manufacturer	Quantitative or qualitative	BV markers
NuSwab® (LabCorp, Burlington, NC)	Semi-quantitative; Qualitative	*Atopobium vaginae,* BVAB2, *Megasphaera-1, L. crispatus* (Cartwright et al., 2012)
SureSwab® real-time PCR assay (Quest diagnostics, Secaucus, NJ)	Quantitative; Qualitative	*Lactobacillus* spp., *Atopobium vaginae, Megasphaera* spp., *Gardnerella vaginalis*
BD MAX™ real-time PCR assay (Becton, Dickinson and company, Franklin Lakes, NJ)	Quantitative; Qualitative	BV Candida group (*C. albicans, C. tropicalis, C. parapsilosis*, and C. dubliniensis), *C. glabrata* or *C. krusei*, and *Trichomonas vaginalis*, *Lactobacillus* spp. (*L. crispatus, L. jensenii*, *Atopobium vaginae, Megasphaera* spp., *Gardnerella vaginalis,* BVAB2 (Gaydos et al., 2017)
BV multiplex assay (Medical Diagnostic Laboratories, Hamilton Township, NJ)	Quantitative; Qualitative	*Lactobacillus* profiling *Atopobium vaginae, Megasphaera* phylotypes 1 and 2, *Gardnerella vaginalis,* BVAB2 (Hilbert et al., 2016)
OSOM® BVBlue® assay (Gryphus Diagnostics, LLC, Knoxville, TN)	Qualitative	Sialidase enzyme activity (Myziuk et al., 2003)
FemExam® card (Litmus Concepts, Santa Clara, CA)	Qualitative	Vaginal pH and amine activity (West et al., 2003)
IMMULITE® immunoassay (Siemens Healthcare, Erlangen, Germany)	Quantitative; Qualitative	Levels of cytokines: TNF-α, IL-6, IP-10 (Zirath et al., 2017)
QuickLine immunoassay (Milenia Biotec, Gießen, Germany)	Quantitative; Qualitative	Levels of cytokines: IL-6, TNF-α (Kunze et al., 2016)

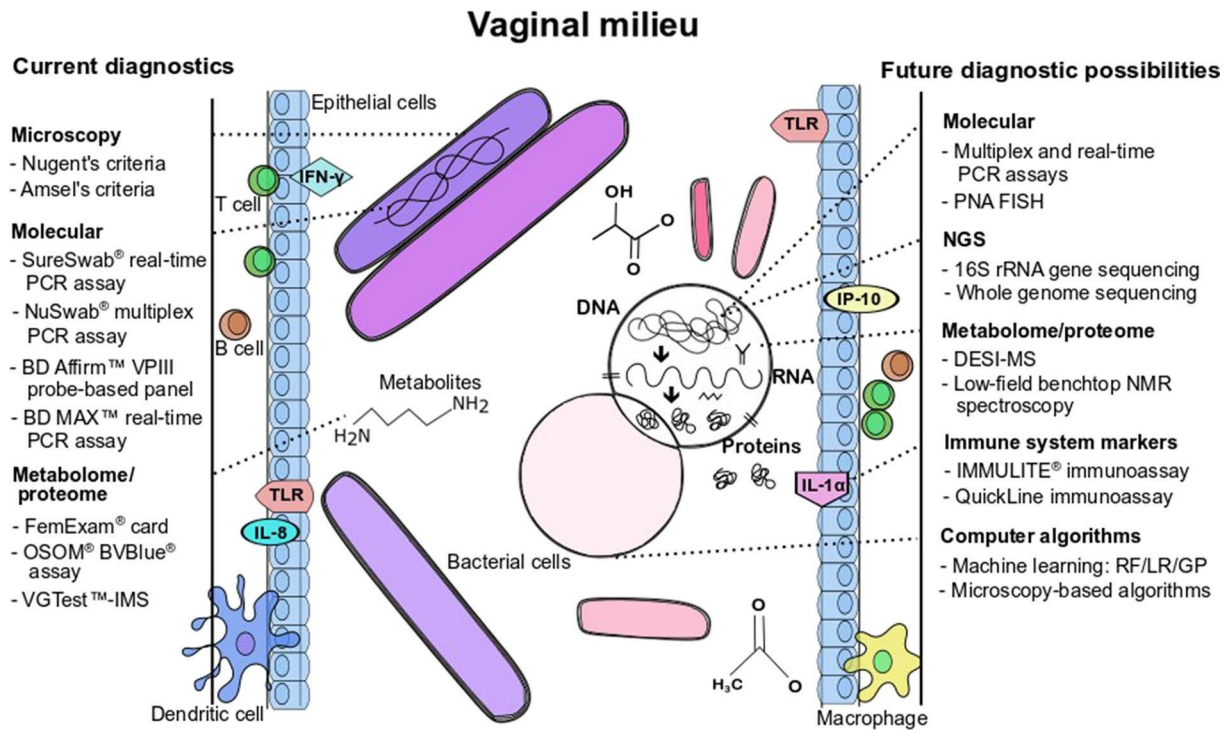

FIGURE 1 | Current diagnostics and future possibilities for BV detection and/or BV pathogenesis characterization. Figure annotations in acronym or abbreviated form are: DESI-MS, desorption electrospray ionization-mass spectrometry; GP, genetic programming; IFN, interferon; IL, interleukin; LR, logistic regression; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PNA-FISH, peptide nucleic acid fluorescence *in situ* hybridization; RF, random forest; TLR, toll-like receptor; VGTest-IMS, VGTest-ion mobility spectrometry.

different populations, study design and co-infection status such as HIV and pregnancy. As suggested by Redelinghuys et al. (2017), before a potential cut-off value can be used, it should first be validated with additional sets of data and in different study populations.

Commercially available molecular diagnostic panels for the diagnosis of BV include the detection of the *Lactobacillus* genus, but do not allow an extensive differentiation of species. Current diagnostic panels only allow differentiation of *L. crispatus* and *L. jensenii* (**Table 1**), but other studies have shown that *L.*

vaginalis and *L. iners* also have been found in a population of African women with a low Nugent score (Jespers et al., 2015). In another study *L. vaginalis* was detected in 8% of Belgian women with BV (Jespers et al., 2012). Different species of the *Lactobacillus* genus interact differently with the vaginal environment, which may suggest that each species may contribute to the development of BV through a different mechanism (Jespers et al., 2015).

A VMB dominated by *L. iners* has been characterized as an unstable, transitional VMB and associated with vaginal dysbiosis (Borgdorff et al., 2016; Amabebe and Anumba, 2018; van Houdt et al., 2018). Janulaitiene et al. (2017) found that a healthy VMB was dominated by multiple *Lactobacillus* species, in contrast to BV positive samples where *L. iners* was the most frequently detected species, alone or in combination with *L. crispatus*. Other findings indicated that this particular species of *Lactobacillus* has been found in women with and without BV, indicating the challenge of using *L. iners* as a marker of BV (Zozaya-Hinchliffe et al., 2010).

The same concept may apply to *G. vaginalis* as marker of BV which has four different clades that each possess unique genetic markers and virulence factors (Ahmed et al., 2012). Clades 1 and 3 have been found more commonly among women with BV in contrast to clade 2, which has been associated with an intermediate vaginal microflora (Balashov et al., 2014; Coleman and Gaydos, 2018). Multiple clades have also been associated with BV, suggesting that a polyclonal *G. vaginalis* might be a risk for BV (Balashov et al., 2014). Genotypic differences between *G. vaginalis* strains could explain different clinical phenotypes such as with asymptomatic BV or the detection of *G. vaginalis* in healthy women (Ahmed et al., 2012). It is also of importance to take into consideration that carriers with a dense colonization of *G. vaginalis*, as seen with a high Nugent score, may remain asymptomatic for BV (Nugent et al., 1991; Ahmed et al., 2012). Due to the challenges of using a single species as a marker of BV and the shortcomings of using microscopy for identification of the VMB in BV, characterization of the VMB and its diversity may play an important role in the diagnosis of BV.

A study utilizing 16S rRNA PCR with clone analysis identified greater bacterial diversity in women with BV in contrast to women without BV, which is expected because of complete *Lactobacillus* dominance in a healthy VMB (Fredricks et al., 2005). This molecular technique enabled the identification of 35 unique bacterial species in women with BV by using the bacterium specific 16S rRNA gene (Fredricks et al., 2005). The question remains as to what the clinical significance of the identified species is, because species such as *Atopobium vaginae* and *Gardnerella vaginalis,* were more frequently detected in women with BV while other bacteria such as *Peptostreptococcus* spp., were less frequently detected in BV (Fredricks et al., 2005).

Molecular techniques utilizing the 16S rRNA gene for taxonomic identification also led to the identification of unculturable and fastidious microorganisms such as BV-associated bacterium 1 (BVAB1), BVAB2, and BVAB3 that were found to be specific indicators for BV (Fredricks et al., 2005). This technique also enabled a better understanding of bacterial morphotypes identified using Gram stain analysis (Srinivasan et al., 2013). Molecular amplification and sequencing of the 16S rRNA gene has its limitations, including the introduction of potential bias by broad-range amplification of the bacterial 16S rRNA gene (Winsley et al., 2012; Twin et al., 2013). Differences in taxa between studies can also be attributed to different sequencing approaches and to universal PCR primer mismatches to certain bacteria such as *Bifidobacterium* and *G. vaginalis* (Verhelst et al., 2004). Although the development of molecular techniques has revealed more bacterial species that form part of the pathophysiology of BV, threshold values and the appropriate number of marker bacterial species included on these panels to remain sensitive, specific and cost effective remain to be determined.

The Potential of High-Throughput Next Generation Sequencing (NGS) for the Diagnosis of BV

Development of a high-throughput technology such as next generation sequencing (NGS) allows the identification of multiple microorganisms that may be present in low abundance in a clinical sample (Hong et al., 2016). Traditional molecular approaches such as targeted Sanger sequencing of the 16S rRNA gene has enabled clinical identification of microorganisms in a polymicrobial sample when compared with traditional culturing methods (Salipante et al., 2013). However, high-throughput sequencing technologies such as targeted metagenomics on an NGS platform have the advantage of simplified identification of multiple microorganisms in a single sequencing approach, compared to interpreting multiple superimposed Sanger sequencing reads (Salipante et al., 2013).

To date, NGS techniques have not been extensively applied in the diagnosis of BV and therefore this section will mainly focus on the potential of the application in the diagnosis and treatment of BV. Next generation sequencing continues to improve the understanding of the complex pathology of BV by enabling the complete and accurate characterization of the diverse VMB associated with BV (Budd et al., 2015). Metagenomic analysis have indicated that bacterial communities associated with BV are highly diverse, either dominated by *G. vaginalis, L. iners* and/or combinations of anaerobic bacteria with or without *Lactobacillus* species in varying proportions (McKinnon et al., 2019). Deep sequencing of the bacterial 16S rRNA gene enabled multiple new bacterial associations with BV, providing new information on the possible role of *Atopobium vaginae* as indicator organism (Fredricks, 2011; Srinivasan et al., 2012; van de Wijgert et al., 2014).

NGS techniques can provide information on the relative proportion and absolute number of all incident bacterial species in the VMB (Ravel et al., 2011; Bostwick et al., 2016). The targeted NGS approach could identify mixed vaginal infections associated with BV and enables a comprehensive investigation of the VMB including the involvement of antibiotic resistance genes (Mullany, 2014). Targeted NGS has been evaluated by Bostwick et al. (2016) as an approach to diagnose and manage BV in clinical practice. The 16S rRNA sequencing approach was evaluated in a case of recurrent BV, where treatment with metronidazole was

unsuccessful and a VMB with 56% of anaerobes was identified. In a second case, the patient immediately received treatment with metronidazole based on a positive diagnosis based on Amsel's criteria. The patient developed recurrent BV symptoms with a mixed BV VMB, a *Candida albicans* co-infection and antibiotic resistance. This patient had a VMB dominated by *L. crispatus* and *L. iners*. The use of broad-spectrum antibiotic treatment can induce shifts in the VMB from a healthy VMB often to a *L. iners* transitional VMB (Mayer et al., 2015). In the third clinical case, NGS results indicated a VMB composed of BVAB, *L. iners* and mixed anaerobic bacteria in a patient that was BV positive by Amsel's criteria and with no *G. vaginalis* present. The possibility of mixed bacterial infection is important to consider with the diagnosis of BV. With the NGS approach, the re-emergence of bacterial infection can also be studied, which may aid in the understanding of recurrent BV (Mayer et al., 2015; Bostwick et al., 2016).

Commercial tests that employ cost-effective, short-read 16S rRNA sequencing include the Ion Torrent 16S™ Metagenomics kit (Thermo Fisher scientific, Waltham, MA, USA) in comparison to the Illumina 16S rRNA metagenomic sequencing (Illumina, San Diego, USA), which has been widely used for research purposes (Malla et al., 2019). Next-generation 16S rRNA sequencing has not been utilized in clinical microbiology practice in resource-limited laboratories because of sequencing cost, procedural challenges to prepare the sequencing libraries and the complexity of analysis (Salipante et al., 2013). Various laboratories have evaluated 16S rRNA sequencing for diagnostic purposes and developed analysis pipelines to characterize multiple hypervariable regions of the 16S rRNA gene (Barb et al., 2016; Watts et al., 2017; Culbreath et al., 2019). However, algorithms specifically for the diagnosis of BV is not yet commercially available. Similar to quantitative methods, the cut-off percentage of bacterial abundance related to clinical significance should also be determined. Standardization is needed in terms of the terminology of microbial communities, the methodologies to describe them, and standardization across sample site should also be considered. Targeted sequencing on the NGS platform has similar shortcomings as the 16S rRNA NAATs described earlier. To overcome limited identification of bacteria on species level, long-amplicon PCR-based approaches of the full 16S rRNA gene (∼1,500 base pairs) with the Oxford Nanopore Technologies can be considered for accurate characterization of microbial communities and analysis of antibiotic resistance gene islands (Cusc et al., 2018; Malla et al., 2019).

As an alternative to 16S rRNA sequencing, whole genome sequencing (WGS) is another approach to investigate for the diagnosis and management of BV. Whole genome sequencing has the potential to generate a large amount of data from a single isolate, including species, strain type, virulence, antibiotic resistance and other information for outbreak and case management (Besser et al., 2018). Whole genome sequencing has been suggested to be used for microbial epidemiology, the surveillance of pathogens and outbreaks and for the prediction of antibiotic resistance (Jackson et al., 2019). Whole genome sequencing or targeted metagenomics (16S rRNA) might be useful for the diagnosis of BV in clinical cases where an intermediate VMB has been identified [Nugent score finding (score 4–6)]. As an intermediate VMB is proposed to represent a transitional state of the VMB to BV or *vice versa*, NGS techniques can be used to monitor slight changes in terms of abundance and composition of the VMB. The development of NGS technologies is ongoing and is suggested to be highly applicable in the future, where personalized medicine approaches will be possible for the treatment of specific bacterial infections (Punina et al., 2015; Jackson et al., 2019). The personalized medicine approach might also be applicable in BV because of a lack of consensus on the structure and composition of the VMB in this complex condition.

Metabolomics and Proteomics

Proteomics is the study of the collection of proteins produced by the host and microbiome that embody the functional activity of the entire bionetwork (Peters et al., 2019). Metabolomics is the study of low molecular weight compounds (<1,500 Da) that are produced as substrates or by-products of enzymatic reactions in response to stimuli in a biological system (Peters et al., 2019). Compared to other components in an ecosystem, the metabolome may be the component that best represent the phenotype of that ecosystem (Guijas et al., 2018). Nonetheless, both proteomics and metabolomics are useful in studying host-microbiome interactions on a functional level and could be used to elucidate the pathogenesis of disease (Peters et al., 2019). Newer, high-throughput technologies have created the opportunity to screen and study metabolites and proteins on a large scale. High-throughput mass spectrometry (MS) and nuclear magnetic resonance (NMR) techniques have been used to explore the vaginal milieu under BV conditions and identify proteins or metabolites as biomarkers of this condition (Yeoman et al., 2013; McMillan et al., 2015; Ferreira et al., 2018; Parolin et al., 2018).

Although characteristic for its polymicrobial nature, the BV microbiome is typically associated with the production of specific metabolites, such as cadaverine, putrescine, tyramine and succinate, which have been linked to the "fishy" or amine odor and increased vaginal pH characteristic of BV (Yeoman et al., 2013; McMillan et al., 2015; Pruski et al., 2017). Lactic acid, for example, forms part of the metabolome signature of a healthy VMB (Stafford et al., 2017). Information on the proteome of cervical-vaginal fluid (CVF) is limited, but the protein content of CVF has been shown to differ between a BV microbiome and a healthy VMB (Zegels et al., 2009; Cruciani et al., 2013; Ferreira et al., 2018). Proteins associated with the immune response are either exclusive to BV or show elevated levels of expression (Cruciani et al., 2013; Ferreira et al., 2018). Distinctive metabolites and proteins could therefore be used as biomarkers of a healthy or BV microbiome, irrespective of the dominant bacterial species. A factor which complicates the diagnostic value of metabolomics and proteomics is that it could prove difficult to link the metabolites to the species producing them, which may or may not be essential for treatment purposes.

The evolution of diagnostics research has seen the field shift to evaluating a variety of combination criteria for the detection of BV. Laghi et al. (2014) investigated the microbiome and metabolome of women affected by BV. In a group of women

treated with a placebo, this study found that high ratios of lactate to BV-associated metabolites and lactobacilli to BV-associated bacteria were likely to experience spontaneous remission in the absence of treatment, indicating that these may not have been true cases of BV. Another study suggested the coupling of acetate, malonate and nicotinate, quantified with proton (^{1}H) NMR, with the VMB species *Atopobium* spp., *M. hominis* and *Prevotella* spp., quantified with qPCR, as diagnostic criteria for BV (Vitali et al., 2015). With the aid of liquid chromatography mass spectrometry (LC-MS), McMillan et al. (2015) have found that higher levels of 2-hydroxyisovalerate (2 HV) and gamma-hydroxy-butyrate (GHB), and reduced levels of lactate and tyrosine, were sensitive and specific for BV. In a validation cohort of 45 pregnant women, 2 HV: tyrosine ratios proved to be the most specific (94%) and sensitive (89%) for BV diagnosis (AUC = 0.946). The combination of a vaginal pH level of more than 4.5 with the detection of specific polyamines has been proposed as diagnostic criteria for BV diagnosis and to assess whether treatment is necessary (Watson and Reid, 2018). The use of high-throughput technologies to screen for biomarkers is still in its infancy (mostly regarding the ease of data analysis) and most of the proposed biomarkers have yet to be validated in different resource-limited settings where they could be implemented as POC tests for BV.

The number of available POC tests that have metabolites or proteins incorporated is limited. The commercial OSOM® BVBlue® test (Gryphus Diagnostics, LLC, Knoxville, TN) is a chromogenic rapid diagnostic test that is based on the detection of activity of the sialidase enzyme, an enzyme that is produced by BV-related bacteria such as *Bacteroides* spp. (Myziuk et al., 2003). Compared to Nugent's criteria, this test has a sensitivity and specificity of around 90% and 96%, respectively; compared to the Amsel criteria the sensitivity and specificity of this test range between 50% and 88%, and between 91% and 100%, respectively (Myziuk et al., 2003; Bradshaw et al., 2005). Although it is reported that a statistically significant correlation exists between a positive BVBlue® test and a raised vaginal pH, it has been found that combining these two criteria for the diagnosis of BV might improve either the sensitivity or specificity of the BVBlue® test, but at the same time could impair the alternative measure (Bradshaw et al., 2005). Another POC test, the FemExam® (Litmus Concepts, Santa Clara, CA) test, is similar to the Amsel's criteria as it measures vaginal pH and amine activity (West et al., 2003). The test is based on a two-card system where card 1 measures vaginal pH and the presence of trimethylamine, where card 2 measures the activity of proline iminopeptidase in *G. vaginalis* (West et al., 2003). West et al. (2003) reported a sensitivity of 91% and a specificity of 62% when the combined test results of the two cards were compared to Nugent's criteria. Both the BVBlue® and FemExam® tests are rapid, easy to perform and objective in comparison to the Amsel and Nugent's criteria. The sensitivity and specificity of these POC tests may be affected or compromised by the polymicrobial nature of BV and the wide spectrum of metabolites and proteins associated with its microbiome. Also, different study populations may harbor varying ratios of species to metabolites and/or proteins, which may or may not be produced in different concentrations.

Direct desorption electrospray ionization mass spectrometry (DESI-MS) analysis could be developed as a POC test as this technique was proven to be successful in identifying mucosal metabolite profiles from swabs that were collected in a clinical setting (Pruski et al., 2017). This technique could be used to explore the mucosal metabolite signature(s) associated with BV and help delineate the bacterial-host interactions at the vaginal mucosa during this condition. Another technology that could be optimized for the POC is low-field benchtop NMR spectroscopy, which involves the use of instruments that operate at frequencies below 100 MHz (Percival et al., 2019). Percival et al. (2019) successfully showed that LF NMR could detect and quantify biomarkers of type 2 diabetes in urine. This study therefore demonstrated the feasibility of the technology to do a biochemical screening of human biofluids.

Immune System Markers

Although several developing countries rely on the syndromic management of BV based on a vaginal discharge syndrome, Mlisana et al. (2012) reported that vaginal discharge and other symptoms are poor predictors of BV with a sensitivity of 10% and a specificity of 94.4%. This study found that asymptomatic women with one or more active STIs had subclinical inflammation and that these women may have increased levels of genital inflammation similar to those of women with symptomatic infections. Bacterial vaginosis has been associated with a range of upregulated genital pro-inflammatory cytokines (Masson et al., 2014; Kyongo et al., 2015) and women with BV may have an increase in the number of HIV target cells in the genital tract with higher expression levels of immune activation markers (Thurman et al., 2015; Gosmann et al., 2017).

The profiling of immune mediators in the genital tract of different groups of women has revealed variation in these profiles according to age, pregnancy status, gestational age and geographical region (Donders et al., 2003; Kyongo et al., 2015). The polymicrobial nature of BV complicates the assignment of a standard signature of immune mediators to a condition that is associated with divergent microbiome profiles. Different species or microbiome profiles may be associated with different immune mediators and/or different levels of inflammation and the risk of acquiring HIV. For example, *L. crispatus* has been associated with significantly lower levels, and *G. vaginalis*, *A. vaginae* and *P. bivia* with significantly higher levels, of the cytokines interleukin (IL)-1α, IL-1β and IL-12p70 (Kyongo et al., 2015). Women with a high-diversity VMB profile with abundant *Prevotella* spp. have been reported to have higher levels of interferon (IFN)-γ and IL-1β in cervicovaginal lavages (Anahtar et al., 2015). Also, young women with diverse VMBs dominated by anaerobes other than *Gardnerella* spp. have been reported to have elevated numbers of activated mucosal CD4+ T cells and an over four-fold higher risk of HIV acquisition compared to women with VMBs dominated by *L. crispatus* (Gosmann et al., 2017). In South African adolescent girls aged 16 to 22 years, Lennard et al. (2018) characterized the VMB and found three distinct microbiome subtypes, one of which was associated with extreme genital inflammation. The authors suggested that the inflammatory state associated with the BVAB1-dominated subtype may be chronic

and that this subtype can be predicted with a sensitivity of 80% and specificity of 88%, based on a Nugent score of ≥9. The high sensitivity and specificity of high Nugent scores to predict this microbiome subtype might explain why high Nugent scores are representative of certain microbiomes and their associated symptoms but not of all VMB signatures. The Nugent scoring diagnostic system is therefore limited in the sense that it has to represent a complex and diverse VMB in terms of its immune mediator profile and bacterial species dominance. Considering the relationship between the VMB and its immune complement, the immune system is another avenue that could be explored for the development and expansion of BV diagnostics.

Microbiome profiles that represent dysbiosis may not always be detectable with conventional diagnostics. High-throughput technologies have enabled the characterization of the VMB on a grand scale and different bacterial species and microbiome profiles that have been associated with inflammation could therefore be easily characterized and used as indirect markers of an inflammatory state. However, characterizing the microbiome may reveal dysbiosis, but it is not an exhaustive approach to indicate the level of inflammation of the genital tract and therefore the risk of acquiring HIV and other STIs and developing reproductive complications (Mlisana et al., 2012; Masson et al., 2018). Consequently, the polymicrobial nature of BV and its association with divergent immune mediator profiles better support the use of direct markers of inflammation, such as immune mediators, as a diagnostic avenue. Compared with normal microflora, BV is often associated with varying levels of the immune mediators IL-1α, IL-1β, IL-6, IL-12(p70), IL-8, interferon gamma-induced protein (IP)-10, tumor necrosis factor (TNF)-β and secretory leukocyte protease inhibitor (SLPI) (Masson et al., 2014, 2016; Kyongo et al., 2015; Jespers et al., 2017).

In cervical lavage (CVL) samples, Masson et al. (2016) identified that the pro-inflammatory cytokine interleukin (IL)-1β and the chemokine interferon-γ-induced protein (IP)-10 could potentially be used at the POC to identify women with STIs or BV. This study reported that, according to STI and/or BV status, a model that comprised IL-1β (direct relationship between its concentration and BV) and IP-10 (inverse relationship between its concentration and BV) could correctly classify 75% of women with a sensitivity of 77% and a specificity of 72%. With the inclusion of another cytokine, IL-1α (direct relationship between its concentration and BV), the fit of the model was significantly improved ($p = 0.0001$) and the model correctly classified 76% of women with a sensitivity of 72% and specificity of 81%. The performance of the three cytokine biomarkers was validated by Masson et al. (2018) in a cohort of women who were recruited irrespective of vaginal symptoms and who were from four different geographical regions in Africa. The diagnostic model performed best with lateral vaginal swabs as genital specimen, correctly classifying 76% of women based on their STI, BV or intermediate VMB status with a sensitivity and specificity of 86% and 64% respectively. This study found that vaginal pH alone had a low specificity (61%) for classifying women according to their STI or VMB status. However, when vaginal pH was combined with the cytokine biomarkers, the accuracy of the diagnostic test was improved and 82% of cases were correctly identified (sensitivity of 86% and specificity of 64%). Interestingly, the authors also found that by combining pH with only IL-1α, the diagnostic model also correctly classified 82% of cases with a sensitivity and specificity of 86% and 68% respectively. Several other studies support the inverse relationship between IP-10 and BV and the direct relationship between BV and IL-1α and IL-1β (Deese et al., 2015; Kyongo et al., 2015; Jespers et al., 2017).

Immune mediators that are associated with a BV profile could be incorporated into diagnostic tests such as automated chemiluminescent enzyme immunoassays or lateral flow-based immunoassays (POC diagnostic assay). Kunze et al. (2016) measured the concentrations of IL-6 in amniotic fluid and TNF-α in vaginal secretions with the IMMULITE® system (Siemens Healthcare, Erlangen, Germany), an automated chemiluminescent enzyme immunoassay, and the QuickLine rapid test (Milenia Biotec, Gießen, Germany), a lateral flow immunoassay. A comparison between the two assays revealed a strong correlation for each marker with BV, with a Spearman correlation coefficient of 0.88 ($p < 0.0001$) for IL-6 and 0.86 ($p < 0.0001$) for TNF-α. The IMMULITE® system offers sensitive biomarker quantification, including an inflammation panel. The IMMULITE® 1,000 system is a small benchtop immunoanalyzer that is easy to use with a low cost of operation (Zirath et al., 2017). Chaemsaithong et al. (2015) compared the concentrations of IL-6 and IP-10 in amniotic fluid determined by standard enzyme-linked immunosorbent assay (ELISA) and a lateral flow-based immunoassay and reported that the results of the POC test strongly correlated with concentrations as determined by ELISA.

Computer Algorithm-Based Diagnosis of BV

Although Amsel's criteria and the Nugent scoring system are considered as the "gold standard" for BV diagnosis, problems still exist because of the "interobserver variability" and the fact that the "intermediate vaginal microbiome" do not necessarily indicate disease progression to BV or *vice versa* (van de Wijgert et al., 2014). The advances in machine learning and its application in other fields have been followed by attempts to apply computer algorithms in BV diagnosis (Baker et al., 2014; Beck and Foster, 2014, 2015; Carter et al., 2014; Song et al., 2017; Jarvis et al., 2018).

Computer algorithms could potentially have a wide range of applications that may help clinicians and researchers to search for models that identify features relevant to BV diagnosis, to assess relative bacterial abundance data (qPCR) to diagnose BV, or to analyze bacterial morphotypes on microscope images for more accurate Nugent scoring results (Beck and Foster, 2014, 2015; Carter et al., 2014; Song et al., 2017; Jarvis et al., 2018). One of the first attempts to apply machine learning algorithms in BV diagnosis was done by Beck and Foster (2014), where the authors first grouped the correlations in microbial relative abundance data from studies by Ravel et al. (2011) and Srinivasan et al. (2012) and built different classification models (based on Amsel's criteria or Nugent scoring) using three different types of machine learning algorithms ["genetic programming" (GP), "logistic regression" (LR) and "random

forest" (RF)]. The classification models using RF and LR obtained accuracy values between 90% and 95% when the models classified potential diagnostic features and bacterial groups associated with Nugent scoring, whereas the models using GP obtained slightly lower accuracy values (above 80%; Beck and Foster, 2014). However, the identified diagnostic features differed between all three machine learning algorithms and a technical difficulty was encountered when the models were deconstructed to identify which diagnostic features influenced the classification accuracy (Beck and Foster, 2014). These observations led to a follow-up study in 2015 where the changes in the accuracy values change were observed when each diagnostic feature was sequentially added to the classification models created by RF and LR (Beck and Foster, 2015). Instead of grouping the VMB according to microbial relative abundances, features were all ranked according to their importance in each classification model (Beck and Foster, 2015). The results revealed that a relatively consistent decrease in classification accuracy was observed as the RF classification model feature ranking decreases, while a more uneven decrease was observed for the LR model feature rankings, suggesting that the RF classification may be more useful in predicting BV (Beck and Foster, 2015). The top 15 important features for BV classification models based on Nugent scoring included several BV-associated bacteria (*Prevotella* spp., *G. vaginalis*, *Dialister* spp., *A. vaginae*, *Megasphaera* spp., *Eggerthella* spp., *Sneathia* spp., *Peptoniphilus* spp.) and clinical features like vaginal pH and "clue cells" (Beck and Foster, 2015).

Computer algorithms can also be incorporated into a molecular diagnostic tool to assess the relative abundance data of BV-associated bacteria for diagnosing BV. In a recent study conducted by Jarvis et al. (2018), a novel qPCR-based diagnostic tool (that detects ten BV-associated bacterial species and four *Lactobacillus* species) with a diagnostic machine learning algorithm (CLS2.0q) was developed. Evaluation of this molecular diagnostic tool with 172 women (149 symptomatic and 23 asymptomatic) resulted in a sensitivity of 93% and a specificity of 90% when compared with Nugent scoring (Jarvis et al., 2018). Although this molecular diagnostic tool currently requires further validation and improvements to become useful in clinical settings, it could be handy in POC settings.

Lastly, computer-based algorithms could be used to automate BV diagnosis based on microscopy results. In a study by Song et al. (2017), different approaches ("Bacteria Regions Segmentation," "Overlapping Bacteria Clumps Splitting" and "Bacteria Morphotypes Learning") were introduced to automate BV diagnosis based on the Nugent scoring system. The automated BV diagnosis procedures were as follows: (i) the selection of images from a slide; (ii) the generation of the superpixels by grouping pixels of different regions of bacteria; (iii) the calculation of superpixel contrast at the region level; (iv) the segmentation of "bacteria regions" by "saliency cut"; (v) the splitting of overlapping bacterial clumps; (vi) the classification of bacterial morphotypes through a machine learning algorithm; and (vii) BV diagnosis using the Nugent scoring system (Song et al., 2017). The experimental results using 105 vaginal smears revealed a sensitivity of 58.3% and a specificity of 87.1%, and the authors presumed the reason for low sensitivity was due to an inability of the algorithm to split heavily overlapped bacteria (Song et al., 2017). However, when the images were manually selected by human readers (experts) before the superpixel generation, sensitivity improved by up to 75% (Song et al., 2017). Again, this approach requires more research and improvements before it can be used in clinical settings, but it could be beneficial in future as it provides a more objective analysis of the vaginal smears and prevents "interobserver variability."

THE ROLE OF THE POLYMICROBIAL BIOFILM IN THE TREATMENT AND DIAGNOSIS OF BV

Bacterial vaginosis can be diagnosed microscopically with the presence of "clue cells," one of the four components of Amsel's criteria, but it was only in 2005, when Swidsinski et al. (2005) described the adherent biofilms found on these squamous vaginal epithelial cells. A spatial organization of bacteria associated with the vaginal epithelium shed new light on the etiology of BV but also led to the definition of BV as a synergistic polymicrobial syndrome with not only *G. vaginalis* playing an important role (Holst, 1990; Sobel, 2000; Swidsinski et al., 2005). Bacterial vaginosis is associated with a polymicrobial biofilm formed by BV-associated *G. vaginalis* and other BV-associated bacteria including *A. vaginae*, *Mobiluncus mulieris* and *Prevotella bivia* (Castro and Cerca, 2015; Castro et al., 2016, 2019; Hardy et al., 2016). This polymicrobial biofilm might be one of the reasons for the high recurrence rate of BV due to the protection of bacteria against H_2O_2, lactic acid, bacteriocins and antibiotics commonly used for treatment (such as metronidazole and tinidazole), but also provides resistance against the host immune system (e.g., prevent macrophage phagocytosis or chemotaxis; Al-Mushrif et al., 2000; Patterson et al., 2007; Alves et al., 2014; Swidsinski et al., 2015; Castro et al., 2019).

Biofilms composed mainly of *G. vaginalis* have the ability to adhere to vaginal epithelial cells, even in the presence of *L. crispatus*, and have also shown to benefit from the colonization of other BV-associated bacteria (*A. vaginae, M. mulieris, P. bivia, Fusobacterium nucleatum*; Machado et al., 2013a,b; Gottschick et al., 2017). Moreover, the adherence of *G. vaginalis* has been found to increase in the presence of *L. iners,* which could be due to the weak protective action exhibited by *L. iners,* although *L. iners* and *L. crispatus* have shown similar inhibitory effects against the adherence of BV-associated bacteria (Machado et al., 2013b). This investigation on the adherence of *L. iners* and *G. vaginalis* on vaginal epithelial cells confirmed that *L. iners* does not exhibit an antagonistic effect against *G. vaginalis* and suggested that the two species may be tolerant toward each other in the vaginal environment (Machado et al., 2013b). Such findings are important to fully understand biofilm formation and structure if the investigation thereof would be used for diagnostic and monitoring purposes and might especially be important in the adjustment of treatment in cases of a high recurrence rate.

Gardnerella vaginalis is known as the initial colonizer in the biofilm formation in BV, accounting for 60% of the bacterial composition. Initial colonization involves the attachment and

formation of a biofilm (Machado and Cerca, 2015; Hardy et al., 2016). Transcriptomic analysis of *G. vaginalis* gene expression (e.g., virulence genes such as vaginolysin and sialidase) in biofilms indicated gene-regulated processes to result in a protected form of bacterial growth with high virulence and low metabolic activity (Castro et al., 2017, 2019). Gene expression of *G. vaginalis* transcripts encoding antimicrobial resistance proteins might be of particular interest, since a polymicrobial biofilm has been suggested to have greater antibiotic tolerance in contrast to a mono-species biofilm (Castro et al., 2019). This phenotype of *G. vaginalis* is another mechanism that may contribute to the recurrent nature of BV and may be a potential biomarker for biofilm formation and potential target for treatment remedies; however, extensive investigation on how such gene expression is influenced still needs to be done.

Both *G. vaginalis* and *A. vaginae* are accepted as important bacterial constituents of the BV biofilm, although single colonization of *A. vaginae* has not been found to initiate biofilm formation. Research findings highlighted higher bacterial loads when both species were present in a biofilm, in contrast to biofilms of only *G. vaginalis,* highlighting the synergistic relationship of these two species in biofilm formationto create favorable conditions for optimal growth and survival (Hardy et al., 2015). Such a synergistic relationship could also play a role in increased antibiotic resistance in BV and it has been demonstrated that different *A. vaginae* strains have different susceptibility profiles *in vitro* to metronidazole and secnidazole (Mendling et al., 2019). With the known increased recurrence rate of BV, antiseptics such as dequalinium chloride (DQC) (Fluomizin®) has been accepted as an alternative treatment for BV. This compound has antimicrobial activity against different pathogens (aerobic and anaerobic bacteria), especially against *G. vaginalis* and *A. vaginae* and has no known safety concerns and mechanisms of resistance (Weissenbacher et al., 2012).

Microbial communities, such as the VMB, is known as reservoirs for numerous antimicrobial resistance genes. Collectively these genes harbored in a microbial community is called the resistome, which can be studied by means of functional metagenomics and targeted (PCR-based) metagenomics, including real-time PCR and sequence-based metagenomics (Penders et al., 2013). Characterization of the resistome allows the investigation of present antimicrobial resistance (AMR) genes in a microbial environment, which might be useful in the investigation of the VMB and biofilms in BV for optimal treatment remedies. Although resistome research has mostly been done with the gut microbiome, and only aerobic biofilm samples of wastewater have been tested, the resistome might be an area of research that may give more insight into antibiotic resistance associated with BV (van Schaik, 2015; Relman and Lipsitch, 2018; Tian et al., 2019).

Another area that may be relevant to investigate in BV is microbiome-drug interactions, more recently termed pharmaco-microbiomics. In pharmaco-microbiomics, individual variation in drug response can be addressed by characterizing the composition of microbial communities and identify the chemical mechanisms in these microbiomes to understand drug metabolism (Guthrie and Kelly, 2019). A relationship has already been established between the VMB and levels of antiretrovirals (ARVs), for instance, in women with a VMB consisting of *G. vaginalis* and other anaerobic bacteria lower levels of tenofovir were detected than in women with a *Lactobacillus*-dominating microbiome (Donahue Carlson et al., 2017; Klatt et al., 2017). The finding was that a non-*Lactobacillus* microbiome rapidly depleted tenofovir before target cells could actively convert it to a pharmacologically active drug (Klatt et al., 2017). Therefore, tenofovir would be less effective in women with a non-*Lactobacillus* or BV type of microbiome. Besides the investigation of the metabolism of ARVs by microbial communities, the question is if such an effect would be suspected with the treatment of BV. Research regarding the metabolism of metronidazole or other antimicrobial drugs by the VMB has not been done to establish the efficacy of drugs with a BV type of microbiome. Moreover, research regarding the metabolism of drugs by biofilms in BV might contribute to additional treatment remedies or alteration of current treatment remedies such as initial drug concentration and mode of delivery.

For an optimal treatment approach for BV, the composition and structure of a biofilm can be investigated by using the fluorescence *in situ* hybridization (FISH) method followed by visualization with fluorescence microscopy, as described by Swidsinski et al. (2005) and Machado et al. (2013a). The presence and adherence of both *G. vaginalis* and *A. vaginae* can be investigated by using peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH), which includes a specific PNA probe specifically selecting for the bacterial species of interest followed by DAPI staining to quantify cells in a mono- and dual-species biofilm (Freitas et al., 2017; Castro et al., 2019). A combination of confocal laser scanning microscopy (CLSM) and FISH has been recommended by Castro et al. (2019) to investigate the spatial distribution of a bacterial population and different architectures of the tested dual-species biofilms. Hardy et al. (2015) developed a PNA probe specific for *A. vaginae* (AtolTM1 probe), with a 67% sensitivity and 89.4% specificity as tested on clinical samples as compared to quantitative PCR (qPCR). The PNA FISH and qPCR can be used to determine the bacterial load of *G. vaginalis* and *A. vaginae* on vaginal epithelial cells, which is relevant in the prediction of the presence of a bacterial biofilm in BV (Hardy et al., 2015). Methods such as PNA FISH may be useful in cases of recurrent treatment failure, where it can be used to detect changes in biofilm composition and simultaneously monitor treatment efficacy. This method may therefore rather form part of a personalized treatment or precision medicine approach, especially in cases of high recurrence of BV with unsuccessful attempts of depletion of the biofilm structure in BV, and not as a method for standard diagnostic purposes.

CONCLUSION

The polymicrobial nature of BV necessitates the use of diagnostic tests that are based on combination criteria. Part of the challenge lies in determining which combination criteria are sensitive and specific enough as diagnostic criteria for BV; the other challenge is to develop cost-effective diagnostic tests, which could

preferably be used at the POC. Although there is a complex interplay between vaginal pH and the concentration of different bacterial species, it is evident that vaginal pH, specifically higher than 4.5, improves the performance of diagnostic tests when combined with other components in CVF.

The efficient and accurate detection of vaginal dysbiosis has always been plagued by factors such as the difference in biomarker levels across populations (e.g., bacterial species) and sample type variations (Kyongo et al., 2015; Masson et al., 2018). Many studies discussed in this review have highlighted the potential of different combination criteria with biomarkers beyond the genetic level to improve BV diagnostics. If the purpose of diagnosis is to treat, the question should be asked whether a perfect equilibrium exists between the VMB and all its related components. That is, does bacterial concentration translate into corresponding levels of metabolites, proteins and inflammatory markers? Nonetheless, the continual reduction in operational costs of high-throughput technologies provide the opportunity to study the vaginal milieu with a systems biology approach on a large scale to map and link potential biomarkers. This review does not necessarily suggest the replacement of diagnostic tools currently available for BV but does highlight the limitations of these tools and calls for the expansion of the BV diagnostics field by exploring the vast array of diagnostic opportunities discussed here.

In many resource-limited settings, however, POC tests for BV are either not available or simply too expensive for routine diagnostic use and healthcare practitioners have to rely on syndromic management of vaginal discharge syndrome. It is therefore imperative that the development and evaluation of new diagnostic tests must include both a cost- and health-benefit analysis in various settings, especially where expensive instrumentation is required. The risk profiles of different populations for adverse sequelae of BV infection, such as increased risk for HIV infection and poor pregnancy outcomes should guide diagnostic test selection. In such at-risk populations, we have to ask the question—what is the cost of cost?

AUTHOR CONTRIBUTIONS

All authors contributed to concept design and layout of the manuscript. MR, JG, and HJ wrote the body of the manuscript and MK contributed as senior author. All authors listed approved the manuscript for publication.

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