Characterizing the genotypic and phenotypic diversity of *Gardnerella vaginalis* from vaginal clinical samples

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In fulfillment of the requirements for the degree

MSc in Medical Virology

Division of Medical Virology

Faculty of Health Sciences

University of Cape Town

August, 2018

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Declaration

I, Kopano Valerie Masete, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Date: 30/08/2018

Acknowledgements

Rémy Froissart, you have been a super incredible supervisor. Nobody ever believed me when I told them stories of all the things you did for me that revealed just how amazing you are. I have really enjoyed interacting with you and learning from you. Merci beaucoup pour tout! ☺

Jo-Ann Passmore and the GEMS team, thank you for availing yourselves to help me whenever I needed it. Iyaloo. Monalisa, Kathy and Hoyam, thank you for all the life advice and laughs. Thandi, I don't see how I would have made it through my masters without you. I'm truly grateful.

Thank you to everyone who supported me financially, including the National Research Foundation/German Academic Exchange Service (NRF/DAAD), French Embassy, Campus France, Jo-Ann Passmore and the University of Cape Town.

But my greatest gratitude goes to my family, Kgomotso, Frank and Kgosi. Thank you for being so understanding and supporting me no matter what. You guys are truly amazing! I love you all so much!

Abbreviations

ARDRA	Amplified ribosomal DNA restriction analysis
ATCC	American Type Culture Collection
bp	Base-pair
BPDE	Benzo[a]pyrene diol epoxide
BV	Bacterial vaginosis
cas	CRISPR-associated
CHU	Centre hospitalier universitaire
CNRS	Centre national de la recherche scientifique
cpn60	Chaparonin-60
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
EPS	Extracellular polymeric substances
erm	Erythromycin resistance methylase
FISH	Fluorescence in situ hybridization
gyrA	DNA gyrase subunit A resistance
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HSV-2	Herpes simplex virus
IgA	Immunoglobulin A
MALDI-tof	Matrix-assisted laser desorption/ionization-time-of-flight
MIC	Minimum inhibitory concentration
Min	Minute
MIVEGEC	Maladies Infectieuses et Vecteurs: Ecologie, Génétique, Evolution et Contrôle
ml	Milliliter
MRS	Man, Rogosa & Sharpe
NC	Negative control

NCBI	National Center for Biotechnology
nim	Nitromidazole resistance
nm	Nanometer
nM	Nano-molar
OD	Optical density
p	P-value
parC	Topoisomerase IV protein resistance
PCR	Polymerase chain reaction
pH	Power of hydrogen
PROM	Premature rupture of membranes
qPCR	Quantitative-PCR
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RFU	Relative fluorescence units
rRNA	Ribosomal ribonucleic acid
sec	Seconds
TEM	Transmission electron microscopy
tuf	Translation elongation factor Tu
UCT	University of Cape Town
umu	UV mutagenesis resistance
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
0%SB	0% blood-supplemented (no blood) Schaedler medium
5%SB	5% blood-supplemented Schaedler medium
xg	Relative centrifugal force
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar

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Abstract

Bacterial vaginosis (BV) is a common vaginal condition affecting reproductive-age women, especially in sub-Saharan Africa. With poor treatment outcomes, BV has been associated with pregnancy complications, pelvic inflammatory disease as well as acquisition and transmission of sexually transmitted diseases. While the etiology of BV is not well characterized, it is understood that *Gardnerella vaginalis* plays a critical role in BV by initiating the formation of the polymicrobial biofilm that characterizes BV and by degrading protective vaginal mucus through the release of sialidase. Recent evidence suggests that the *G. vaginalis* species is more heterogeneous that initially thought and that not all *G. vaginalis* may be involved BV. The aim of this study was thus to characterize the genotypic and phenotypic diversity of *G. vaginalis* isolates. This was achieved *in vitro*, using 109 *G. vaginalis* isolates that were previously purified from vaginal samples of 109 French women who were BV-positive (n = 75), BV-intermediate (n = 20) or BV-negative (n = 14), as diagnosed by Nugent scoring. To determine the genotypic diversity of *G. vaginalis* isolates, 90 isolates were successfully genotyped using their chaperonin-60 (*cpn60*) sequences, revealing the presence of four phylogenetic clades (subgroups A-D) made up of 13 subgroup A, 17 subgroup B, 58 subgroup C and 2 subgroup D isolates. To determine the phenotypic diversity of *G. vaginalis* isolates, sialidase activity, biofilm formation and susceptibility to antibiotics used to treat BV were measured. Sialidase activity was not detected in subgroup A and D isolates but was detected, at similar levels, in subgroup B and C isolates. Isolates from all subgroups of *G. vaginalis* could form similar amounts of biofilm. *G. vaginalis* isolates (n = 45) were largely resistant to metronidazole (71%), but sensitive to clindamycin (100%), moxifloxacin (91%) and augmentin (100%). The presence of prophages in *G. vaginalis* isolates was also investigated, revealing the presence of bacteriophage (phage)-like particles that could not be classified into any known phage families, whose phage status remains to be confirmed. In conclusion, *G. vaginalis* subgroup B and C isolates were the only ones that formed biofilm as well as had detectable sialidase activity suggesting that *G. vaginalis* subgroups B and C are most likely to be involved in BV. These results contribute to our knowledge of BV and could be useful in future studies that aim to design better treatment strategies for BV.

Chapter 1: Introduction

1.1 Bacterial vaginosis (BV)

BV is a common vaginal condition affecting reproductive-age women globally, with a prevalence ranging from 4.5-24% in Europe and 6-58% in sub-Saharan Africa during 1984-2011 (1). A recent study in 2015 revealed a 31% prevalence rate of BV in South African women (2). However, these statistics underestimate the actual prevalence of BV as more than half of BV-positive women are asymptomatic and thus do not get tested or are misdiagnosed as BV-negative when they are in fact BV-positive (3). BV is often considered a minor condition as the only symptoms are abnormal vaginal discharge with foul odor. However, BV is recurrent and can severely diminish women's self-esteem, sexual relationships and quality of life (4). Moreover, BV puts women at a high risk of experiencing pregnancy complications and acquiring and transmitting human immunodeficiency virus (HIV) as well as other sexually transmitted infections (5). This stresses the need for effective clinical strategies to manage BV. Unfortunately, this is not the case as the current poor treatment outcomes of BV reflect our poor understanding of the etiology of BV (6). It is understood that BV is characterized by a dysbiosis of the vaginal microbiota, during which a plethora of facultative or strictly anaerobic bacteria exist in a polymicrobial biofilm (7). However, a few fundamental question are yet to be addressed, including: (i) what causes the shift from normal to diseased state; (ii) is there one or a set of bacteria that serve as the etiological agent(s) while the rest of the BV-associated bacteria are merely opportunistic and (iii) which bacterial targets would lead to the eradication of the polymicrobial biofilm that forms during BV? This study is concerned with the latter question. Answering these questions will help us better understand BV pathogenesis, providing knowledge that could be helpful in research efforts aimed at improving the current poor treatment outcomes of BV.

1.1.1 Microbiological definition of BV

There is still much debate on how to define BV in the microbiological context. However, BV is often defined as characterized by changes in the vaginal microbiota, that is, a decrease in commensal lactic acid-producing bacteria (mostly *Lactobacillus* species), coupled with an increase in number and diversity of facultative or strictly anaerobic BV-associated bacteria existing in a polymicrobial biofilm (**Figure 1.1**). This polymicrobial biofilm is composed primarily of *Gardnerella vaginalis* and *Atopobium vaginae* but also contains species such as *Prevotella* species (*P. bivia, P. dentalis, P. buccalis, P. timonensis* and *P. amii)*, *Megasphaera* species (*M. elsdenii* and *M. micronuciformis*), *Sneathia sanguinegens*, *Eggerthella hongkongensis*, *Mobiluncus mulieris*, *Fusobacterium gonidiaformans*, *Leptotrichia amnionii*, *Aerococcus christensenii*, *Parvimonas micra*, *Peptoniphilus* species (*P. lacrimalis*, *P. asaccharolyticus* and *P. methioninivorax*), *Porphyromonas asaccharolytica*, *Dialister micraerophilus,* as well as novel BV-associated bacteria (BVAB) 1-3 belonging to the *Clostridiales* order (8-11). The broad relevance of this definition has been brought into question by studies revealing that, while the vaginal tracts of healthy white and Asian women are dominated by *Lactobacillus* species, the vaginal tracts of many healthy black or Hispanic women are not, but are in fact dominated by what are described as BV-associated bacteria (10, 12, 13). In addition to producing hydrogen peroxide and bacteriocins that are toxic to BV-associated bacteria, *Lactobacillus* species maintain acidic vaginal conditions through the production of lactic acid, providing a healthy vaginal environment that does not support the growth of pathogenic bacteria (14). However, some BV-associated bacteria such as *Atopobium* and *Leptotrichia* species also produce lactic acid (15, 16), suggesting a possible mechanism by which vaginal health can be maintained in a non-lactobacilli-dominated vaginal tract (13, 17). These findings suggest a need to redefine BV in the context of ethnic diversity, as the current definition enables a reality where healthy black and Hispanic women are misdiagnosed as BV-positive, contributing to the misuse of antibiotics.

A hypothesis that has been posed to explain the ethnic differences in vaginal microbial profiles associated with health is the possibility that some BV-associated bacteria can play dual roles as commensal microbes or pathogens (18), depending on other factors such as the presence or absence of *Lactobacillus* species, availability of glucose and/or BV status. Another possible hypothesis is that intra-species diversity separates commensal from pathogenic BV-associated bacteria, and that healthy black or Hispanic women are colonized by commensal subtypes of BV-associated bacteria. It is thus important to study in detail, the diversity of each BV-associated species as this could shed light on ethnicity-based differences in microbial profiles of healthy women, as well as help us better understand the etiology of BV.

1.1.2 BV symptoms and diagnosis

BV is most commonly diagnosed using Amsel's criteria (19) or Nugent scoring (20). Amsel's criteria considers BV-positive as presenting with at least three of the following: (i) vaginal pH above 4.5, (ii) thin homogeneous vaginal discharge (iii) containing epithelial cells coated with bacilli (clue cells) and/or (iv) releasing a fishy odor upon treatment with 10% potassium hydroxide (whiff test). A limitation of Amsel's criteria is that it cannot be used to diagnose asymptomatic BV-positive women, with one study finding a 63% (34/54) false negative rate (21). Nugent scoring is thus more commonly used to diagnose BV, that is, scoring vaginal Gram-stained smears from 0-10 based on the presence of large Gram-positive rods (*Lactobacillus* morphotype, scored 4-0), small Gram-variable rods (*G. vaginalis* or *Bacteroides* morphotype, scored 0-4), and curved Gram-negative rods (*Mobiluncus* morphotype, scored 0-2) with scores of 0-3, 4-6 and 7-10 indicating BV-negative, BV-intermediate and BV-positive, respectively (20). While Nugent scoring can diagnose asymptomatic BV-positive women, it also has its shortcomings as it can result in false positives in particularly black or Hispanic women, whose healthy microbiota is not dominated by *Lactobacillus* species (10, 12, 13). Another tested strategy to diagnose BV is using polymerase chain reaction (PCR) primers specific for BV-associated bacteria (22-25); although, these sensitive nucleic

acid amplification tests have the same limitation as Nugent scoring (high rates of false positives) as this strategy is based on the false assumption that there exists a core microbial signature, differentiating healthy from BV-positive microbiota (10). Another method used to diagnose BV is using the Osem BVBlue® test, a rapid BV diagnostic test that detects levels of sialidase activity in vaginal secretions (26). Sialidase, a mucolytic enzyme secreted by *G. vaginalis* and *Prevotella* species, is elevated in 69-84% of vaginal secretions from BV-positive women (27-29). However, the detection of sialidase activity alone is not adequate enough to diagnose BV as sialidase activity is also detected in 3.2-6% of BV-negative women (29, 30). A combination of these tests have been used in effort to increase the sensitivity and specificity of BV diagnosis (31); although, there is still an urgent need to develop a more accurate test that can diagnose BV in the context of ethnic differences.

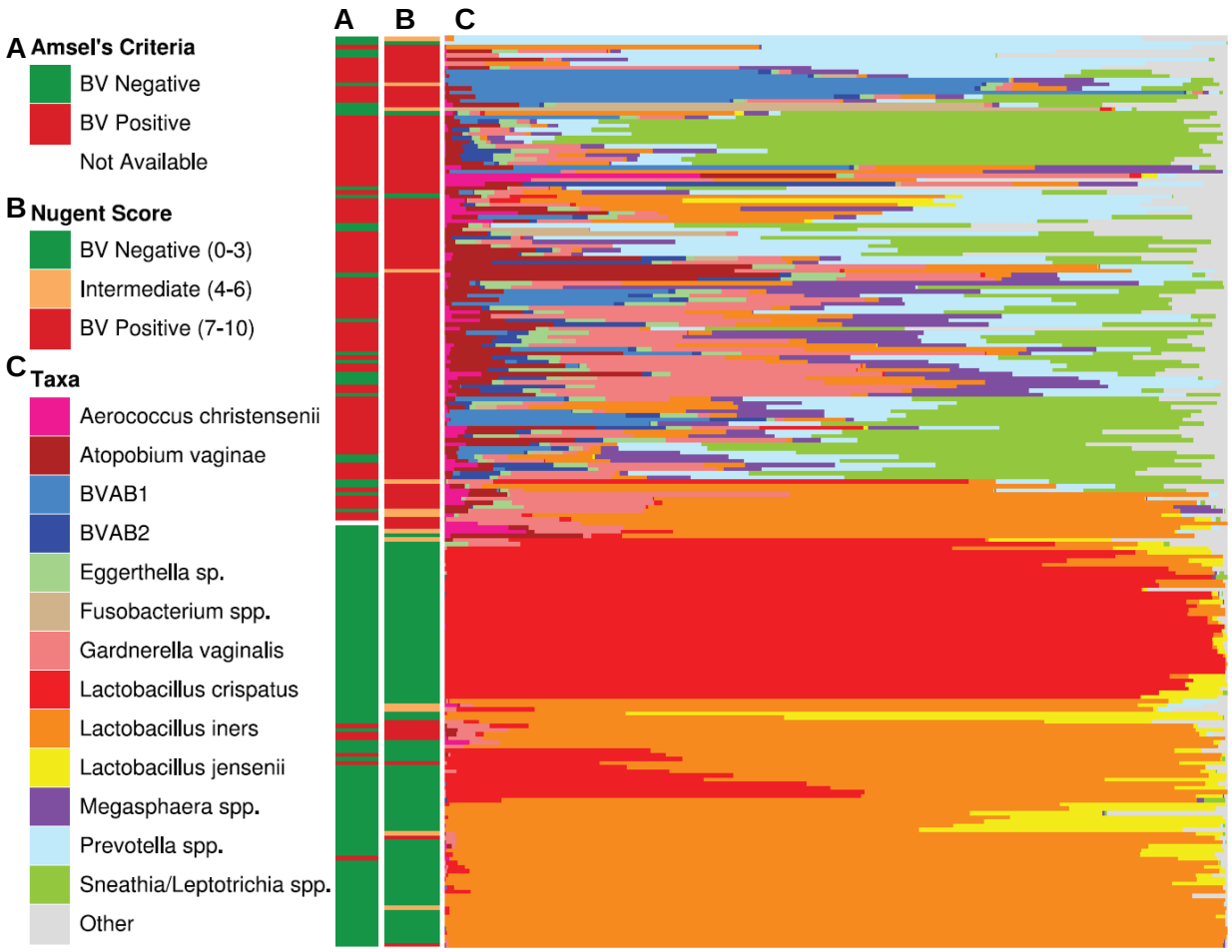


Figure 1.1: Vaginal bacterial composition in BV-positive and BV-negative women. BV status according to **(A)** Amsel's criteria and **(B)** Nugent score is shown in the two vertical bars upon which **(C)** bacterial taxa (according to 16S rRNA genes) are stratified. Figure adapted from Srinivasan et al. (9).

1.1.3 Risk factors for BV

In addition to ethnic differences (10, 13), there are several other risk factors that have been suggested for BV. A meta-analysis of 43 studies conducted between 1992-2007 suggested that risky sexual behaviors such as having a new or multiple sexual partners and a lack of condom use were associated with a modest increased risk of BV acquisition, suggesting that BV is sexually associated (32). Considering the lack of empirical evidence suggesting BV to be a sexually transmitted condition, while also not ignoring the association of BV with risky sexual behavior, BV was termed to be a

sexually-enhanced condition (33). Menstruation has been described as a risk factor for BV as an increase in *G. vaginalis* and decrease in *L. crispatus* during menses has been observed (11, 34, 35). However, these were transient changes, which lasted as long as the duration of menstruation; thus, this claim remains to be confirmed. Douching has also been associated with BV, presumably due to the bacterial disequilibrium and/or inflammation (chemical or physical) brought about by douching (36). Interestingly, smoking has also been associated with increased risk of BV. This is thought to be due to the action of benzo[a]pyrene diol epoxide (BPDE) from cigarette smoke in the female genital tract, which may promote induction of prophages of *Lactobacillus* species *in vitro*, leading to their decline (37). It is difficult to determine the direct mechanisms by which these events contribute to BV as the etiology of BV is poorly understood; thus, most of these risk factors are still being debated.

1.1.4 Treatment of BV

The recommended first-line regimens for BV antibiotic treatment are: (i) 500mg oral metronidazole twice a day for 7 days; (ii) 5g of 0.75% metronidazole intra-vaginal gel once a day for 5 days or (iii) 5g of 2% clindamycin intra-vaginal cream at bedtime for 7 days (38). However, BV treatment failure rates are substantial, with one study finding that, of 104 women treated with either metronidazole or clindamycin antibiotics, 49% experienced treatment failure within one month (6). In addition, BV recurrence occurs at an alarming rate with another study showing that more than 50% of 130 women had at least one recurrent BV episode within a year (39). One reason for this treatment failure could be antibiotic resistance. Indeed, it is becoming increasingly clear that some BV-associated bacteria have acquired resistance to metronidazole and/or clindamycin (40-43). In addition to antibiotic resistance, the biofilm forming ability of BV-associated bacteria could also be contributing to the high BV recurrence rate as antibiotic-sensitive bacteria may be protected inside an antibiotic-impenetrable biofilm, rendering antibiotic treatment ineffective (44). There is thus a great need to explore alternative BV

treatments, which is particularly difficult in the case of BV as its etiology is poorly understood.

1.1.5 Complications of BV

BV has been associated with poor reproductive health outcomes (45). BV may increase risk of developing pelvic inflammatory disease, possibly due to BV-associated bacteria from the lower female genital tract ascending to the upper genital tract causing inflammation of the uterine lining and/or fallopian tubes, thus possibly linking BV to infertility (46). This ascension is particularly problematic in pregnant women as BV has been linked to adverse pregnancy outcomes including preterm delivery, premature rupture of membranes (PROM) and postpartum endometritis (45, 47). BV has also been associated with the acquisition and/or transmission of several sexually transmitted viral infections, including HIV, cervical human papillomavirus (HPV) and herpes simplex virus-2 (HSV-2), as well as bacterial infections caused by *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (48-52). Several hypotheses have been proposed to explain the increased susceptibility of BV-positive women to sexually transmitted infections, including: (i) higher genital inflammation during BV resulting in infiltration of HIV target cells (53, 54); (ii) mucolytic sialidase activity is higher in BV-positive women, thus, the protective mucus lining that coats the epithelial cell layer would be diminished during BV, enhancing access of HPV and HSV-2 to their epithelial cell targets (50, 51) and (iii) lack of lactobacilli-produced hydrogen peroxide during BV, which would otherwise be toxic to pathogens such as *N. gonorrhoeae* and *C. trachomatis* (52). With such grave potential consequences, it is necessary to design effective prophylaxis and treatment strategies for BV.

1.2 *Gardnerella vaginalis*

G. vaginalis is well accepted to be BV-associated, being present in the vaginal tracts of 97-100% of BV-positive women (8, 9). They are Gram-positive, facultative anaerobic, 0.3-0.5 μm wide, 1-3 μm long bacilli that form 0.05-0.2mm diameter colonies on blood-

supplemented agar after 48 h at 37°C (55, 56). Initially classified as *Haemophilus vaginalis* by Gardner and Dukes in 1955, they were thought to be the cause of nonspecific bacterial vaginitis (55), the condition now referred to as BV. After DNA-DNA hybridization assays in the 1980's revealed no genetic relationship between *Haemophilus vaginalis* and other members of this genera, *Haemophilus vaginalis* was reclassified as *G. vaginalis* (56). In addition to being present in the vaginal tract, *G. vaginalis* has been isolated from various female mucosal surfaces, including the endometrium (57), anus (58), urinary tract (59) and oral cavity (60). Notably, *G. vaginalis* has also been isolated from male urethra and penile skin; moreover, monogamous heterosexual couples were shown to share the same strains of *G. vaginalis*, genotyped using unique sequence variants of the *G. vaginalis* 16S rRNA gene (61). This led to the speculation that *G. vaginalis* is sexually transmitted; however, this remains to be confirmed.

1.2.1 Association of *G. vaginalis* with BV

G. vaginalis is thought to play a central role in BV pathogenesis (7, 44). While there is no core set of microbes that define all cases of BV (10), *G. vaginalis* is found in virtually all (97-100%) BV-positive women (8, 9). While *G. vaginalis* comprises only up to 5% of 16S rRNA genes (represented in the peach colour, **Figure 1.1**), *G. vaginalis* comprises on average 47% of all transcripts in BV, making *G. vaginalis* the most transcriptionally active BV-associated bacteria (62). One study revealed the presence of *G. vaginalis* to be absolutely necessary for BV pathogenesis (63). This is probably because *G. vaginalis* plays a critical role in sialidase production and biofilm formation, two hallmark traits of BV (reviewed later in detail in sections 1.2.4 and 1.2.5, respectively). During BV, *G. vaginalis* can act in synergy with other BV-associated bacteria. For example, synergy between *G. vaginalis* and *P. bivia* was described with regards to energy source cycling, where it was postulated that *P. bivia* produces ammonia, which is utilized by *G. vaginalis,* which subsequently produces amino acids, which are utilized by *P. bivia* in order to produce ammonia (64). In a dual-species biofilm assay, growth of *G. vaginalis*

was increased by the presence of *A. vaginae, F. nucleatum, M. mulieris and P. bivia*, while in turn, growth of *P. bivia* was increased by the presence *G. vaginalis* (65). In a subsequent dual-species biofilm assay, performed by the same researchers, *G. vaginalis* strains isolated from BV-negative women showed synergy with *M. mulieris* (a BV-associated pathogen), while those from BV-positive women antagonized *M. mulieris*, a curious observation that was contrary to what was expected, which warrants further research (66).

Although well-described in BV pathogenesis, *G. vaginalis* can also be found in genital tracts of BV-negative women (10). To quantify *G. vaginalis* bacterial loads in BV-negative and BV-positive women, Balashov *et al*. (67) used the *tuf* gene, which encodes the translation elongation factor, Tu, as it was shown to be more reliable than 16S rRNA in quantifying *G. vaginalis* bacterial load because *G. vaginalis* harbors only one copy of the *tuf* gene as opposed to multiple copies of the 16S rRNA gene. This study revealed an important observation that, while BV-positive women harbor high *G. vaginalis* bacterial loads, BV-negative women can harbor either high or low *G. vaginalis* bacterial loads, suggesting that there is subset of *G. vaginalis* that are not involved in BV, even if present at high concentrations (**Figure 1.2**). However, phenotypic traits differentiating these subsets are yet to be established.

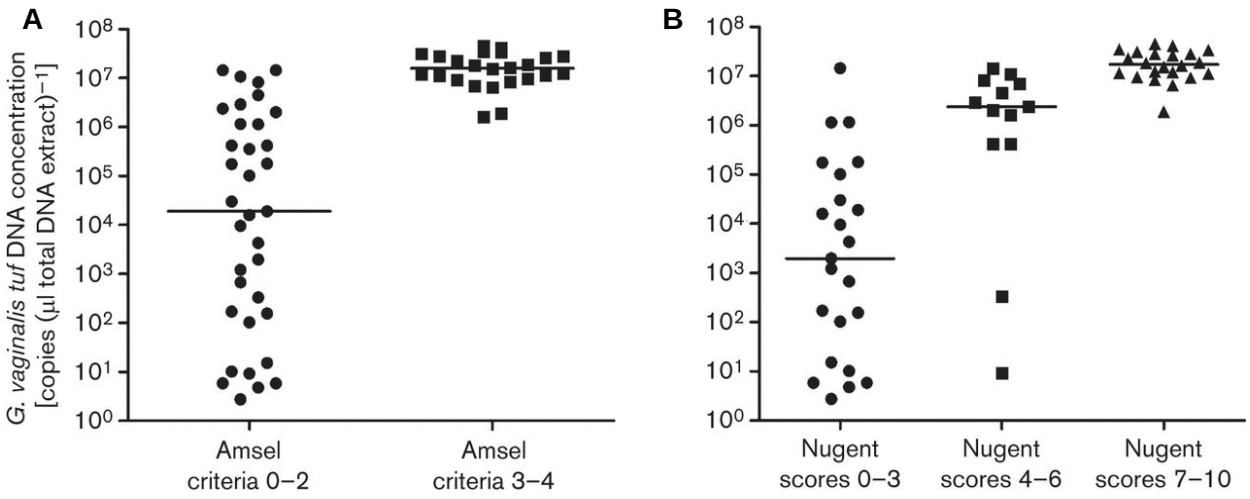


Figure 1.2: High *G. vaginalis* load is associated with BV. DNA concentration of 60 *G. vaginalis* isolates as characterized according to **(A)** Amsel criteria and **(B)** Nugent scores. Figure adapted from Balashov et al. (67).

1.2.2 Phenotypic heterogeneity of *G. vaginalis*

The heterogeneity of *G. vaginalis* was initially elucidated through the use of a biotyping scheme designed by Piot *et al.* (68) that was based on biochemically testing *G. vaginalis* isolates for hippurate hydrolysis, β-galactosidase and lipase activity. Using this biotyping scheme, 8 biotypes were identified with more than 80% of the screened 359 *G. vaginalis* isolates belonging to three biotypes. However, since the chosen biochemical markers were not related to BV virulence, no biotypes were found to be predictive of BV. Nonetheless, this biotyping scheme has been used multiple times since, with opposing conclusions with regards to associations of some biotypes with BV (67, 69-72). Furthermore, differences in methodology used to test activities of the three biochemical markers, particularly lipase activity, were shown to greatly influence results, leading to erroneous biotyping (73). Recently, this biotyping scheme has been abandoned for tests based on relevant BV phenotypes, such as sialidase and biofilm formation, discussed later in detail (sections 1.2.4 and 1.2.5).

1.2.3 Genotypic heterogeneity of *G. vaginalis*

In an effort to genotype *G. vaginalis,* traditional assays such as amplified ribosomal DNA restriction analysis (ARDRA) and random amplified polymorphic DNA (RAPD) analysis revealed the presence of up to three genotypes of *G. vaginalis* (67, 71, 72, 74, 75). While none of these studies revealed any genotype-specific associations with BV, only two out of the three genotypes of *G. vaginalis* demonstrated sialidase activity, implicating the two genotypes in BV pathogenesis (75). These DNA fingerprinting methods have recently been replaced with modern sequence-based methods of genotyping.

It has been well accepted that there is only one species of *Gardnerella,* that is *G. vaginalis*; however, whole genome sequencing of 17 *G. vaginalis* isolates revealed the presence of four clades of *G. vaginalis*, with sufficient divergence from each other to be considered four distinct species (57). Although, phenotypic traits differentiating the clades would need to be established before reclassifying *G. vaginalis* as having four species (76). The same four clades of *G. vaginalis* could be discriminated by phylogenetic analysis of a 552 base-pair (bp) region of the chaparonin-60 (*cpn60*) housekeeping gene, into subgroups A, B, C and D by Jayapraesh *et al.* (72) corresponding to whole genome sequence clades 4, 2, 1 and 3 in Ahmed *et al.* (57), respectively. Multiple studies have since genotyped *G. vaginalis* into two to four subgroups using *cpn60* sequence analysis (27, 67, 77). Generally, studies have shown that most women harbor multiple subgroups of *G. vaginalis*, with subgroup diversity being predictive of BV; although, there is still debate over which subgroups of *G. vaginalis* are associated with BV (67, 72, 78, 79). Jayaprakesh *et al.* (72) conducted a study using 44 vaginal samples, 20 (45%) of which were from BV-positive women and found subgroup B to be the only BV-associated subgroup. However, Balashov *et al.* (67) used 59 vaginal samples, 22 (37%) from BV-positive women, and found subgroups C and D to be BV-associated. Using a larger sample size of 75 vaginal samples, 29 (27%) of which were from BV-positive women, Janulaitiene *et al.* (78) found subgroup B

and C to be BV-associated. One reason for the discrepancies between the studies could be the differences in sample size and prevalence of each subgroup, that is, low prevalence of some *G. vaginalis* subgroups may limit the statistical power to test the association between these *G. vaginalis* subgroups and BV. For example, Jayaprakesh *et al*. and Janulaitiene *et al.,* who both found subgroup B to be BV-associated, detected this subgroup in 97.7% (43/44) and 42.7% (32/75) of their samples, respectively. In contrast, Balashov *et al*, who did not find subgroup B to be BV-associated, detected subgroup B in only 25.4% (15/59) of their samples, a figure that was reduced even further after separating the subgroup B samples according to their BV-negative, BV-intermediate and BV-positive status, thus further limiting this subgroup's statistical power. Further studies using larger cohorts are required to test these associations as they are imperative in identifying which *G. vaginalis* subgroups to target in BV therapy.

1.2.4 Role of *G. vaginalis* sialidase activity in BV pathogenesis

G. vaginalis contributes to the elevated sialidase activity levels seen in BV-positive versus BV-negative women (27-29). *G. vaginalis* is thought to secrete sialidase to cleave immunoglobulin A (IgA) sialic acids residues as well as the sialic acids that make up the protective mucus layer that lines vaginal epithelium (80). Additionally, sialidase-activity-positive *G. vaginalis* isolates internalize, catabolize and deplete vaginal sialic acids (80). It is important to note that not all *G. vaginalis* harbor the sialidase gene as it was detected only 49-75% of *G. vaginalis* isolates screened across different studies (27, 75, 81). Moreover, not all *G. vaginalis* isolates with the sialidase gene demonstrate sialidase activity (72, 75, 80). Jayaprakesh *et al.* (27) showed that, while the sialidase gene was detected in all 33 subgroup B, 35 subgroup C and 8 subgroup D isolates, the sialidase gene was detected in only 1/36 subgroup A isolates. Additionally, while all 33 subgroup B isolates had detectable sialidase activity, only a minority of subgroup C isolates (3/35) and none of the subgroup A and D isolates had detectable sialidase activity. Notably, as subgroup B was previously associated with BV (72), the finding that

all isolates from this subgroup had detectable sialidase activity strongly implicates subgroup B as actively involved in BV (27).

A recent study by Hardy *et al.* (81) found an association between BV and high quantitative-PCR (qPCR) loads of the *G. vaginalis* sialidase gene in vaginal specimens. Moreover, Hardy *et al.* are the first to show an association between *G. vaginalis* sialidase gene loads and biofilm formation. They postulated that *G. vaginalis* uses sialidase to break down protective vaginal mucus, allowing it easier access to vaginal epithelium to form adherent biofilms, a phenomenon that has been described for other organisms such *as Pseudomonas aeruginosa* and *Streptococcus pneumoniae* (82, 83). It is thus necessary to test sialidase activity and biofilm formation in the same isolates that are genotyped into the four subgroups of *G. vaginalis* as this will provide novel information that could identify *G. vaginalis* subgroups involved in BV.

1.2.5 Role of *G. vaginalis* biofilm formation in BV pathogenesis

An adherent polymicrobial biofilm is a hallmark trait of BV, being present on vaginal epithelial cells of 90-97% of biopsy specimens from BV-positive women but only 4-10% from BV-negative women (63, 84). It is estimated that *G. vaginalis* contributes 60-95% of the biofilm's biomass. In an assay measuring epithelial cell adherence of BV-associated bacteria co-cultured with *L. crispatus*, *G. vaginalis* was found to be more effective at displacing *L. crispatus* than the other BV-associated bacteria, including *A. vaginae, P. bivia* and *M. mulieris* (85). Similarly, another study showed that *G. vaginalis* displayed the highest initial adhesion to epithelial cells and the highest biofilm forming ability compared to 29 other BV-associated bacteria (86). These data suggest that *G. vaginalis* may be an early colonizer, initiating the formation of the biofilm, which subsequently allows other BV-associated bacteria to colonize the female genital tract, forming the polymicrobial biofilm that characterizes BV (44).

G. vaginalis biofilms are thought to play a number of distinct roles in the context of BV. *G. vaginalis* biofilms are strongly associated with BV recurrence (87), discussed later in detail (section 1.2.6). Compared to in planktonic form, *G. vaginalis* in biofilm form survived up to five times better in the presence of toxic lactobacilli-produced lactic acid and hydrogen peroxide (88), which is possibly the mechanism that *G. vaginalis* uses to survive in healthy lactobacilli-dominated vaginal tracts (10, 84). In addition to the vaginal tract, *G. vaginalis* has also been shown to form biofilm in the endometrium of BV-positive women, possibly linking *G. vaginalis* to the adverse pregnancy outcomes associated with BV (7).

With regards to the differences in biofilm forming ability amongst the four subgroups of *G. vaginalis,* there has only been one report, which revealed that all four subgroups are capable of forming biofilm (72). This was a report of a qualitative assay that detected the absence or presence of biofilm thus, it remains to be seen if some subgroups produce more biofilm than others. This information could be useful in determining which subgroups play an important role in the formation of biofilm and thus, which subgroups to target when aiming to eliminate the polymicrobial biofilm that forms during BV.

1.2.6 Role of *G. vaginalis* in poor BV treatment outcomes

As discussed earlier (section 1.1.4), the current standard of care for BV is antibiotic treatment with metronidazole or clindamycin (38), but this treatment fails up to 50% of the time (6) and recurrence is common (39). Recurrence could be due to the antibiotic-impenetrable biofilm that forms during BV (87), that is, antibiotics are not always able to reach the center of biofilms as they can get used up by bacteria in the exterior layers of biofilms (89). BV biofilms are thought to be initiated by *G. vaginalis* (44); moreover, *G. vaginalis* biofilms have been shown to persist in women after they were treated for BV with metronidazole (87). Therefore, strategies targeting *G. vaginalis* biofilms may alleviate the current high BV recurrence rates.

As BV is a recurrent condition, women often have to get retreated multiple times (6). It is thus not surprising that BV-associated bacteria such as *G. vaginalis* have acquired resistance to antibiotics used in the treatment of BV. This is particularly true for metronidazole, with reported resistance rates ranging from 61-68% amongst *G. vaginalis* isolates (41-43). Moreover, the majority of metronidazole-resistant *G. vaginalis* isolates are highly resistant, that is, require more than 256 µg/ml metronidazole to inhibit growth (41, 42). A study that compared metronidazole susceptibility amongst *G. vaginalis* subgroups, using 87 isolates, showed that all subgroup A and D isolates were metronidazole-resistant (22/22 and 14/14 isolates, respectively) compared to only 35% (16/37) subgroup C and 7% (1/14) subgroup B isolates (41). Compared to metronidazole, clindamycin resistance among *G. vaginalis* isolates is lower, ranging from 1.5-6% (42, 43) and is thus considered a good alternative in the event of metronidazole treatment failure. In addition to clindamycin, moxifloxacin and augmentin could be considered good alternatives in the event of metronidazole treatment failure as they have demonstrated good BV treatment outcomes (90, 91); although, *G. vaginalis* susceptibility to these antibiotics is not well studied. It could be useful to study clindamycin, moxifloxacin and augmentin susceptibility of *G. vaginalis* and to compare it to metronidazole susceptibility, using the same set of isolates, as this could yield information on which antibiotics could serve as the best alternative to metronidazole in the management of *G. vaginalis* biofilms present during BV.

1.2.7 Bacteriophages of *G. vaginalis*

Genomes of *G. vaginalis* are known to harbor clustered regularly interspaced short palindromic repeats (CRISPR) loci and their associated *cas* genes (59, 62, 92). Bacteria use their CRISPR-cas systems to mount an immune response against foreign DNA, especially against DNA of bacteriophage (phages), which are viruses that infect bacteria (93). CRISPR loci contain spacer sequences, sequences that are homologous to foreign DNA, which are acquired and accumulate with every phage infection (93). CRISPR loci can thus be used as evidence for the existence of phages that have

previously targeted particular bacteria. Up to 70% of *G. vaginalis* spacer sequences could not be matched to any known bacterial or viral sequences on the GenBank database, suggesting that the majority of *G. vaginalis* phages have yet to be isolated and characterized (92).

Phages are most commonly classified as either virulent or temperate, based on the types of lifecycles the can undergo after gaining entry into a host cell (94). Virulent phages exclusively undergo the lytic lifecycle: a short cycle of replication, assembly of phage proteins and host cell lysis upon exit. Temperate phages can undergo the lysogenic lifecycle, a state whereby they integrate their phage DNA (prophage) into their host's genome, latently multiplying with the host until environmental stress induces them into the lytic lifecycle (94). While virulent phages against *G. vaginalis* have yet to be reported, there have been several reports of the presence of prophage sequences within *G. vaginalis* genomes (59, 95, 96). One of the earliest references of *G. vaginalis* phages was made in 2010, where 10 phage-associated genes (including integrase and assembly genes) were found in three *G. vaginalis* strains (95). Later, another study by Malki *et al*. (59) did a comprehensive analysis of *G. vaginalis* sequences from the National Center for Biotechnology (NCBI) database, which revealed the presence of 442 prophage sequences, with a median length 939bp, from 39 genomes of *G. vaginalis* from the bladder, vagina and endometrium. The number of phage sequences per *G. vaginalis* genome ranged from 0-33. A total of 104 clusters of orthologous prophage genes were identified based on sequence similarity. Half of the 104 prophage clusters showed little to no matches to any phage sequences in the GenBank database. Of the prophage clusters that could be matched, most resembled an array of different bacterial taxa while a few clusters resembled a single bacterial taxon including *Bacillus, Mycobacterium* and *Staphylococcus* species. All the phages belonged to the *Siphoviridae* family. While prophage homologue sequences in large clusters were highly conserved, 49 clusters were made up of a single prophage gene sequence, suggesting that *G. vaginalis* frequently acquires phage sequences independently. Based on their estimates of rate of phage acquisition, Malki *et al*. hypothesized that *G. vaginalis*

prophages can be acquired both through vertical inheritance (from parent to offspring) and horizontal gene transfer (from donor to recipient). Their analysis also showed that *G. vaginalis* isolated from the same host can differ in prophage gene content suggesting intra-host prophage gene acquisition/loss.

Recently, prophages of *G. vaginalis* were identified by whole genome sequencing in another study by Miller-Ensminger *et al.* (96), where they were able to identify prophages and nonintegrated phages, that is, possible virulent phages and prophages existing as extra-chromosomal plasmids. The number of phage sequences per *G. vaginalis* genome ranged from zero to two while for other bacterial taxa, the highest number of phage per genome was 10. Of the 21 isolates of *G. vaginalis* that were sequenced, almost half (10/21) had low quality or no prophage detection; while the remainder had one integrated phage (9/21), one nonintegrated phage (1/21) or both an integrated and a nonintegrated phage detected. It is worth noting that far fewer *G. vaginalis* phages were identified in the study by Miller-Ensminger *et al.* (average one phage per genome; range 0-2; 21 genomes) compared to the study by Malki *et al*. (average 11 phages per genome; range 0-33; 39 genomes). This is most likely due to different methods used to identify prophages in the two studies, serving as caution to future studies to consider using various phage annotation tools in order to avoid underestimating phage populations. Nevertheless, these studies provide strong bioinformatic evidence for the presence of *G. vaginalis* phages, despite the current lack of empirical laboratory evidence.

Since there is evidence that prophages of *G. vaginalis* exist, it is possible that *Lactobacillus* species suppress the growth of *G. vaginalis* by releasing substances that induce prophages of *G. vaginalis.* This hypothesis follows an observation made in a previous study where *Streptococcus pneumonia* supernatant contained hydrogen peroxide, which caused the induction of prophages of *Staphylococcus aureus* (97). As *Lactobacillus* species such as *L. gasseri* are known hydrogen peroxide-producers (98),

the supernatant of *L. gasseri* could be tested for its ability to induce prophages of *G. vaginalis.* Mitomycin C, ciprofloxacin and ultraviolet (UV) radiation are commonly used to induce prophages of other bacteria as they are DNA-damaging agents that lead to the activation of the SOS response, which can trigger the inactivation phage repressor genes, resulting in prophage induction (99). As in other studies, these prophage-inducing agents can be tested for their use as positive controls in the induction of *G. vaginalis* prophages.

1.3.1 Aim

To characterize the genotypic and phenotypic diversity of vaginal *G. vaginalis* isolates.

1.3.2 Objectives

1. To genotype *G. vaginalis* isolates using *cpn60* sequence analysis.
2. To phenotype *G. vaginalis* isolates with regards to sialidase activity, biofilm formation and susceptibility to antibiotics used to treat BV.
3. To screen a panel of *G. vaginalis* isolates for the presence of prophages.

1.3.3 Rationale

G. vaginalis is thought to contribute to BV pathogenesis by: (i) secreting sialidase, which degrades protective vaginal mucus; (ii) forming biofilms that support the growth of other BV-associated bacteria and (iii) resisting the antibiotic effects of metronidazole. While *G. vaginalis* is present in virtually all BV-positive women, many BV-negative women can harbor high *G. vaginalis* bacterial loads suggesting that not all *G. vaginalis* are involved in BV. Four phylogenetically distinct subgroups of *G. vaginalis* have recently been identified and it is thought that not all subgroups are involved in BV. This study thus aims to investigate the diversity of *G. vaginalis* isolates with regards to sialidase activity, biofilm formation and susceptibility to antibiotics used to treat BV. It is possible that *Lactobacillus* species may suppress the growth of *G. vaginalis* through releasing hydrogen peroxide, which induces prophages of *G. vaginalis.* Therefore, this study further aims to screen a panel of *G. vaginalis* isolates for the presence of prophages. This is the first study to use the same collection of *G. vaginalis* isolates to quantify sialidase activity, biofilm formation and antibiotic susceptibility of *G. vaginalis* subgroups. These results will elucidate the roles the different subgroups of *G. vaginalis* play in the context of BV and could thus identify which subgroups of *G. vaginalis* to target in BV treatment studies.

Chapter 2: Materials and Methods

2.1 Samples

One hundred and nine isolates of *G. vaginalis,* purified from vaginal samples of women routinely visiting Hôpital Arnaud-De-Villeneuve (CHU de Montpellier, France), were kindly provided by Dr. Rémy Froissart from the Institut de recherche pour le développement (IRD), France (Collaboration Agreement UCT-MIVEGEC, Reference: CNRS 174773). The *L. gasseri* isolate used in this study was kindly provided by Mrs. Hoyam Gamieldien (Division of Medical Virology, University of Cape Town), purified from a vaginal sample of a woman in Cape Town, previously enrolled in the now completed Women's Initiative for Sexual Health (WISH) study (UCT HREC267/2013) that aimed to determine factors affecting HIV susceptibility in the adolescent genital tract (100). All women consented to their samples being collected, stored, used and shared for research purposes. Ethics approval to conduct this study was obtained from UCT's Human Research Ethics Committee (HREC202/2018). To obtain pure isolates, vaginal samples were streaked as to obtain single colonies on plates of nalidixic acid-supplemented Columbia blood agar (Biomérieux, Marcy-l'Étoile, France) for *G. vaginalis* and MRS (Sigma-Aldrich, Missouri, United States of America) agar (BD Biosciences, New Jersey, United States of America) for *L. gasseri* and grown anaerobically (using Oxoid AnaeroGen Sachet, Thermo Fisher Scientific, Massachusetts, United States of America) for 48 h at 37°C. Colonies were picked and regrown on Columbia blood agar (Biomérieux, Marcy-l'Étoile, France) if confirmed to be *G. vaginalis* or *L. gasseri* by MALDI-TOF (Bruker Daltonics, CHU de Montpellier, France). The isolates were stored at -80°C for the duration of this study in 20% (v/v) glycerol (Sigma-Aldrich, Missouri, United States of America) 5% (v/v) horse blood (Primate Unit and Delft Animal Center, Cape Town, South Africa)-supplemented Schaedler medium (Oxiod, Hampshire, United Kingdom) for *G. vaginalis* and 20% (v/v) glycerol MRS medium for *L. gasseri*.

2.2 DNA extraction

Isolates of *G. vaginalis* from -80°C freezer stocks were grown anaerobically in 5% (v/v) horse blood-supplemented Schaedler (5%SB) agar plates for 48 h at 37°C. DNA was extracted from bacterial colonies using InstaGene matrix (Bio-Rad, California, United States of America) according to the manufacturer's instructions. Briefly, bacterial cells were washed in phosphate buffered saline (PBS, Sigma-Aldrich, Missouri, United States of America), resuspended in 200 µl InstaGene matrix, incubated for 30 min at 56°C and then 100°C for 8 min before being stored at -20°C. The supernatant containing extracted DNA was used for PCR of the *cpn60* gene (section 2.3) and sialidase gene (section 2.4).

2.3 *cpn60* PCR

To genotype *G. vaginalis* isolates, a 552bp DNA region of the *cpn60* housekeeping gene, which was previously used to genotype *G. vaginalis* isolates (27, 72), was amplified through PCR and sequenced. *cpn60* PCR reactions were carried out using forward primer H729 5'-CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCIGGIGAYGGIA-CIACIAC-3' and reverse primer H730 5'-AGCGGATAACAATTTCACACAGGAYKIYKIT-CICCRAAICCIGGIGCYTT-3', from a previous study (72). Amplifications were done in a 50 µl reaction mix made up of 20 µl extracted DNA, 250 µM PCR grade nucleotide mix, 800 nM of each primer, PCR reaction buffer (2.5 mM MgCl2, 50 mM KCl, 10 mM Tris.HCl, pH 8.3) and 2.5 units of Taq DNA polymerase (Roche, Basel, Switzerland). The PCR reactions ran for 3 min at 94°C, followed by 40 cycles of 30 sec at 94°C, 1 min at 52°C and 60 sec at 72°C, with a final extension time of 10 min at 72°C. The *G. vaginalis* isolates, with a detectable ~600bp DNA band visible on 2% (w/v) agarose gel (Eurogentec, Liège, Belgium), were sequenced using Sanger sequencing (Genoscreen, Lille, France) with M13 (-47) forward and M13 (-48) reverse sequencing primers (underlined sequences). *G. vaginalis* isolates with poor quality reads were re-sequenced using Sanger sequencing (Eurofins Genomics, Ebersberg, Germany) with the same primers.

2.4 Sialidase PCR

To screen *G. vaginalis* isolates for the presence of the sialidase gene, a sialidase 682bp DNA region was amplified using forward primer sia1-F 5'-ATGGAACGTCGTTCAACGA-AG-3' and reverse primer sia1-R 5'-GATACGCGTTTTATGTCTCTTGC-3', from a previous study (71). Amplifications were done in 25 µl reaction mix made up of 10 µl extracted DNA, 200 µM PCR grade nucleotide mix, 200 nM of each primer, PCR reaction buffer mix (2.5 mM MgCl2, 50 mM KCl, 10 mM Tris.HCl, pH 8.3) and 1.25 units of Taq DNA polymerase. The reactions ran for 3 min at 94°C, followed by 28 cycles of 30 sec at 94°C, 30 sec at 52°C and 50 sec at 72°C, with a final extension time of 10 min at 72°C. The *G. vaginalis* isolates with a detectable ~700bp DNA band visible on 2% (w/v) agarose gel were sequenced using Sanger sequencing (Genoscreen, Lille, France).

2.5 Phylogenetic analysis

All phylogenetic analyses were performed using Geneious software (version 10.2.3, Biomatters). A *cpn60* phylogenetic tree was constructed with 90 sequences obtained from isolates in this study, as well as five sequences from the online database, cpnDB (cpnDB_nr; www.cpndb.ca), of which four sequences were used to delineate the four subgroups of *G. vaginalis* that were previously described (72), while *Bifidobacterium asteroides* PRL2011 was used to root the tree. A sialidase phylogenetic tree was constructed with 75 sequences obtained from isolates in this study, while a *Bifidobacterium bifidum* ATCC 15696 571bp sialidase gene region from NCBI was used to root the tree. Sequences were aligned using Geneious pairwise aligner with a 51% similarity cost matrix; the genetic distance model was calculated using HKY and the tree was built using the neighbor-joining method from bootstrap calculations of 100 replicates.

2.6 Sialidase activity quantification

Since sialidase gene presence is not always predictive of sialidase gene activity (27), the sialidase activity of all 109 *G. vaginalis* isolates was quantified. Isolates of *G. vaginalis* from -80°C freezer stocks were grown anaerobically on 5%SB agar plates for 48 h at 37°C. A loopful (10 µl) of bacteria were grown anaerobically in 1.5 ml 0.1% (w/v) starch (Sigma-Aldrich, Missouri, United States of America), 0.5% (w/v) yeast (BD Biosciences, New Jersey, United States of America)-supplemented brain heart infusion (sBHI, Sigma-Aldrich, Missouri, United States of America) medium for 48 h at 37°C. The optical density at a wavelength of 600 nm (OD600nm) of bacteria was measured using a cell density meter (model WPA CO8000, Biochrom, Cambridge, United Kingdom) and 50 µl of the culture was added to triplicate wells of a 96 well black flat bottom plate (Sigma-Aldrich, Missouri, United States of America) containing 100 µl of 460 µM 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (4MUsia, Sigma-Aldrich, Missouri, United States of America) in 0.1M sodium acetate (Sigma-Aldrich, Missouri, United States of America), pH 5.5. Sialidase activity was quantified by measuring fluorescence (excitation at 365 nm and emission at 440 nm), one well at a time, reading the plate 20 times within a total of 45 min using the Glomax Multi Detection system (Promega, Wisconsin, United States of America). The rate of substrate conversion was reported as relative fluorescence units (RFU), which were obtained by averaging out the 20 readings of each triplicate and adjusting for cell concentration.

2.7 Biofilm formation quantification

As biofilm is the hallmark trait of BV (44, 63, 84), the amount of biofilm formed by *G. vaginalis* isolates was quantified. Isolates of *G. vaginalis* from -80°C freezer stocks were grown anaerobically on 5%SB agar plates for 48 h at 37°C. A loopful of bacteria were grown anaerobically in 1.5 ml 5%SB for 48 h at 37°C. After standardizing the bacteria to an OD600nm of 1, 200 µl of the cultures were distributed into 8 replicate wells of a 96 well round bottom plate (Corning, New York, United States of America) and grown

anaerobically for 48 h at 37°C. Planktonic cells were carefully pipetted out and the biofilm was stained for 15 min in 225 µl 1% (v/v) crystal violet dye (Sigma-Aldrich, Missouri, United States of America), which was diluted in distilled water. Plates were washed three times by carefully adding distillated water, at a low water pressure, to the wells, inverting the plate and placing it firmly above an empty pipette box, gently moving it from left to right as to remove the water, while preventing loss of biofilm, and finally, gently blotting the plate dry against a paper towel. Crystal violet dye-bound biofilm was dissolved in 200 µl of 30% (v/v) acetic acid (Sigma-Aldrich, Missouri, United States of America), which was diluted in distilled water. Biofilm was quantified at OD550nm using a VersaMax microplate reader and its SoftMax PRO software (version 5, Molecular Devices, California, United States of America).

2.8 Antibiotic susceptibility testing

The susceptibility of *G. vaginalis* isolates to antibiotics used to treat BV, including metronidazole, clindamycin, augmentin and moxifloxacin, was tested. Isolates of *G. vaginalis* from -80°C freezer stocks were grown anaerobically on 5%SB agar plates for 48 h at 37°C. The turbidity of the bacterial cultures were standardized to McFarland unit of 1 in saline before being streaked to form a lawn (merged bacterial colonies) on 5% horse blood-supplemented Brucella agar plates (BD Biosciences, New Jersey, United States of America). Bacteria were grown anaerobically for 48 h at 37°C, in the presence of metronidazole, clindamycin, augmentin and moxifloxacin antibiotic minimum inhibitory concentration (MIC) test strips (Liofilchem, Roseto degli Abruzzi, Italy) impregnated with a concentration gradient of 0.016-256 µg/ml of the various antibiotics. The MIC was determined as being the lowest antibiotic concentration along the strip inhibiting *G. vaginalis* growth.

2.9 *G. vaginalis* prophage induction

Since the presence of prophage sequences in *G. vaginalis* genomes has been reported (59, 96), it was hypothesized that *L. gasseri* supernatant could induce prophages of *G.*

vaginalis, as *L. gasseri* is known to inhibit *G. vaginalis* growth (98) and produce hydrogen peroxide, a prophage-inducing agent (97). The ability of *L. gasseri* supernatant to induce prophages of *G. vaginalis* was thus tested. The *L. gasseri* supernatant was obtained from an overnight culture of *L. gasseri* in 0%SB that was centrifuged at 3500xg for 10 min before sterile-filtering (0.2 µm, GVS, Sanford, United States of America) the supernatant. Furthermore, UV-C radiation at 254 nm (TUV TL 8W Mini lamp, Phillips, Amsterdam, Netherlands), mitomycin C (Sigma-Aldrich, Missouri, United States of America) and ciprofloxacin (Sigma-Aldrich, Missouri, United States of America) were tested for their ability to induce prophages of *G. vaginalis* isolates, as they are commonly used to induce prophages of other bacteria (99). Prophage-inducing agents are commonly introduced to growing bacterial cultures during early log phase as this maximizes the chances of prophage induction (101). To this end, the growth kinetics of *G. vaginalis* needed to be determined.

2.9.1 Growth kinetics of *G. vaginalis*

Isolates of *G. vaginalis* from -80°C freezer stocks were grown anaerobically on 5%SB agar plates for 48 h at 37°C. A loopful of bacteria were grown anaerobically in 1.5 ml 5%SB for 48 h at 37°C. Cultures were standardized to an OD600nm of 0.1, in duplicate, in 15 ml or 50 ml 5%SB or Schaedler medium without blood (0%SB) and grown anaerobically for 48 h at 37°C. The growth of *G. vaginalis* was monitored by measuring the OD600nm of 100 µl of the growing cultures at 9 time points.

2.9.2 Prophage induction using *L. gasseri* supernatant

To optimize experimental conditions for prophage induction of *G. vaginalis* isolates using *L. gasseri* supernatant, dilutions of *L. gasseri* supernatant (0-50%) were performed on 9 h log-phase (OD600nm 0.7, range 0.5-0.8) *G. vaginalis* cultures that had started growing from an OD600nm of 0.1 in 0%SB. As a control, the same dilutions were performed using only 0%SB. Bacteria were grown anaerobically, in duplicate 150 µl volumes, in 96 well round bottom plates. Growth of *G. vaginalis* was monitored until

18 h after the addition of the *L. gasseri* supernatant by removing 50 µl of the growing culture at various time points to measure its OD600nm. In a subsequent experiment to induce prophages in a larger volume, *G. vaginalis* were subcultured in 50 ml 0%SB for 9 h (OD600nm of 0.34, range 0.3-0.4) before 25 ml *L. gasseri* supernatant was added to 25 ml of this culture. As a control, 25 ml log-phase cultures of *G. vaginalis* were grown with 25 ml Schaedler medium. Growth of *G. vaginalis* was quantified at 6, 12 and 20 h after the addition of the *L. gasseri* supernatant by removing 100 µl of the growing culture to measure its OD600nm. To determine the viability of the isolates grown in the presence of 50% *L. gasseri* supernatant, after the isolates had grown for a total of 29 h, a cotton swab was inoculated with the various cultures, streaked to form a lawn on 0%SB agar plates and incubated for 48 h at 37°C.

2.9.3 Prophage induction using UV radiation

To optimize experimental conditions for prophage induction of *G. vaginalis* isolates using UV radiation, 1.5 ml of 9 h log-phase bacteria in 0%SB were centrifuged at 589xg for 5min, 1.2 ml of the supernatant was discarded, 300 l of the log-phase *G. vaginalis* isolates were streaked on 0%SB agar plate and placed under UV radiation for 10-60 sec before collecting the cells with 1.5 ml 0%SB. A control of *G. vaginalis* isolates not irradiated with UV was included. Bacteria were grown anaerobically, in duplicate 150 µl volumes, in 96 well round bottom plates. Growth of *G. vaginalis* was quantified at 12 h and 18 h by removing 50 µl of the growing culture to measure its OD600nm.

2.9.4 Prophage induction using mitomycin C

For prophage induction using mitomycin C, *G. vaginalis* isolates were subcultured in 50 ml 5%SB for 16-18 h (OD600nm 4, range 3.5-4.5), upon where 0.5 µg/ml mitomycin C (which was diluted in 0.1mm sterile-filtered water, Sigma Aldrich, Missouri, United States of America) was added to the growing cultures. As a control, *G. vaginalis* isolates were subcultured in 50 ml 5%SB without mitomycin C. Growth of *G. vaginalis* was quantified at 24 h and 36 h after inoculation by removing 100 µl of the growing cultures

to measure their OD600nm. To determine the viability of the isolates grown in the presence of 0.5 µg/ml mitomycin C, after the isolates had grown for a total of 36 h, a cotton swab was inoculated with the various cultures, streaked to form a lawn on 5%SB agar plates and incubated for 48 h at 37°C. In a subsequent experiment to optimize experimental conditions for prophage induction using mitomycin C, two-fold serial dilutions of mitomycin C (0.5-16 µg/ml) were performed on 9 h log-phase *G. vaginalis* cultures (OD600nm 0.7, range 0.5-0.8) that had started growing from an OD600nm of 0.1 in 0%SB. As a control, *G. vaginalis* isolates were subcultured without mitomycin C. Growth of *G. vaginalis* was quantified 12 h and 18 h after the addition of mitomycin C, by removing 50 µl of the growing cultures to measure their OD600nm.

2.9.5 Prophage induction using ciprofloxacin

To optimize experimental conditions for prophage induction of *G. vaginalis* isolates using ciprofloxacin, two-fold serial dilutions of ciprofloxacin [0.06-20.5-16 µg/ml, which was diluted in 0.1M HCl (Sigma-Aldrich, Missouri, United States of America)] were performed on 9 h log-phase *G. vaginalis* cultures (OD600nm 0.7, range 0.5-0.8) that had started growing from an OD600nm of 0.1 in 0%SB. As a control, *G. vaginalis* isolates were subcultured without ciprofloxacin. Growth of *G. vaginalis* was quantified 12 h and 18 h after the addition of ciprofloxacin, by removing 50 µl of the growing culture to measure its OD600nm. In a subsequent experiment to induce prophages in a larger volume, *G. vaginalis* were subcultured in 15 ml 0%SB for 12 h (OD600nm of 0.6, range 0.5-0.7), upon where 2 µg/ml ciprofloxacin was added to the growing cultures. A control of growing *G. vaginalis* cultures without ciprofloxacin was included. Growth of *G. vaginalis* was quantified at 12, 24 and 36 h after the addition of ciprofloxacin, by removing 100 µl of the growing culture to measure its OD600nm. To determine the viability of the isolates grown in the presence of 2 µg/ml ciprofloxacin, after the isolates had grown for a total of 48 h, a cotton swab was inoculated with the various cultures, streaked to form a lawn on 0%SB agar plates and incubated for 48 h at 37°C.

2.9.6 Transmission electron microscopy of *G. vaginalis* phages

To visualize temperate phages following prophage induction, *G. vaginalis* supernatants were purified of bacterial cells and observed using transmission electron microscopy (TEM). A volume of 15 ml of sample was centrifuged at 3500xg for 10 min at 4°C to remove bacteria. The supernatant was treated with 10% (v/v) chloroform (Sigma-Aldrich, Missouri, United States of America) for 15 min to kill residual bacteria before centrifuging the supernatant, as before, to remove any remaining bacterial debris. Supernatants were sterile-filtered (0.2 µm) to further purify any phages that were present. A total of 12 ml per sample was centrifuged at 20 800xg for 60 min at 4°C before washing the pellet twice in sterile-filtered (0.2 µm) 0.1 M NH_4-acetate (Sigma-Aldrich, Missouri, United States of America), pH 7, which was diluted in distilled water. The pellet was resuspended in 50 µl 0.1 M NH_4-acetate. A volume of 10 µl of sample was incubated for 10 min on an ionized TEM grid (Agar Scientific, London, United Kingdom) and stained with 10 µl 2% (w/v) uranyl acetate (SPI Supplies, Pennsylvania, United States of America) before being analyzed under TEM (using an FEI Tecnai T20 TEM, Eindhoven, Netherlands), with the help of TEM specialist, Mr Mohammed A. Jaffer at the Aaron Klug Centre for Imaging and Analysis, University of Cape Town.

2.10 Statistical analysis

Pie charts were constructed using Microsoft Excel (Microsoft Corporation). All graphs and statistical analyses were performed using Prism, version 5 (GraphPad). Nonparametric, unpaired Mann-Whitney U tests and Fischer's exact tests were used to test for significance. A p-value of less than 0.05 ($p < 0.05$) was considered significant.

Chapter 3: Results

Recent evidence suggests that there is significant heterogeneity within the species of *G. vaginalis* and that not all genotypes of *G. vaginalis* may be involved in BV (27, 57, 67, 72). Thus, the genotypic and phenotypic diversity of 109 vaginal *G. vaginalis* isolates (kindly provided by Dr Rémy Froissart, Institut de Recherche pour le Développement, France) were characterized in this study. Genotypic diversity was investigated through *cpn60* phylogenetic analysis (section 3.1), while phenotypic diversity was investigated through evaluating sialidase activity (section 3.2), biofilm formation (section 3.3) and susceptibility to antibiotics used to treat BV (section 3.4). The presence of prophages in a panel of *G. vaginalis* isolates was also investigated (sections 3.5). Of the 109 *G. vaginalis* isolates used in this study, 75 (69%) were from BV-positive women (Nugent 7-10), 20 (18%) were from BV-intermediate women (Nugent 4-6) and 14 (13%) were from BV-negative women (Nugent 0-3, **Figure 3.1 B**).

3.1 Genotyping *G. vaginalis* isolates using *cpn60* sequence analysis

Previous whole genome sequence analysis as well as sequence analysis of a 552bp region of the *cpn60* housekeeping gene revealed that *G. vaginalis* isolates cluster in four subgroups, termed subgroups A-D (27, 57, 72). To genotype the 109 *G. vaginalis* isolates used in this study, *cpn60* phylogenetic analysis was performed (**Figure 3.1 A**). Included in the phylogenetic analysis were four reference sequences (obtained from cpnDB_nr; www.cpndb.ca), each previously determined to cluster in one of the four subgroups of *G. vaginalis* (72), including N143 from subgroup A, N153 from subgroup B, ATCC 14019 from subgroup C and 101 from subgroup D. Of the 109 isolates used in this study, 94 (86%) produced PCR products of the expected size (~600bp) and were sequenced. Of these, 90/94 (96%) produced high quality reads of sufficient size for alignment and phylogenetic analysis.

cpn60 sequences of the 90 *G. vaginalis* isolates clustered into four subgroups, with 13 subgroup A (14%), 17 subgroup B (19%), 58 subgroup C (64%) and 2 subgroup D (2%) isolates being detected in this collection (**Figure 3.1 A**). Subgroups A, B and C included isolates from women of all BV states, while subgroup D included isolates from BV-positive women only (**Figure 3.1 B**). Isolates from BV-positive women were the most prevalent across all subgroups. In summary, isolates from this study cluster in four subgroups, with subgroup D isolates being the least prevalent, while the majority of isolates cluster in subgroup C.

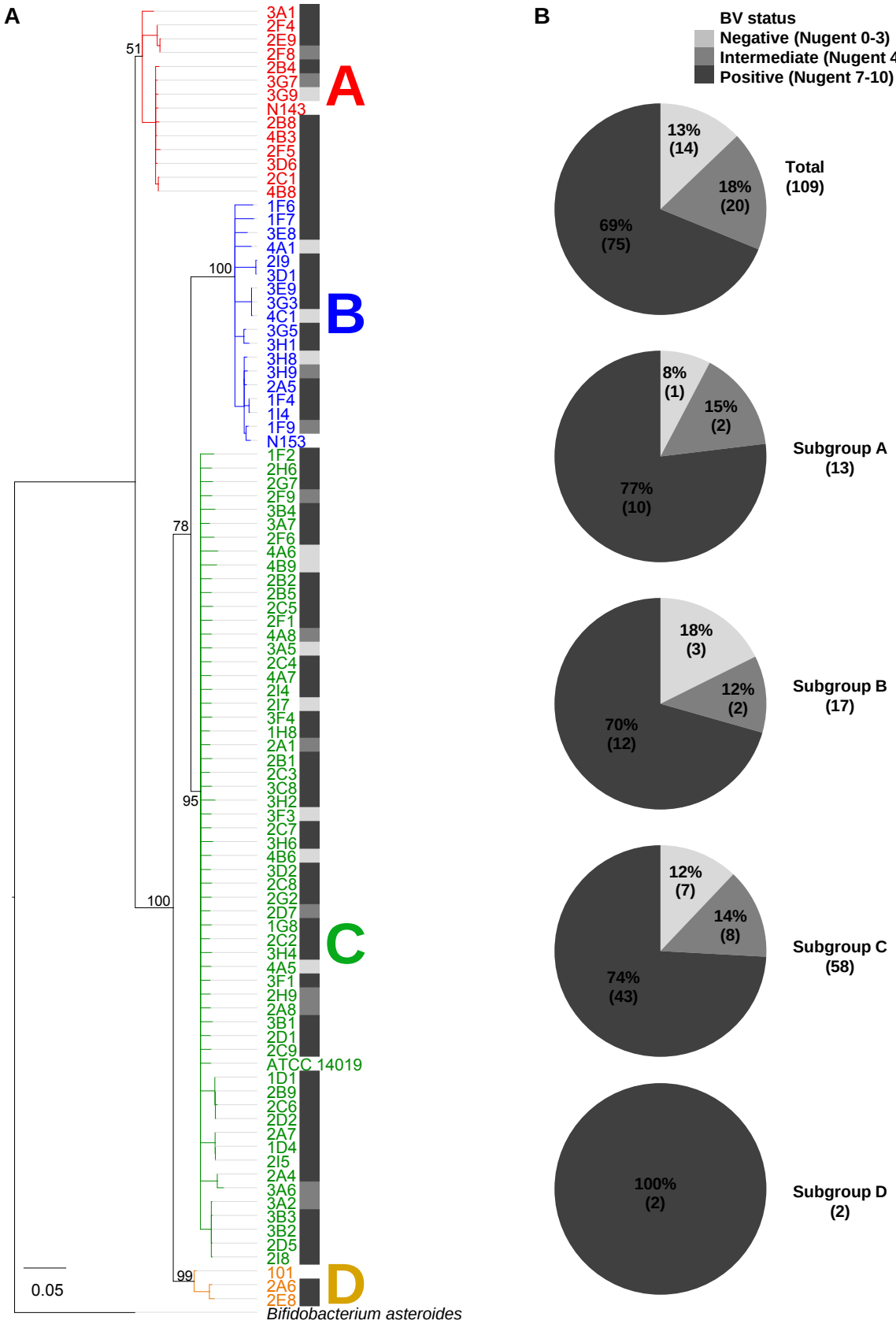

A
3A1
2F4
2E9
2F8
2B4
3G7
3G9
N143
2B8
4B3
2F5
3D6
2C1
4B8
1F6
1F7
3E8
4A1
2I9
3D1
3E9
3G3
4C1
3G5
3H1
3H8
3H9
2A5
1F4
1I4
1F9
N153
1F2
2H6
2G7
2F9
3B4
3A7
2F6
4A6
4B9
2B2
2B5
2C5
2F1
4A8
3A5
2C4
4A7
2I4
2I7
3F4
1H8
2A1
2B1
2C3
3C8
3H2
3F3
2C7
3H6
4B6
3D2
2C8
2G2
2D7
1G8
2C2
3H4
4A5
3F1
2H9
2A8
3B1
2D1
2C9
ATCC 14019
1D1
2B9
2C6
2D2
2A7
1D4
2I5
2A4
3A6
3A2
3B3
3B2
2D5
2I8
101
2A6
2E8
Bifidobacterium asteroides
51
100
78
95
100
99
A
B
C
D
0.05
B
BV status
Negative (Nugent 0-3)
Intermediate (Nugent 4-6)
Positive (Nugent 7-10)
13% (14)
18% (20)
69% (75)
Total (109)
8% (1)
15% (2)
77% (10)
Subgroup A (13)
18% (3)
12% (2)
70% (12)
Subgroup B (17)
12% (7)
14% (8)
74% (43)
Subgroup C (58)
100% (2)
Subgroup D (2)

Figure 3.1: *cpn60*-based phylogenetic analysis of *G. vaginalis* isolates. (A) *cpn60* sequences of the 90 isolates used in this study cluster in four clades (subgroups). Four sequences from a publically available *cpn60* database (cpnDB_nr, www.cpndb.ca) were included in the analysis to delineate subgroup A (N143, red), B (N153, blue), C (ATCC 14019, green), and D (101, gold) *G. vaginalis* clades, described in a previous study (72), while *Bifidobacterium asteroides* PRL2011 (also from cpnDB_nr) was used to root the tree. The tree was generated using the neighbor-joining method and 100 bootstrap replicates on Geneious (version 10.2.3, Biomatters). Super-imposed is a vertical bar indicating BV status, corresponding to the colour code in B. **(B)** Frequency of *G. vaginalis* isolates from BV-negative (Nugent 0-3), BV-intermediate (Nugent 4-6) and BV-positive women (Nugent 7-10). The first pie chart (Total) characterizes the entire collection of 109 *G. vaginalis* isolates while the rest of the pie charts characterize a subset (90/109) of the collection that is made up of isolates which *cpn60* was detected by PCR and sequenced. Number of isolates is indicated in brackets.

3.2.1 Sialidase gene presence in *G. vaginalis* isolates

G. vaginalis is known to produce sialidase, a mucolytic enzyme that cleaves sialic acids that make up the protective mucus layer lining the vaginal epithelium (80). Sialidase activity is detected in 69-84% of vaginal secretions from BV-positive women (27-29), in contrast to only 3.2-6% of vaginal secretions from BV-negative women (29, 30). It was hypothesized that during BV, sialidase breaks down protective vaginal mucus, allowing pathogenic bacteria to adhere to vaginal epithelium and form biofilms (81). It was recently shown that not all subgroups of *G. vaginalis* harbor the sialidase gene (27). To screen for the presence of the sialidase gene in *G. vaginalis* isolates in this study, PCR was carried using *G. vaginalis* sialidase-specific primers from previous studies (71, 72). Of the 109 *G. vaginalis* isolates used in this study, 75 produced PCR products of the expected size (~700bp) and were sequenced. All 75 isolates produced high quality reads of sufficient size for alignment and phylogenetic analysis.

The sialidase gene was detected at similar frequencies in 13/17 (76%) subgroup B and 53/58 (91%) subgroup C isolates ($p = 0.2$, Fischer's exact test), but was not detected in any of 13 subgroup A or 2 subgroup D isolates (**Figure 3.2 A**). Sialidase sequences of subgroup C isolates clustered together, separately from sialidase sequences of subgroup B isolates (**Figure 3.2 C**). The sialidase gene was detected at similar frequencies in *G. vaginalis* isolates from BV-negative (79%, 11/14), BV-intermediate

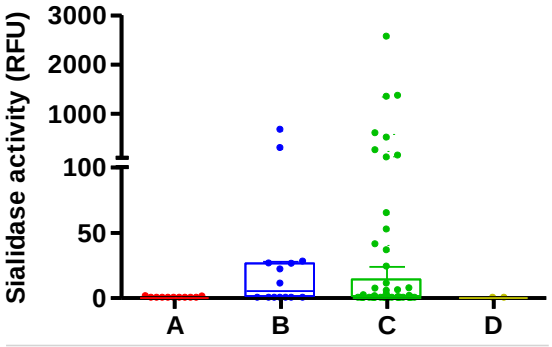
(50%, 10/20) and BV-positive (72%, 54/75) women ($p > 0.05$ for all comparisons, Fischer's exact tests, **Figure 3.2 B**). In summary, the sialidase gene was not detected in subgroup A and D isolates, was detected in the majority of subgroup B and C isolates and was detected at similar frequencies in isolates from women of different BV states.

3.2.2 Quantifying sialidase activity of *G. vaginalis* isolates

It was previously shown that sialidase gene presence is not predictive of sialidase enzymatic activity, particularly for *G. vaginalis* subgroup C isolates where only 9% (3/35) of sialidase-PCR-positive isolates had detectable sialidase activity (27). The sialidase activity of this collection of 109 *G. vaginalis* isolates was measured by quantifying the rate of hydrolysis of a fluorescent sialidase substrate. Sialidase activity was detected in only 36% (27/75) of sialidase-PCR-positive isolates (**Figure 3.2 B**). There was no significant difference in frequency of *G. vaginalis* isolates with detectable sialidase activity between subgroup B (41%, 7/17) and C (31%, 18/58) isolates ($p = 0.56$; Fischer's exact test, **Figure 3.2 A**). None of the subgroup A or D isolates had detectable sialidase activity, consistent with the lack of sialidase gene detection in these subgroups. Sialidase activity was detected at similar frequencies in *G. vaginalis* isolates from BV-negative (21%, 3/14), BV-intermediate (20%, 4/20) and BV-positive (27%, 20/75) women ($p > 0.05$ for all comparisons, Fischer's exact tests, **Figure 3.2 B**).

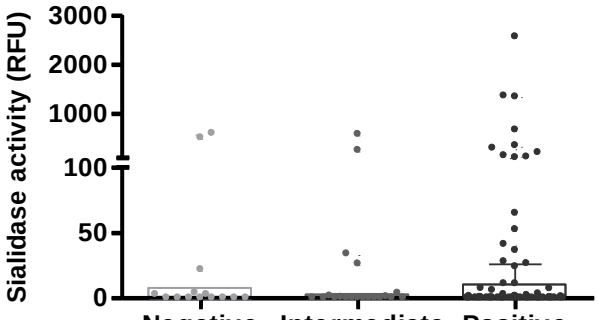
As sialidase activity was not detected in the majority of isolates, there was no significant difference between median sialidase activity of isolates from different subgroups or from women with different BV states ($p > 0.05$ for all comparisons, Mann-Whitney U tests). This was true even when statistical analysis was performed only on isolates that had detectable sialidase activity. In summary, the detection of the sialidase gene was only 36% predictive of sialidase activity. Additionally, sialidase activity was detected at similar levels in subgroup B and C isolates only, as well as isolates from women of different BV states.

A



	A	B	C	D
Activity +	0	7	18	0
PCR +	0	13	53	0
PCR -	13	4	5	2
#Isolates	13	17	58	2

B



	Negative	Intermediate	Positive
Activity +	3	4	20
PCR +	11	10	54
PCR -	3	10	21
#Isolates	14	20	75

C

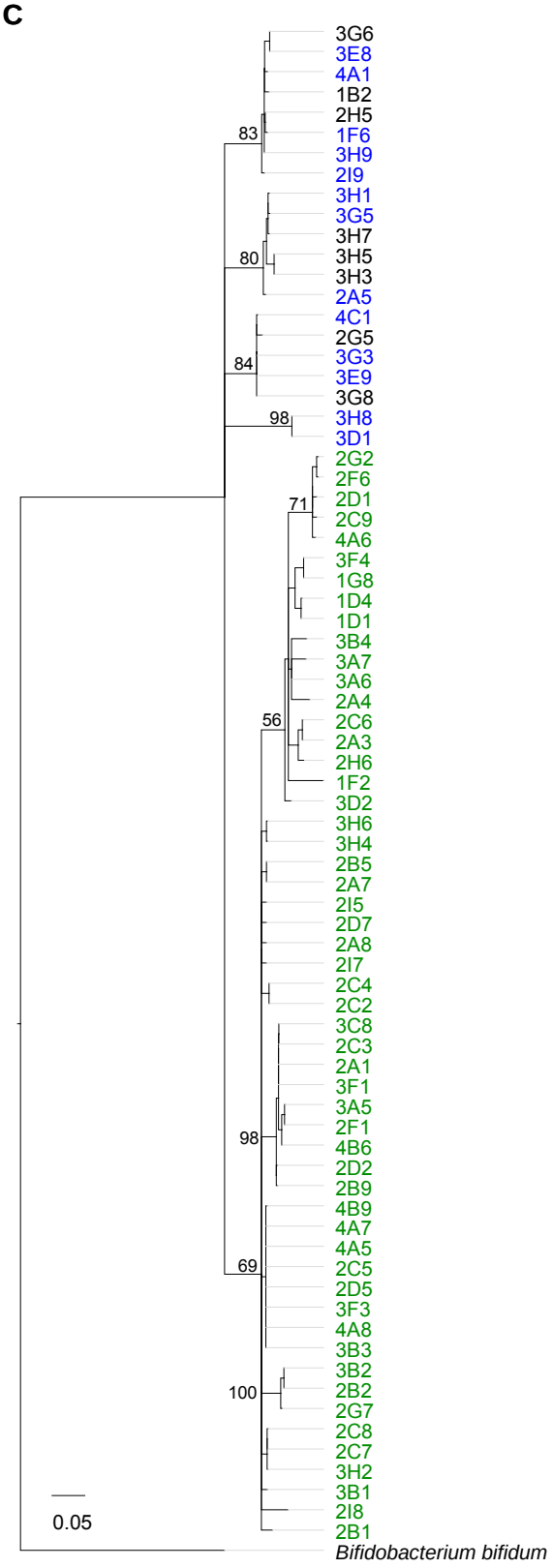


Figure 3.2: Sialidase gene presence and activity of *G. vaginalis* isolates. Screening *G. vaginalis* isolates for the detection of sialidase gene and activity from **(A)** different subgroups and **(B)** BV-negative (Nugent 0-3), BV-intermediate (Nugent 4-6) and BV-positive women (Nugent 7-10). Sialidase activity of stationary-phase *G. vaginalis* was measured using a fluorescent sialidase substrate and reported in relative fluorescence units (RFU) representing an average of 20 triplicate fluorescence readings per isolate, taken over 45 min and adjusted for cell concentration. Turkey box and whisker plots are shown. Each point represents an average of triplicate readings of one isolate. **(C)** Phylogenetic tree of 75 *G. vaginalis* sialidase sequences. *Bifidobacterium bifidum* ATCC 15696 from NCBI was used to root the tree. Different colours indicate *G. vaginalis cpn60*-based subgroups, with blue indicating subgroup B, green indicating subgroup C and black indicating *cpn60* PCR-negative isolates. The tree was generated using the neighbor-joining method and 100 bootstrap replicates on Geneious (Biomatters).

3.3 Quantifying biofilm formation of *G. vaginalis* isolates

G. vaginalis is thought to play a critical role during BV pathogenesis by initiating the formation of biofilm, which subsequently allows other pathogenic bacteria to colonize the female genital tract, forming a polymicrobial biofilm (44). A previous qualitative study revealed that isolates from all subgroups of *G. vaginalis* are able to form biofilm; although, the amount of biofilm formed was not quantified (72). To quantify biofilm formation in this study, *G. vaginalis* biofilms in 96 well plates were stained with crystal violet dye and measured at OD550nm, as previously described (102). Biofilm formation of subgroup A (median 0.16, range 0.10-1.96), subgroup B (median 0.22, range 0.10-1.75) and subgroup C isolates (median 0.15, range 0.07-2.15) was similar ($p > 0.05$ for all comparisons, Mann-Whitney U tests, **Figure 3.3 A**). Biofilm formation of the two subgroup D isolates was also measured (average 1.36, range 0.97-1.78) but could not be statistically compared to the other subgroups as a minimum of three values is required to perform a Mann-Whitney U test. Biofilm formation of isolates from BV-negative women (median 0.30, range 0.1-1.7), BV-intermediate women (median 0.25, range 0.1-2.0) and BV-positive women (median 0.20, range 0.1-2.1) was similar ($p > 0.05$ for all comparisons, Mann-Whitney U tests, **Figure 3.3 B**). Taken together, these data suggest that isolates from all subgroups of *G. vaginalis* as well as isolates from women of all BV states could form similar amounts of biofilm.

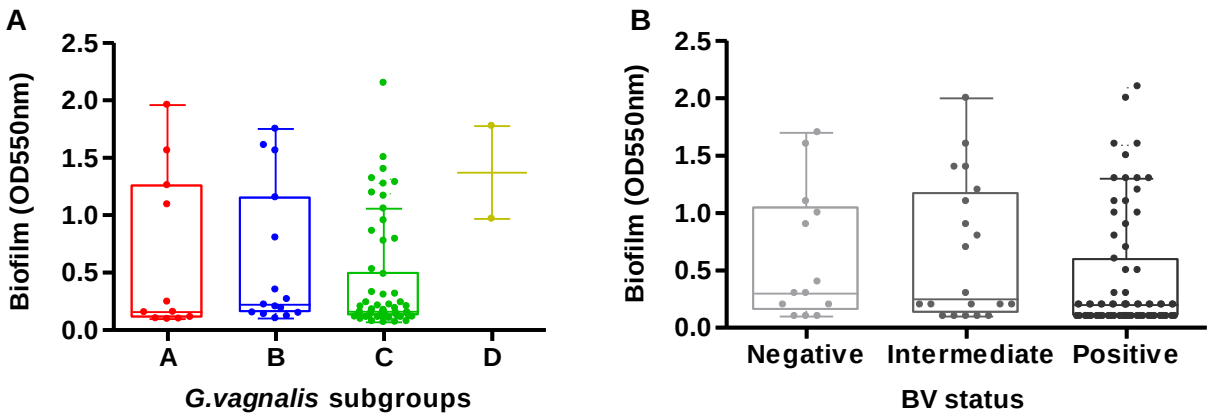


Figure 3.3: Biofilm formation of *G. vaginalis* isolates. Biofilm formation of stationary-phase *G. vaginalis* isolates was stained with crystal violet dye and measured. **(A)** Biofilm formation of *G. vaginalis* subgroups. **(B)** Biofilm formation of *G. vaginalis* isolates from BV-negative (Nugent 0-3), BV-intermediate (Nugent 4-6) and BV-positive women (Nugent 7-10). Turkey box and whisker plots are shown. Each point represents a reading of one isolate, obtained from an average of three independent experiments, each with 8 replicates per isolate.

3.4 Antibiotic susceptibility of *G. vaginalis* isolates

As *G. vaginalis* plays such a central role in BV, its susceptibility to antibiotics used to treat BV influences treatment outcomes (87). The antibiotic susceptibility of *G. vaginalis* isolates to metronidazole and clindamycin, the first-line recommended antibiotics for the treatment of BV (38), was thus tested. Given the substantial amount of metronidazole and clindamycin BV treatment failure as well as high BV recurrence rates (6, 39), alternative antibiotics to treat BV are being explored, including moxifloxacin and augmentin, which have both demonstrated clinical efficacy in the treatment of BV (90, 91). Thus, the antibiotic susceptibility of *G. vaginalis* isolates to moxifloxacin and augmentin was also tested. Antibiotic susceptibility was tested by growing *G. vaginalis* isolates in the presence of MIC test strips (containing 0.016-256 µg/ml of the various antibiotics), in a lawn, on 5% blood-supplemented Brucella agar, as recommended by the manufacturer. However, antibiotic susceptibility was measured for only 45/109 *G. vaginalis* isolates as the remainder of the isolates could not form a lawn on 5% blood-supplemented Brucella agar.

Metronidazole had the highest median MIC (256 µg/ml) for the 45 tested *G. vaginalis* isolates, followed by moxifloxacin (0.25 µg/ml), clindamycin (0.094 µg/ml), and then augmentin (0.047 µg/ml, $p < 0.0001$ for all comparisons, Mann-Whitney U tests, **Figure 3.4 A**). Previous studies described breakpoints for *G. vaginalis* resistance to metronidazole as 32 µg/ml (41), clindamycin as 4 µg/ml (42), moxifloxacin as 0.5 µg/ml (103), and augmentin as 8 µg/ml (42). Using these breakpoints for resistance, 71% (32/45) of isolates were resistant to metronidazole, 9% (4/45) were resistant to moxifloxacin, while none were resistant to clindamycin or augmentin (**Figure 3.4 A**). Of the isolates that were resistant to metronidazole, 84% (27/32) were highly resistant, as defined as having an MIC ≥ 256 µg/ml, the highest concentration of antibiotic along the MIC test strip. This level of resistance was not observed for clindamycin, moxifloxacin or augmentin. Metronidazole resistance was observed across all subgroups of *G. vaginalis*, while moxifloxacin resistance was only observed in subgroup C isolates (**Figure 3.4 B**). Similarly, metronidazole resistance was observed across isolates from women of all BV states, while moxifloxacin resistance was only observed in isolates from BV-positive women (**Figure 3.4 C**). Taken together, these data reveal that the majority of *G. vaginalis* isolates were resistant to metronidazole, the most commonly used antibiotic to treat BV, while being sensitive to clindamycin, moxifloxacin and augmentin.

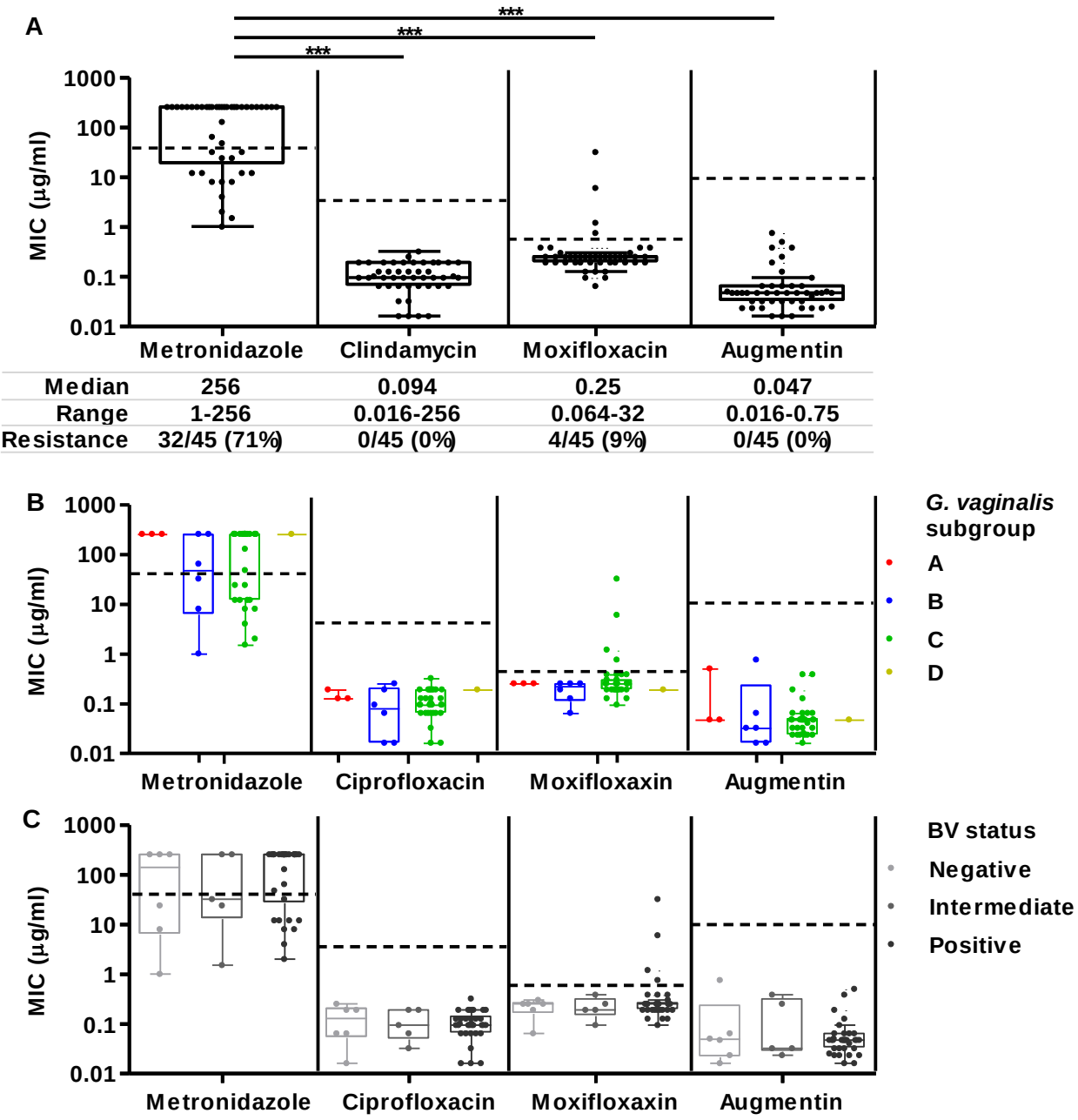


Figure 3.4: Antibiotic susceptibility of *G. vaginalis* isolates. Metronidazole, clindamycin, moxifloxacin and augmentin MICs of *G. vaginalis* isolates grown on 5% blood-supplemented Brucella agar were determined using MIC test strips for **(A)** a total of 45 *G. vaginalis* isolates; **(B)** 3, 6 and 33 and 1 isolate from subgroup A, B, C and D, respectively and **(C)** 6, 5 and 34 isolates from BV-negative, BV-intermediate and BV-positive women, respectively. Turkey box and whisker plots are shown. Each point represents one isolate. Horizontal broken lines mark the breakpoints for *G. vaginalis* resistance to metronidazole (32 µg/ml), clindamycin (4 µg/ml), moxifloxacin (0.5 µg/ml) and augmentin (8 µg/ml), as described in previous studies (41, 42, 103). Fischer's exact tests ($p < 0.0001$) were used to test for statistical significant differences in frequency of resistant isolates per antibiotic.

3.5 Prophage induction of *G. vaginalis*

Prophage sequences have previously been found in genomes of *G. vaginalis* (59, 96); however, phages of *G. vaginalis* have never been isolated. *L. gasseri* produce hydrogen peroxide, a prophage-inducing agent known to inhibit *G. vaginalis* growth (97, 98). It was thus hypothesized hydrogen peroxide released by *L. gasseri* inhibits *G. vaginalis* growth by inducing prophages of *G. vaginalis*. The ability of *L. gasseri* culture supernatant to induce prophages of *G. vaginalis* was thus tested. Additionally, mitomycin C, ciprofloxacin and UV radiation are commonly used to induce prophages of other bacteria (99, 104); thus, these prophage-inducing agents were tested for their use as positive controls in the induction of *G. vaginalis* prophages

3.5.1 Optimizing experimental conditions for prophage induction of *G. vaginalis*

In order to induce prophages of *G. vaginalis,* isolates were initially grown in 5% blood-supplemented Schaedler medium, in the presence or absence of prophage-inducing agents. However, mitomycin C, ciprofloxacin and *L. gasseri* supernatant appeared to cause red blood cells lysis as they changed the colour of the blood-supplemented medium from red to yellow. It was thus necessary to grow *G. vaginalis* isolates in Schaedler medium without blood in subsequent prophage induction experiments. In our laboratory, prophage induction experiments are often conducted using 50 ml culture volumes as this volume is large enough to yield enough phage particles to observe under TEM and perform phage DNA extraction for whole genome sequencing. However, only 15 ml is required to yield enough phage particles to observe under TEM. It was thus decided that the prophage induction optimization experiments would be conducted using 15 ml culture volumes until productive prophage induction was suspected and phage DNA needed to be extracted.

Because prophage-inducing agents are commonly added to early log-phase bacterial cultures (101), the growth kinetics of *G. vaginalis* needed to be determined. To do this, three *G. vaginalis* isolates, 3G7 (red) from subgroup A, 3H8 (blue) from subgroup B and

3B3 (green) from subgroup C, were standardized to an OD600nm of 0.1 in 5% blood-supplemented Schaedler medium (5%SB) or Schaedler medium without blood (0%SB) and their growth was monitored over 48 h. At stationary phase, growth of all three *G. vaginalis* isolates was two times higher in the presence (**Figure 3.5 A**) than in the absence (**Figure 3.5 B**) of blood. Growth kinetics of *G. vaginalis* did not seem to be affected by volume as, when grown in the absence of blood, growth kinetics of *G. vaginalis* were similar in 50 ml (**Figure 3.5 B**) and 15 ml (**Figure 3.5 C**) culture volumes. In the presence of blood, *G. vaginalis* isolates had a lag phase of 7 h, grew exponentially between 7-18 h, slowing down at 18-36 hours, before reaching stationary phase after 36-48 h (**Figure 3.5 A**). In contrast, the absence of blood, *G. vaginalis* growth was more modest during the exponential phase (7-18 h), after which no further growth was evident between 18-48 h (**Figure 3.5 B-C**). It was concluded that, even though *G. vaginalis* isolates grew to higher cell density in the presence of blood, isolates grew satisfactorily in the absence of blood. Thus, isolates were grown in the absence of blood in subsequent prophage induction experiments.

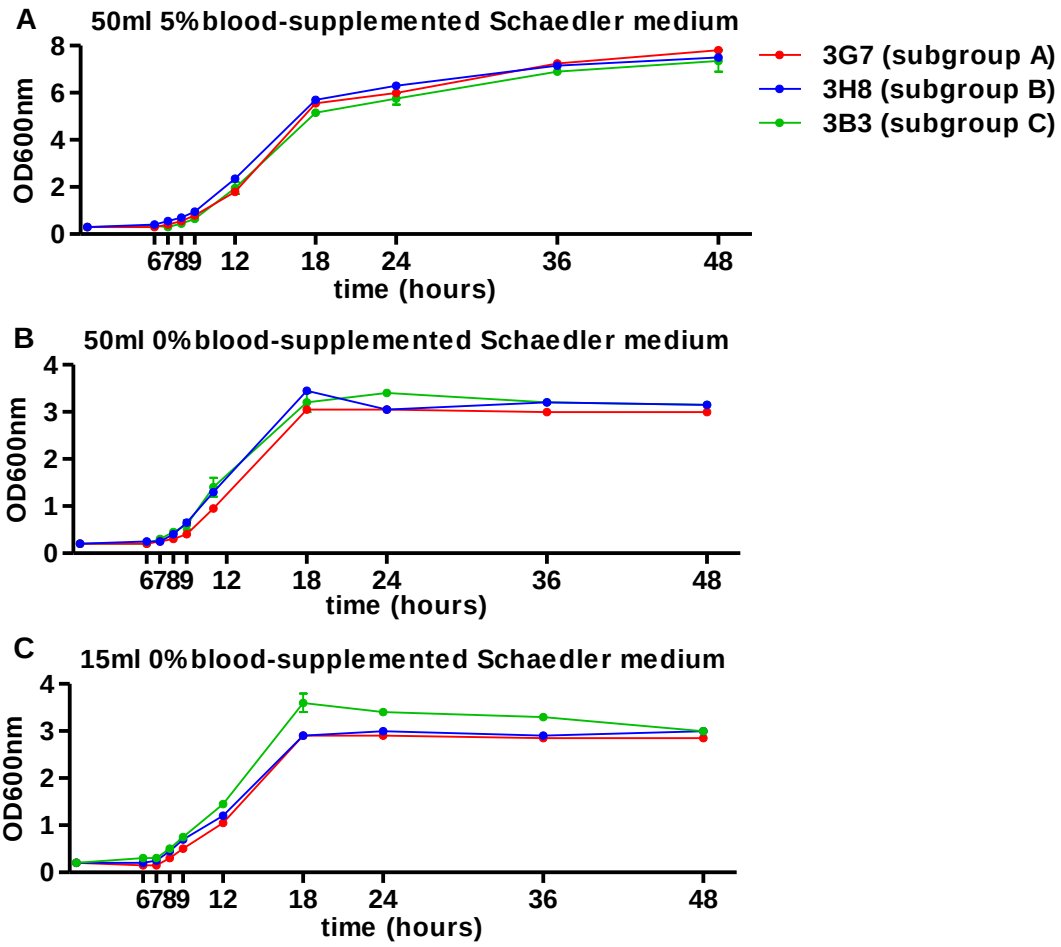


Figure 3.5: Growth kinetics of *G. vaginalis* isolates. *G. vaginalis* standardized to an OD600nm of 0.1 were subcultured in Schaedler medium with or without blood for 48 h. Growth kinetics of *G. vaginalis* in **(A)** 50 ml 5%SB, **(B)** 50 ml 0%SB and **(C)** 15 ml 0%SB were measured at 9 time points for three isolates from subgroup A (3G7, red), B (3H8, blue) and C (3B3, green). Graphs represent an average of two independent experiments. Error bars represent SEM.

3.5.2 *G. vaginalis* prophage induction using *L. gasseri* supernatant

The culture supernatant of one *L. gasseri* isolate, known to produce ~10µM hydrogen peroxide (determined by Ms Anna Happel; PhD candidate, Division of Medical Virology, University of Cape Town), was tested for its ability to induce prophages of *G. vaginalis*. *L. gasseri* supernatant at a final concentration of 50% (v/v) inhibited *G. vaginalis* growth

(79% average, range, 68-88% at 12 h; 74% average, range 73-75% at 18 h) but didn't affect *G. vaginalis* growth at lower concentrations of 0.03-33% (**Figure 3.6 A-B**).

Inhibition of *G. vaginalis* growth in the presence of 50% *L. gasseri* supernatant may have been due to *G. vaginalis* being most dilute in this dilution (50% *G. vaginalis*) compared to the other dilutions (67%-100% *G. vaginalis*), thus resulting in *G. vaginalis* being unable to grow as high as in the other dilutions. To investigate this, the experiment was repeated using 50% *L. gasseri* supernatant as well as 50% Schaedler medium control. As there was no reduction in *G. vaginalis* growth in the 50% Schaedler medium control (**Figure 3.6 C**), it was concluded that the reduction in *G. vaginalis* growth in the 50% *L. gasseri* supernatant dilution was due to *L. gasseri* supernatant contents, possibly hydrogen peroxide.

Since the 50% *L. gasseri* supernatant concentration was the most potent *G. vaginalis*-inhibiting concentration, the ability of 50% *L. gasseri* supernatant to induce prophages of *G. vaginalis* was tested using larger 50 ml culture volumes as to potentially increase the number of any present phage particles in order to characterize them under TEM. Growth of log-phase cultures of *G. vaginalis* was inhibited by the presence of 50% *L. gasseri* supernatant, while growth of log-phase cultures of *G. vaginalis* was unaffected by the presence of 50% Schaedler medium (**Figure 3.6 D**). All three *G. vaginalis* isolates grown in the presence of 50% *L. gasseri* supernatant failed to grow when subcultured on 0%SB agar; in contrast, all isolates grown in the presence of 50% Schaedler medium grew (data not shown). TEM analysis did not reveal the presence of phages in any of the log-phase cultures of *G. vaginalis* grown in the presence of 50% *L. gasseri* supernatant.

To successfully induce prophages, prophage-inducing agents need to be used at concentrations that are high enough to induce prophages but are sub-lethal to the bacterial host (101). The observation that *G. vaginalis* did not grow between 12 and 18

h after log phase in the 50% *L. gasseri* supernatant dilution (**Figure 3.6 A-B**) suggests that this concentration of *L. gasseri* supernatant was too high to allow *G. vaginalis* growth. It was also observed that 33% *L. gasseri* supernatant was too low to decrease *G. vaginalis* growth, as its growth was similar to the 0% *L. gasseri* supernatant control (**Figure 3.6 A-B**). It was thus hypothesized that there could exist a concentration of *L. gasseri* supernatant between 33-50% that would be high enough to induce prophages but not too high as to inhibit *G. vaginalis* growth. Additionally, since growth was monitored for the first time at 6 h after the introduction of *L. gasseri* supernatant (**Figure 3.6 D**), it is possible that *G. vaginalis* growth peaked and decreased between 0-6 h, leading to the false conclusion that *G. vaginalis* did not grow between the first 6 h after the introduction of *L. gasseri* supernatant. To address both concerns, several dilutions of *L. gasseri* supernatant (ranging between 33-50%) were introduced to log-phase cultures of *G. vaginalis* and growth was monitored after 2, 4, 6, 12 and 18 h. *G. vaginalis* steadily increased in growth from 2-18 h at all tested concentrations of *L. gasseri* supernatant (**Figure 3.6 E**), albeit at a slower rate than the 0% *L. gasseri* supernatant control (**Figure 3.6 F**). From 6 h after the introduction of *L. gasseri* supernatant, there was a trend of higher *G. vaginalis* growth with decreasing *L. gasseri* supernatant concentration (**Figure 3.6 E**). There was no difference in growth kinetics of *G. vaginalis* at all time points in all Schaedler medium control dilutions (50-33%) compared to the 0% Schaedler medium (100% *G. vaginalis*) control (**Figure 3.6 F**). Altogether, these data suggest that *L. gasseri* supernatant did not induce prophages of *G. vaginalis* isolates under the tested experimental conditions.

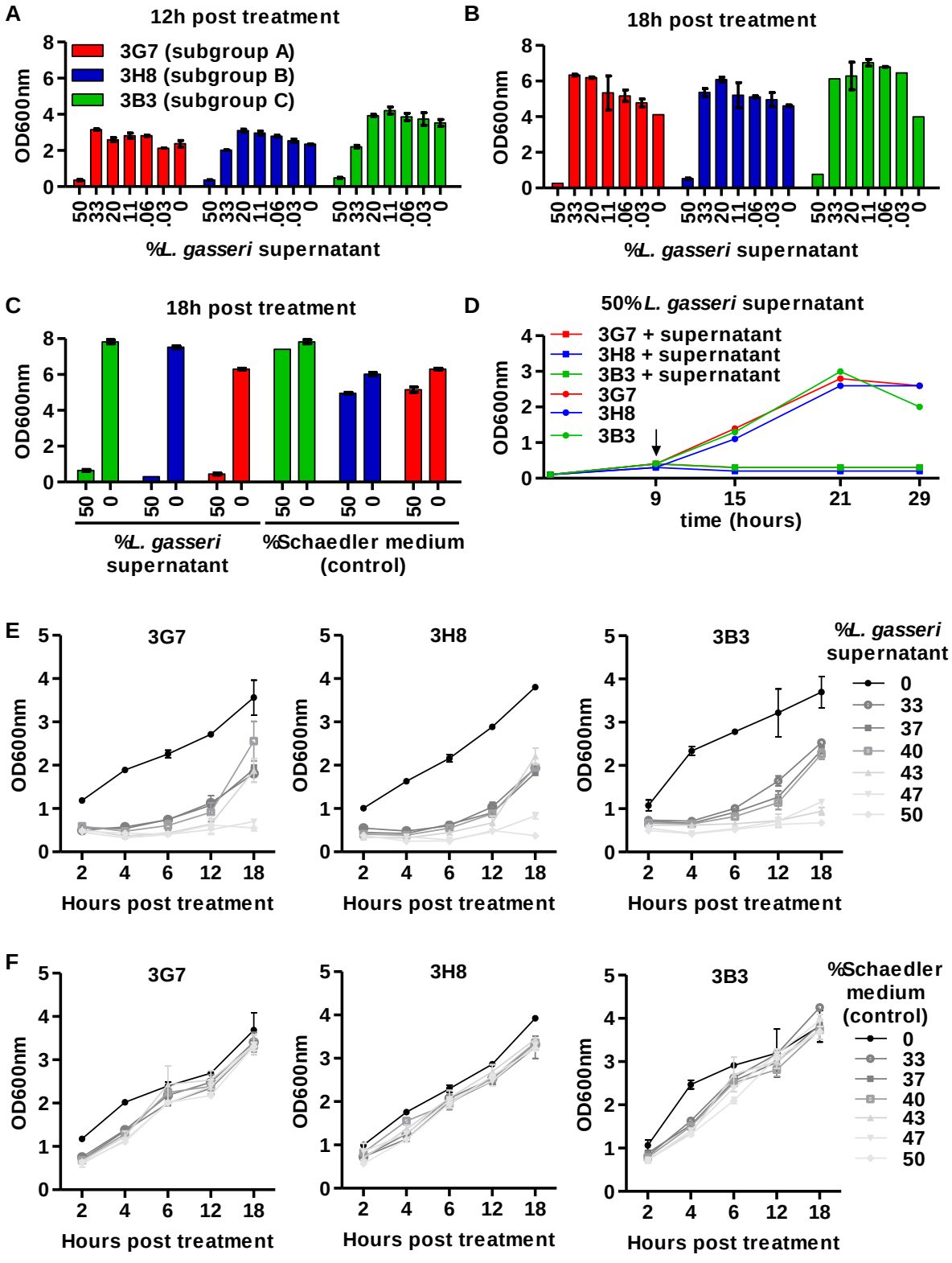

A
12h post treatment
3G7 (subgroup A)
3H8 (subgroup B)
3B3 (subgroup C)
OD600nm
%L. gasseri supernatant
B
18h post treatment
OD600nm
%L. gasseri supernatant
C
18h post treatment
OD600nm
%L. gasseri supernatant
%Schaedler medium (control)
D
50% L. gasseri supernatant
3G7 + supernatant
3H8 + supernatant
3B3 + supernatant
3G7
3H8
3B3
OD600nm
time (hours)
E
3G7
3H8
3B3
OD600nm
Hours post treatment
%L. gasseri supernatant
0
33
37
40
43
47
50
F
3G7
3H8
3B3
OD600nm
Hours post treatment
%Schaedler medium (control)
0
33
37
40
43
47
50

Figure 3.6: Optimizing experimental conditions for *G. vaginalis* prophage induction using *L. gasseri* supernatant. Growth of three *G. vaginalis* isolates, 3G7 (red) from subgroup A, 3H8 (blue) from subgroup B and 3B3 (green) from subgroup C, in the presence of 0.03-50% *L. gasseri* supernatant was measured after **(A)** 12 h and **(B)** 18 h. In a subsequent experiment, **(B)** growth of *G. vaginalis* isolates in the presence of 50% *L. gasseri* supernatant or 50% Schaedler medium control was measured after 18 h. **(D)** Growth kinetics of *G. vaginalis* isolates, in the presence (squares) or absence (circles) of 50% *L. gasseri* supernatant, was monitored over 29 h. A volume of 25 ml *L. gasseri* supernatant was introduced to 25 ml *G. vaginalis* cultures at log-phase (black arrow). In a subsequent experiment, growth kinetics of three *G. vaginalis* isolates was monitored for 2-18 h after the introduction of 33-50% **(E)** *L. gasseri* supernatant or **(F)** Schaedler medium. In all experiments, *L. gasseri* supernatant was introduced to log-phase (9 h, OD600nm 0.3-0.4) cultures of *G. vaginalis* and grown in 150-500 µl culture volumes in Schaedler medium unless otherwise stated. Error bars represent SEM of duplicate cultures.

3.5.3 *G. vaginalis* prophage induction using UV radiation

UV radiation is known to be potent inducer of prophages of other bacteria (101); thus, its ability to induce prophages of *G. vaginalis* isolates was tested. Log-phase cultures of three *G. vaginalis* isolates were irradiated with UV light at 254 nm for 10-60s. There appeared to be no difference in growth of UV-irradiated isolates compared to non-irradiated controls at all tested durations, both at 12 h and 18 h after UV irradiation (**Figure 3.7 A-B**), suggesting that *G. vaginalis* isolates were not sensitive to UV radiation and that *G. vaginalis* prophages could not be induced under the tested experimental conditions.

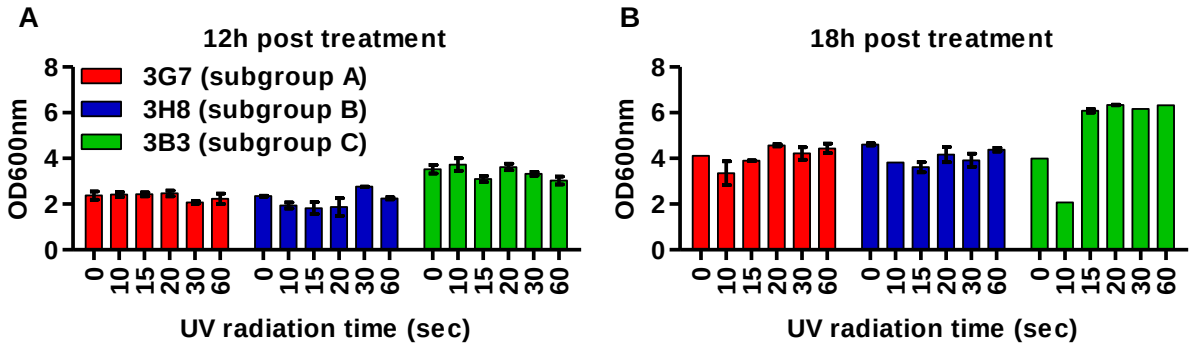


Figure 3.7: Optimizing experimental conditions for *G. vaginalis* prophage induction using UV radiation. Three *G. vaginalis* isolates, 3G7 (red) from subgroup A, 3H8 (blue) from subgroup B and 3B3 (green) from subgroup C, cultured in 150 µl 0%SB, were irradiated with UV light at 254 nm for 10-60s during log phase (9 h, OD600nm 0.3-0.4). Growth was measured at **(A)** 12 h and **(B)** 18 h after UV irradiation. Error bars represent SEM of duplicate cultures.

3.5.4 *G. vaginalis* prophage induction using mitomycin C

Mitomycin C is one of the most commonly used prophage-inducing agents (101), having recently induced prophages of *Bifidobacterium* species (105), the closest relatives of *G. vaginalis* (106). Initially, 0.5 µg/ml mitomycin C was tested for its ability to induce prophages of *G. vaginalis* in 50 ml 5%SB, as this is the most commonly used mitomycin C concentration to induce prophages of other bacteria in our laboratory. Based on optical density measurements, growth kinetics of all three isolates grown in the presence of 0.5 µg/ml mitomycin C were similar to growth kinetics of isolates grown in the absence of mitomycin C (**Figure 3.8 A**). All three isolates grown in the presence of 0.5 µg/ml mitomycin C failed to grow when subcultured on 5%SB agar; in contrast, control isolates grown in the absence of mitomycin C grew (data not shown). TEM analysis did not reveal the presence of phages in any of the isolates grown in the presence of 0.5 µg/ml mitomycin C.

It was hypothesized that this concentration of mitomycin C (0.5 µg/ml) may have been too low to induce prophages of *G. vaginalis* isolates. To optimize experimental conditions for prophage induction of *G. vaginalis* isolates using mitomycin C, higher concentrations of mitomycin C (two-fold dilution series, 0.5-16 µg/ml) were tested for their ability to induce prophages of *G. vaginalis* isolates in 150 µl 0%SB. All isolates displayed a general trend of decreasing growth with increasing mitomycin C concentrations (**Figure 3.8 B-C**). Although, this was likely not due to prophage induction, as all isolates continued to grow between 12 h and 18 h. As with *G. vaginalis* isolates grown in the presence 0.5 µg/ml mitomycin C, TEM analysis of *G. vaginalis* isolates grown in the presence of 8 µg/ml mitomycin C in 15 ml 0%SB did not reveal the presence of phages.

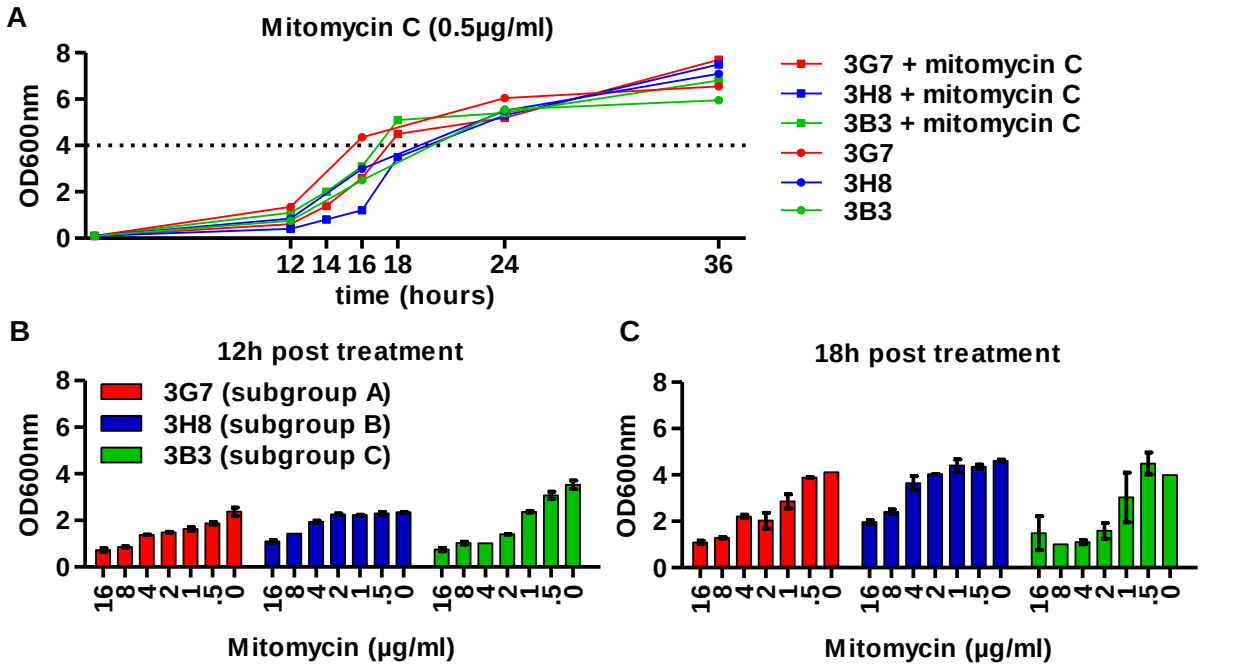


Figure 3.8: Optimizing experimental conditions for *G. vaginalis* prophage induction using mitomycin C. Three *G. vaginalis* isolates, 3G7 (red) from subgroup A, 3H8 (blue) from subgroup B and 3B3 (green) from subgroup C were cultured in **(A)** 50 ml 5%SB, in the presence (squares) and absence (circles) of 0.5 µg/ml mitomycin C introduced at log phase (dotted line, 16-18 h, OD600nm 4, range 3.5-4.5) and growth was monitored over 36 h. In a subsequent experiment, growth of three *G. vaginalis* isolates, cultured in 150 µl 0%SB was measured at **(B)** 12 h and **(C)** 18 h after the introduction of 0.5-16 µg/ml mitomycin C during log phase (9 h, OD600nm 0.3-0.4). Error bars represent SEM of duplicate cultures.

3.5.5 *G. vaginalis* prophage induction using ciprofloxacin

Ciprofloxacin is a fluoroquinolone, a family of antibiotics commonly used to induce prophages of other bacteria (101). To optimize experimental conditions for prophage induction of *G. vaginalis* isolates using ciprofloxacin, various concentrations of ciprofloxacin (two-fold dilution series, 0.06-2 µg/ml) were tested for their ability to induce prophages of *G. vaginalis* isolates in 150 µl 0%SB. Compared to lower concentrations, 2 µg/ml ciprofloxacin was the most potent *G. vaginalis*-inhibiting concentration (**Figure 3.9 A-B**). Therefore, the ability of 2 µg/ml ciprofloxacin to induce prophages of *G. vaginalis* was tested using larger 15 ml culture volumes.

In the presence of ciprofloxacin, *G. vaginalis* isolates grew slower and to a lower cell density compared to control isolates grown in the absence of ciprofloxacin (**Figure 3.9 C**). All three isolates grown in the presence of 2 µg/ml ciprofloxacin failed to grow when subcultured on 0%SB agar; in contrast, control isolates grown in the absence of ciprofloxacin grew (data not shown).

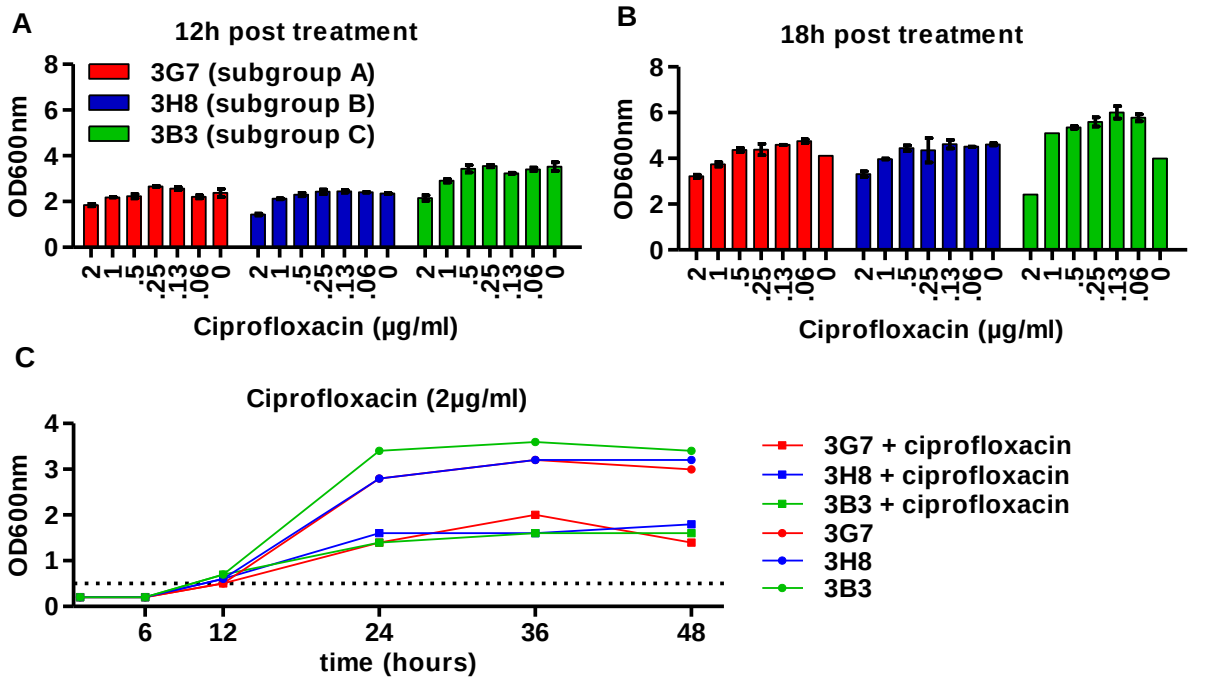


Figure 3.9: Optimizing experimental conditions for *G. vaginalis* prophage induction using ciprofloxacin. Growth of three *G. vaginalis* isolates, 3G7 (red) from subgroup A, 3H8 (blue) from subgroup B and 3B3 (green) from subgroup C, cultured in 150 µl 0%SB was measured at **(A)** 12 h and **(B)** 18 h after the introduction of 0.03-2 µg/ml ciprofloxacin during log phase (9 h, OD600nm 0.3-0.4). Error bars represent SEM of duplicate cultures. **(C)** *G. vaginalis* isolates were cultured in 15 ml 0%SB, in the presence (squares) and absence (circles) of 2 µg/ml ciprofloxacin introduced at log phase (dotted line, 12 h, OD600nm 0.6, range 0.5-0.7) and growth was monitored over 48 h.

The supernatants of the 15 ml cultures of *G. vaginalis* isolates grown in the presence of 2 µg/ml ciprofloxacin were investigated for the presence of temperate phages under TEM, revealing the presence of phage-like particles from at least one (3G7) out of three tested *G. vaginalis* isolates (**Figure 3.10 A-B**). These phage-like particles most

resembled phages belonging to the family of *Podoviridae*, which shown in **Figure 3.10 C** adapted from Lawrence *et al.* (107), because similar to *Podoviridae* phages, these phage-like particles display a head and a short tail with a length of ~30 nm, which is within the typical 10-46 nm range of *Podoviridae* tails (108). While *Podoviridae* tails often have tail-spikes as can be seen in **Figure 3.10 C**, tail spikes were not observed in any of the micrographs of the phage-like particles obtained in this study. This observation was also made in a previous study by Garcia-Heredia *et al.* (109), where phages that were shown to belong to the *Podoviridae* family by whole genome sequence analysis did not display tail-spikes on their micrographs (**Figure 3.10 D**). With the exception of one phage-like particle displayed in **Figure 3.10 A**, the phage-like particles from this study displayed an irregular-shaped head (**Figure 3.10 B**), and not the icosahedral head (**Figure 3.10 C**) that characterizes *Podoviridae* phages (108). Moreover, the head diameter of these phage-like particles was ~50 nm, which is 10 nm below the 60-70 nm range of *Podoviridae* heads (108). Therefore, these phage-like particles could not be confidently classified into any known phage families. Eight *G. vaginalis* isolates could not be infected by these phage-like particles (data not shown). To confirm the phage status of these particles, a suitable host to propagate them would need to be identified and whole genome sequencing of the phages would need to be performed in order for the phages to be characterized.

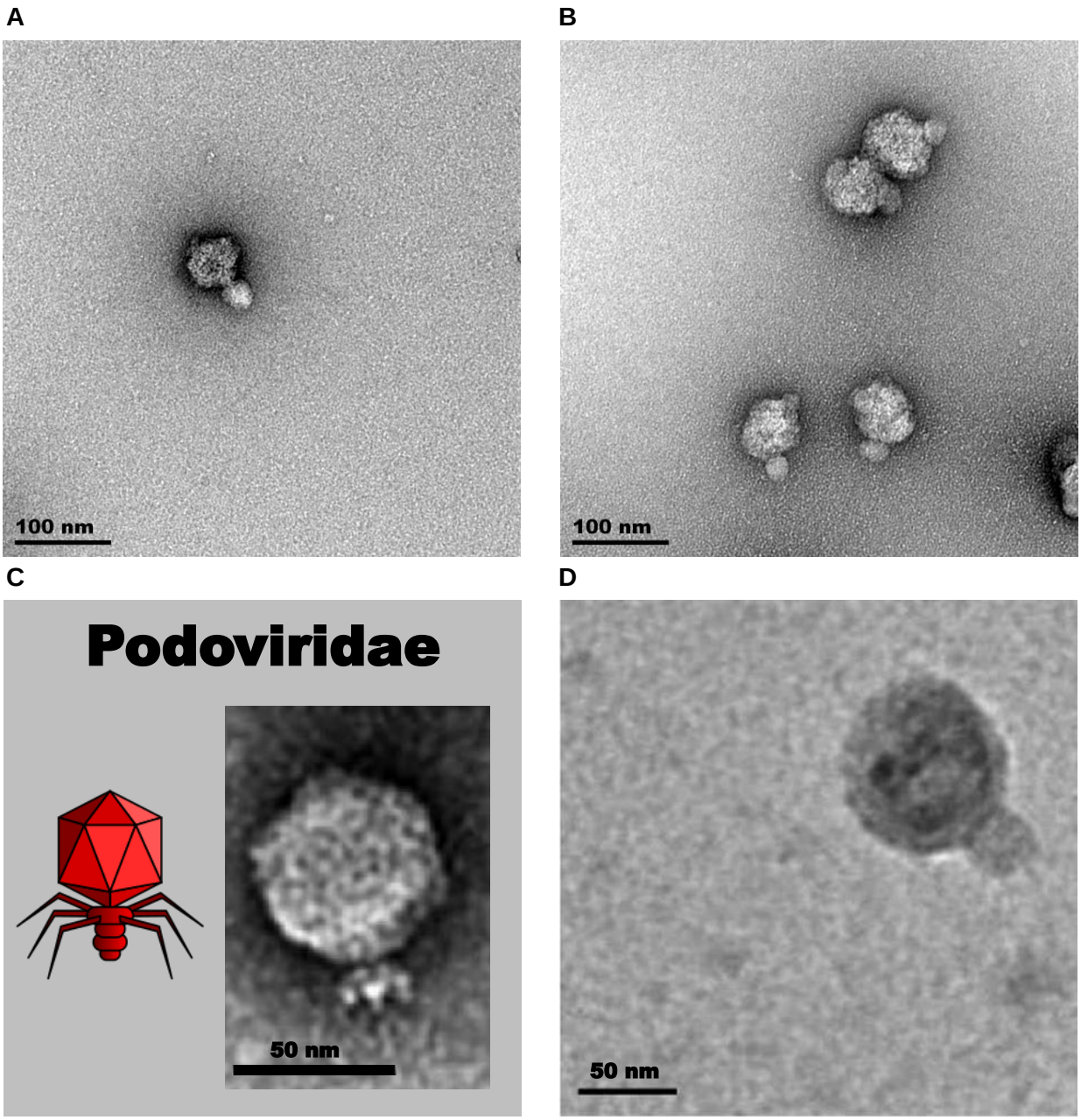


Figure 3.10: Micrographs *G. vaginalis* phage-like particles and *Podoviridae* phages. Micrographs of phage-like particles stained with 2% uranyl acetate. These phages-like particles were isolated from one *G. vaginalis* isolate (3G7 from subgroup C) following induction with 2 µg/ml ciprofloxacin. Two images show **(A)** 1 and **(B)** 4 phage-like particles captured from different fields of view. **(C)** General morphology of *Podoviridae* phages, adapted from Lawrence *et al.* (107). **(D)** Micrograph of AltAD45-P1, a *Podoviridae* phage isolated by Garcia-Heredia *et al*. (109).

Chapter 4: Discussion

BV is the most common vaginal condition affecting women between the ages of 15-44 (110). The symptoms of BV are abnormal vaginal discharge with foul odor; although, up to 84% of BV-positive women can be asymptomatic (111). Having BV increases a woman's risk of acquiring sexually transmitted diseases, pelvic inflammatory disease and adverse pregnancy outcomes (45-49). These grave potential consequences stress the need for effective BV treatment. However, this is currently not the case as up to ~50% of BV-positive women can experience treatment failure within a month or BV recurrence within a year (6, 39). Developing effective treatment strategies for BV is particularly difficult as its etiology is poorly understood. Although, *G. vaginalis* is thought to play an vital role in the initiation of this polymicrobial condition (44, 63). It is thus important to further characterize the role of *G. vaginalis* in BV, as this could help us better understand and treat this condition.

4.1.1 *G. vaginalis* isolates cluster in four subgroups

cpn60 phylogenetic analysis of 90 *G. vaginalis* isolates used in this study revealed the presence of four clades. These four clades were previously termed subgroups A, B, C and D (27, 72), corresponding to *G. vaginalis* whole genome sequence clades 4, 2, 1 and 3 (57), respectively. In this study, subgroup D isolates were the least prevalent, while the majority of isolates clustered in subgroup C. Schellenberg *et al.* (27) found that Belgian women had an equal prevalence of subgroup A, B and C isolates (33%, 6/18 for each), with an absence of subgroup D isolates. Moreover, they revealed that subgroup D isolates were the least prevalent in Canadian (3%, 1/40) as well as Kenyan women (13%, 7/54). It is thus not surprising that only 2% (2/90) of *G. vaginalis* isolates from this study clustered in subgroup D, as it seems to be the least prevalent of the four subgroups across various geographical locations. This was the first study to characterise the prevalence of *G. vaginalis* within the French population. Previous

studies have shown that *G. vaginalis* subgroup distribution differs by geographical location, with subgroups A and C being most prevalent in women from the United States and Canada, while subgroup B being most prevalent in Kenyan women (27, 67, 79). As the French law forbids any racial parameters within medical files, the diversity of *G. vaginalis* could not be characterized in the context of racial groups. More studies are needed to determine *G. vaginalis* subgroup diversity in more countries, as the prevalence of specific *G. vaginalis* subgroups may explain why BV prevalence is high in women of African ancestry but low in women of European ancestry (1).

Based on *cpn60* sequences, *G. vaginalis* from a study by Albert *et al*. (77) clustered into only two subgroups, subgroups A and C. Similarly, *G. vaginalis* from a study by Balashov *et al*. (67) also clustered into only two subgroups, subgroups C and D. Using four *G. vaginalis* clade-specific primers, Balashov *et al*. were able to detect subgroup A and B isolates in 33% (13/39) of their collection, suggesting that 33% of *G. vaginalis* isolates could not be detected by *cpn60* PCR. A similar *G. vaginalis cpn60* false-negative rate (35%, 32/91 isolates) was reported by Janulaitiene *et al.* (78). While both of these studies used different *cpn60* primers to the ones used in this study, that targeted a different region of the *cpn60* gene, 14% (15/109) of *G. vaginalis* isolates in this study were also *cpn60*-PCR-negative. Since *cpn60* is a housekeeping gene, it is unlikely that it is absent in some *G. vaginalis* isolates. What is more likely is that the primers used were not able to bind to all *cpn60* sequences. It is therefore possible that more sensitive *cpn60* primers would improve sensitivity. As an alternative, if time had permitted, the four clade-specific primers designed by Balashov *et al*. could have been used to genotype the 15 *cpn60*-PCR-negative *G. vaginalis* isolates in this this study, as these clade-specific primers have successfully been used to genotype *G. vaginalis* into four subgroups, which correspond to the four *cpn60*-based subgroups, with a false-negative rate of only 2-5% (41, 67, 78, 79).

4.1.2 *G. vaginalis* subgroups associated with BV

The majority of *G. vaginalis* isolates (69%, 75/109) used in this study were from BV-positive women, with each isolate being unique to one woman. These isolates were purified from vaginal samples collected from women visiting a hospital (Hôpital Arnaud-De-Villeneuve, CHU de Montpellier, France), that were complaining about their vaginal health, thus providing a possible explanation for the bias towards BV-positive women. Acknowledging this selection bias, the majority of isolates across all subgroups came from BV-positive women, even though subgroups A, B and C also included isolates from BV-intermediate and BV-negative women. Additionally, as a result of this bias towards isolates from BV-positive women, it was not possible to determine which subgroups of *G. vaginalis* were associated with BV. Based on previous studies, it is still unclear which subgroups of *G. vaginalis* are most predictive of BV. Subgroups B and C have been reported as BV-associated two times across three independent studies (67, 72, 78), while subgroup D has been reported as BV-associated once (67). There is general consensus that most women harbor multiple subgroups of *G. vaginalis*, with subgroup diversity being predictive of BV (67, 72, 78, 79). Since only one isolate was obtained from each woman in this study, subgroup diversity could not be investigated.

4.2 Sialidase activity detected in *G. vaginalis* subgroup B and C isolates

The sialidase gene was detected in 69% (75/109) of isolates in this study, consistent with previous reports of sialidase gene detection in 69-75% of *G. vaginalis* isolates (27, 81). The sialidase gene was detected in subgroup B and C but not subgroup A or D isolates. The lack of sialidase gene detection in subgroup A isolates (97%, 34/35) was also reported by Schellenberg *et al.* (27); however, Schellenberg *et al.* detected the sialidase gene in all (8/8) subgroup D isolates. As only a few subgroup D isolates were present in this study and the one by Schellenberg *et al*., it is possible that future studies with a larger subgroup D sample size might reveal that some but not all subgroup D isolates harbor the sialidase gene. Furthermore, Schellenberg *et al.* detected the sialidase gene in all 33 subgroup B and 35 subgroup C isolates. However, in this study,

the sialidase gene was detected in only 76% (13/17) of subgroup B and 92% (53/58) of subgroup C isolates. The same PCR conditions and primers used by Schellenberg *et al.* were used in this study, thus making it unlikely that experimental differences caused these discrepancies. Nonetheless, different sialidase primers could be used to test for the presence of the sialidase gene in the sialidase-PCR-negative isolates in this study. A total of 10/34 isolates that failed the sialidase PCR also failed the *cpn60* PCR, suggesting that another reason for the lack of sialidase gene detection could be due to PCR failure as a result of the bad quality of the extracted DNA. Checking the quality of the DNA extracted from the *G. vaginalis* isolates may have given some insight into why some of the isolate's sialidase PCR was negative.

Sialidase activity was detected in 25% (27/109) of *G. vaginalis* isolates, which is consistent with a previous report by Schellenberg *et al.* (27) of sialidase activity detection in 35% (36/112) of *G. vaginalis* isolates. Sialidase activity was detected in 36% (27/75) of sialidase-PCR-positive isolates, again consistent with the previously reported sialidase activity detection in 47% (36/77) of sialidase-PCR-positive isolates (27). While Schellenberg *et al.* found that sialidase activity was disproportionately detected in subgroup B (100%, 33/33) and C (9%, 3/35) isolates, sialidase activity was detected at similar frequencies in subgroup B (41%, 7/17) and subgroup C (31%, 18/58) isolates in this study. Schellenberg *et al.* remarked that all subgroup C isolates with detectable sialidase activity were sampled from Belgian women but not from any of the Kenyan or Canadian women included in their study. This suggests that sialidase activity of *G. vaginalis* subgroups may be cohort-dependent. In this study, subgroup C sialidase sequences cluster together, separately from subgroup B sequences. The detection of similar sialidase activity levels between subgroup B and C isolates in this study suggests that the phylogenetic distinctness of subgroup B and C sialidase sequences merely reflect evolutionary divergence between the two subgroups, rather than functional differences in sialidase activity. Consistent with the lack of sialidase gene detection by PCR in all subgroup A and D isolates, sialidase activity was also not

detected in these subgroups. This suggests that *G. vaginalis* subgroups A and D may not be actively involved in BV, as sialidase activity is considered a marker of BV (26).

The reason behind the detection of sialidase activity in only 36% of the sialidase-PCR-positive *G. vaginalis* isolates in this study is yet to be established. This lack of sialidase activity does not seem to be related to BV status, as sialidase activity was detected at similar frequencies in isolates from women of all BV states. However, it is important to note that this may differ *in vivo.* Others have hypothesized that sialidase expression may require stimulation by unknown factors that can be turned on or off based on *in vivo* environmental conditions; although, this hypothesis is yet to be investigated (81). Comparing the sialidase gene promoter regions of *G. vaginalis* isolates could help identify such factors. As *G. vaginalis* subgroups B and C isolates are phylogenetically closely related, the detection of sialidase activity in isolates from these subgroups (but not subgroups A and D) suggests that sialidase activity may be a shared ancestral virulence trait, specific for *G. vaginalis* subgroups B and C.

4.3 Biofilm formation by *G. vaginalis* isolates from all subgroups

This study is the first to quantify and compare biofilm formation of *G. vaginalis* subgroups, revealing that all subgroups appear to be similar in their ability to form biofilm. These data support a previous qualitative study that detected the presence of biofilm in isolates from all *G. vaginalis* subgroups (72). The ability of *G. vaginalis* subgroup B and C isolates to form biofilm, as well as the detection of sialidase activity in these subgroups suggests *G. vaginalis* subgroups B and C as the most likely to be involved in BV (26, 84)

G. vaginalis isolates from both BV-negative (Nugent 0-3) and BV-positive women (Nugent 7-10) were able to form biofilm at similar quantities. This observation was also made in a previous study by Castro *et al.* (112), who further reported that *G. vaginalis*

isolates from BV-positive women were five times more adherent to epithelial cells than *G. vaginalis* isolates from BV-negative women. While *G. vaginalis* isolates from both groups of women appear to be able to form biofilms, the findings by Castro *et al.* suggest that biofilms formed by BV-negative women may be easier to excrete as they are less adherent to the vaginal epithelial wall compared to biofilms formed by BV positive women, which are able to adhere to the vaginal epithelium and form stable biofilms that support the growth of other pathogenic BV-associated bacteria.

Bacteria are known to form biofilms on the surfaces of polypropylene microcentrifuge tubes (113). In this study, *G. vaginalis* isolates were cultured in microcentrifuge tubes and then subcultured in 96 well microtiter plates before biofilm formation was quantified (102). By visual inspection, it was clear that virtually all *G. vaginalis* isolates could form biofilm on the surfaces of microcentrifuge tubes. However, despite multiple optimization attempts, the protocol used underestimated biofilm formation, especially by weak biofilm producers, as biofilm could easily detach from the wells during the protocol's two washing steps. This protocol was chosen as it has been used multiple times to quantify *G. vaginalis* biofilms (66, 72, 88, 112, 114). A fixation step should be investigated in future experiments, as this could minimize biofilm loss. Another method that is commonly used to detect *G. vaginalis* biofilms is fluorescence *in situ* hybridization (FISH), whereby biofilms are detected by confocal microscopy, using florescent probes that bind rDNA or rRNA sequences that are species-specific. This method was not used in this study as it is typically used to detect the presence or absence rather than the quantity of biofilm (7, 81, 85, 87, 115).

A limitation of this study was that biofilm was measured in only one growth medium (5%-blood-supplemented Schaedler medium) and it is known that biofilm formation is growth medium-dependent. Previous studies have noted that *G. vaginalis* formed substantially more biofilm in brain heart infusion broth supplemented with 0.25% (w/v) maltose or 1% (w/v) glucose compared to other tested growth mediums, including Todd

Hewitt broth and ATCC broth #1685 (72, 112, 116). In this study, *G. vaginalis* biofilm was measured in 5% blood-supplemented Schaedler broth, as *G. vaginalis* isolates grew faster and to a higher optical density in this growth medium compared to in brain heart infusion medium supplemented with 0.1% (w/v) starch and 0.5% (w/v) yeast (data not shown). Nonetheless, in future studies, biofilm should be quantified using different growth mediums, preferably ones most representative of *in vivo* conditions.

Another limitation of this study was that biofilm was only measured at pH 7.4, the pH of 5%-blood-supplemented Schaedler medium. Gottschick *et al*. (114) recently noted that *G. vaginalis*' biofilm forming ability increases with increasing pH, being lowest at pH 4.5 and highest at pH 7, the upper and lower limits of their tested pH range. This observation is particularly relevant in the context of BV, as BV is characterized by an increase in vaginal pH from 4-5 towards a pH of 6-7 (10, 117). It is important to further investigate the effect of pH on *G. vaginalis*' biofilm forming ability and compare if there are any differences seen in isolates from different subgroups. If it is found that there are subgroups that are able to form biofilm at low pH levels, this would suggest these subgroups to be the pioneers of biofilm formation that subsequently promote the growth of other BV-associated bacteria, outcompeting commensal lactic-acid-producing species that maintain the acidic vaginal environment, thus serving as a potential explanation for the shift from low to high pH.

4.4 Antibiotic susceptibility of *G. vaginalis* isolates

Antibiotic susceptibility of *G. vaginalis* isolates to metronidazole and clindamycin was tested, as these are the first-line recommended antibiotics for the treatment of BV (110). In addition, antibiotic susceptibility of *G. vaginalis* isolates to moxifloxacin and augmentin were also tested, as these antibiotics are being explored as alternative treatments for BV (90, 91), in light of the emergence of metronidazole and clindamycin resistance (40, 62). Of the 109 *G. vaginalis* isolates used in this study, 64 could not form a lawn on 5% blood-supplemented Brucella agar, thus antibiotic susceptibility was

measured for only 45 *G. vaginalis* isolates. It is unknown why more than half of the isolates in this study could form lawns. Various variables including the type of agar medium, dilution medium (saline versus Schaedler and Müller Hinton), temperature of agar at the time of plating the lawn, and anaerobic sachet lot were investigated as potential reasons but none of these variables appeared to be the cause. Increasing or decreasing the inoculum (that is, McFarlane unit of 0.5 or 2) did not change the results. Even though it was unlikely that the isolates had become less viable over time, new isolate cultures were produced but even they were unable to form lawns. These experiments were conducted with a time limit of two months as they were carried out during an internship in France. It is possible that further experiments testing other variables could have elucidated why some isolates were not able to form lawns.

G. vaginalis isolates were the most frequently resistant to metronidazole (71%, 32/45) compared to all the other antibiotics, which reflects the fact that metronidazole is the most commonly used antibiotic to treat BV (110). Using 32 µg/ml as a breakpoint for resistance as in this study, a previous study also noted a similarly high amount of metronidazole resistance (61%, 53/87) amongst *G. vaginalis* isolates (41). Moreover, 60% (27/45) of isolates were highly resistant to metronidazole (MIC ≥ 256 µg/ml), a common phenomenon that has been observed in ~55% of isolates in other studies (41, 42). Metronidazole resistance was present across all subgroups of *G. vaginalis* tested in this study. However, small sample size limited the statistical power to compare metronidazole resistance across the four subgroups of *G. vaginalis*. Schuyler *et al*. (41) recently found all 22 subgroup A and 15 subgroup D isolates to be resistant to metronidazole, compared to only 7% (1/14) of subgroup B and 43% (16/37) of subgroup C isolates. As *G. vaginalis* biofilms are often recovered from the genital tracts of BV-negative women after metronidazole treatment (87), it is likely that the *G. vaginalis* biofilms that persist in these women belong to the intrinsically resistant subgroups A and D. Persistence of these metronidazole-resistant biofilms would most likely support the growth of other BV-associated bacteria, leading to the re-emergence of the polymicrobial biofilm associated with BV and subsequently, BV recurrence.

Metronidazole is part of the nitromidazole group of antibiotics, whose mechanism of action requires the reduction of their nitro group (typically by anaerobes as they have a higher reducing potential than aerobes) before oxidizing bacterial DNA, causing DNA strand breaks (118). Several mechanisms of resistance by anaerobes to nitromidazoles have been proposed, including: (i) decreased activation or increased inactivation of the drug; (ii) decreased uptake and increased efflux of the drug and (iii) increased DNA damage repair by bacteria (119). Seven genes conferring resistance to nitromidazoles, termed *nim*A – *nim*G, have been identified, which encode a reductase that inactivates nitromidazole into a non-toxic derivative. A previous study found that, while these genes were absent from all 145 vaginal samples from BV-negative women, the were found in only 1.4% (2/144) of samples from BV-positive women (40). It is thus unlikely that *nim* genes are the reason for the high level of *G. vaginalis* metronidazole resistance observed in this study. A recent study suggested that *G. vaginalis* may use its *cas* genes to mitigate the DNA-damaging effects of metronidazole, as 7 *cas* genes (*cas1* to *cas3* and *casA* to *casD*) were found to be up-regulated in the genital tracts of women who experienced BV treatment failure compared to those who were cured (62). *Cas* genes are nucleases, best known for their role in genome editing as part of the CRISPR-*cas* system (93). However, *cas1* proteins can also play a role during DNA excision repair (120). The functional role of the up-regulated *G. vaginalis cas* proteins following metronidazole treatment remains to be investigated (62).

None of the *G. vaginalis* isolates that were tested in this study were resistant to clindamycin. Using 4 µg/ml as a breakpoint for resistance (as in this study), 1.5-6% of *G. vaginalis* isolates were found to be resistant to clindamycin in previous studies (42, 43). Clindamycin is part of the lincosamides group of antibiotics, which bind to the large ribosomal subunit, inhibiting protein synthesis (121). Bacterial clindamycin resistance genes are called *erm* genes, which encode rRNA methylases that change the structure of the antibiotic binding site (40). *Erm* genes were found to be present in 62% (90/144) of vaginal samples from BV-positive women, while being present in only 14% (20/145) of samples from BV-negative women (40). Moreover, *G. vaginalis* are known to harbor

erm genes (122). These reports are inconsistent with the absent to low level of clindamycin resistance of *G. vaginalis* isolates observed in this study as well as others (42, 43). It is possible that the transcription of *erm* genes is not constitutive. Alternatively, *G. vaginalis* isolates in this collection may lack *erm* genes. Both hypotheses could be tested by running qPCR on this collection of *G. vaginalis* isolates, using *erm*-gene-specific primers. Nonetheless, the absent to low level of clindamycin resistance compared to metronidazole resistance amongst *G. vaginalis* isolates suggests that clindamycin remains an acceptable (and perhaps even a better) alternative to metronidazole in the treatment of BV.

Only 9% (4/45) of *G. vaginalis* isolates were resistant to moxifloxacin using 0.5 µg/ml as the breakpoint for resistance. While this breakpoint for resistance is not as conservative as the breakpoints for resistance for the other tested antibiotics, it is the only moxifloxacin breakpoint for *G. vaginalis* resistance that could be found in previous literature (103). Moxifloxacin is part of the fluoroquinolone group of antibiotics, which inhibit DNA gyrase and topoisomerase IV activity, enzymes that stabilize the bacterial DNA helix as it unwinds during DNA replication (123). The most studied mechanisms of bacterial resistance to moxifloxacin are *gyr*A and *par*C mutations, which alter the moxifloxacin binding sites of DNA gyrase and topoisomerase IV proteins (124). Sequencing the *gyr*A and *par*C genes of *G. vaginalis* could shed light on whether *gyr*A and *par*C mutations are the mechanisms *G. vaginalis* uses to resist moxifloxacin activity. This is not the first study to investigate moxifloxacin in the context of BV. Swidinski *et al.* (90) reported that 75% (15/20) of BV-positive women were successfully treated for BV with 400mg moxifloxacin taken orally for 5 days. This, in addition to the observation made in this study that moxifloxacin was superior to metronidazole with regards to *G. vaginalis* susceptibility, prompts the suggestion that moxifloxacin should be considered as an alternative to metronidazole in the treatment of BV.

All *G. vaginalis* isolates tested in this study were sensitive to augmentin. This is consistent with a previous report where, using the same 8 µg/ml breakpoint for resistance as in this study, all 67 tested *G. vaginalis* isolates were sensitive to augmentin (42). Augmentin is a combination of amoxicillin and clavulanic acid. Amoxicillin is part of the penicillin group of antibiotics, which inhibit cell wall synthesis (125). Some bacteria can resist the activity of penicillins using β-lactamases, enzymes that degrade the β-lactam ring of penicillins (125). Clavulanic acid inactivates β-lactamases thereby preventing them from degrading amoxicillin. However, bacteria can also develop resistance to clavulanic acid by overexpressing β-lactamases or modifying the β-lactamase active site of clavulanic acid (126). Nonetheless, augmentin appears to be superior to amoxicillin in the treatment of BV, as in a previous study, 37% (3/8) of BV-positive women treated with amoxicillin experienced treatment failure, while all 6 women treated with augmentin were cured (91). Taken together, augmentin should be further studied in the context of BV, as it is an excellent candidate as an alternative to metronidazole in the treatment of BV.

4.5 Prophage induction of *G. vaginalis*

There have been reports of an abundance of prophage sequences in the genomes of *G. vaginalis* (59, 96); however, phages of *G. vaginalis* have never been isolated. One of the ways *Lactobacillus* species (particularly, *L. gasseri*) are thought to suppress the growth of *G. vaginalis* is by producing hydrogen peroxide (98). Hydrogen peroxide is a reactive oxygen species that generates hydroxyl radicals, molecules that oxidize and damage DNA by causing DNA strand breaks, intra- and inter-strand crosslinking and base modifications (127). Upon encountering DNA damage, bacteria activate their SOS response, a network of least 40 genes that repair damaged DNA (128). The SOS response can trigger the inactivation of prophage repressor genes, resulting in prophage induction (99). Hydrogen peroxide is known to induce prophages (97). It was thus hypothesized that hydrogen peroxide released into the supernatants of *L. gasseri* cultures could induce prophages of *G. vaginalis* isolates.

Not all DNA-damaging agents lead to prophage induction and different prophage-inducing agents can induce different prophages from a single bacterial isolate (101, 129). This is probably because prophage-inducing agents damage DNA using a variety of mechanisms. UV radiation, mitomycin C and ciprofloxacin are other DNA-damaging agents known to induce prophages (99). UV radiation crosslinks adjacent pyrimidine DNA bases, preventing them from binding to their complementary bases (130). Mitomycin C is an antitumor antibiotic that alkylates and crosslinks DNA strands (131). As with moxifloxacin, ciprofloxacin is part of the fluoroquinolone group of antibiotics that destabilize the DNA helix as it unwinds during DNA replication, resulting in double-stranded DNA breaks (132). The ability of *L. gasseri* supernatant, UV radiation, mitomycin C and ciprofloxacin to induce prophages of *G. vaginalis* isolates was tested. UV radiation, mitomycin C and ciprofloxacin were chosen as they were found to be the most commonly used prophage-inducing agents in literature; however, other known prophage-inducing agents that can be tested for their ability to induce prophages of *G. vaginalis* in future studies are quinolone antibiotics such as norfloxacin, levofloxacin, moxifloxacin, nalidixic acid and oxolinic acid as well as agents that generate reactive oxygen species such as paraquat (129, 133).

Prophage-inducing agents that cause DNA damage are usually added to early log-phase bacterial cultures that are undergoing DNA replication, in order to maximize DNA damage, and thus, maximize the rate of prophage induction (101). In this study, early log-phase growth of *G. vaginalis* cultures in Schaedler medium was determined to be around 9 h. For other bacterial species, productive prophage induction is usually suggested by a transient increase followed by a decrease in bacterial cell density, which occurs soon after the addition of a prophage-inducing agent (101). Since prophages against *G. vaginalis* have never been induced, it is unknown when this increase and decrease in cell density occurs. Therefore *G. vaginalis* isolates were monitored for

evidence of prophage induction until they were well into their stationary phase, that is, after at least 24 h.

Log-phase cultures of *G. vaginalis* isolates did not grow in the presence of 50% *L. gasseri* supernatant, while isolates cultured in the presence of lower concentrations of *L. gasseri* supernatant (0.03-33%) grew similarly to isolates cultured without *L. gasseri* supernatant. *G. vaginalis* isolates cultured in the presence of 50% *L. gasseri* supernatant were analyzed under TEM but no temperate phages were observed. To successfully induce prophages, DNA-damaging agents need to be used at concentrations that are high enough to induce prophages but are sub-lethal to the bacterial hosts, as to allow bacteria to mitigate DNA damage long enough for successful prophage induction to occur (101). The specific *L. gasseri* isolate used in this study produces ~10μM hydrogen peroxide, which is lower than hydrogen peroxide concentrations (50-1000μM) that have successfully been used to induce prophages of other bacterial species (97, 134, 135). If time had permitted, different concentrations of hydrogen peroxide alone could have been tested for their ability to induce prophages of *G. vaginalis*, as it is possible that hydrogen peroxide in the 50% *L. gasseri* supernatant was too low to cause DNA damage and prophage induction of *G. vaginalis* isolates. Other virulence factors, such as lactic acid and bacteriocins produced by *L. gasseri* (136), could be responsible for the observed inhibition of *G. vaginalis* growth by the *L. gasseri* supernatant.

G. vaginalis isolates exposed to up to 60 sec of UV radiation grew similarly to isolates that were not exposed to UV radiation, suggesting that UV radiation neither caused prophage induction nor inhibited cell division. It is unlikely that this was due to suboptimal duration of isolates under UV radiation as isolates were irradiated for 5-60 sec and optimal duration for prophage induction using UV radiation is usually around 5-15 sec for other bacteria (101). While UV-C radiation at 254 nm has previously been used to induce prophages of other bacteria (137), it is possible that *G. vaginalis* is

insensitive to UV radiation at 254 nm and that a stronger wavelength is needed to cause DNA damage and prophage induction. Prophage induction by UV radiation has been extensively studied in *Escherichia coli* and it is apparent that having mutations in bacterial genes involved in the SOS response to UV radiation (termed *umuA-C* genes) can make cells insensitive to UV DNA damage, leading to unsuccessful prophage induction attempts (138). *G. vaginalis* isolates could be screened for the presence of such mutations in the future, as this could explain why *G. vaginalis* growth was unaffected by UV radiation.

G. vaginalis isolates cultured in the presence of 0.5 µg/ml mitomycin C grew similarly to isolates cultured without mitomycin C, suggesting that *G. vaginalis* isolates were insensitive to mitomycin C at this concentration. Higher concentrations of mitomycin C (1-16 µg/ml) decreased *G. vaginalis* growth in a dose-dependent manner, suggesting that *G. vaginalis* isolates were sensitive to the DNA-damaging effects of mitomycin C. However, since isolates cultured in the presence of all tested mitomycin C concentrations continued to grow for 18 h after the addition of mitomycin C, it was unlikely that prophage induction had occurred since a decrease, and not an increase, in cell density shortly after the addition of a prophage-inducing agent signals productive prophage induction (101). The absence of temperate phages in *G. vaginalis* isolates cultured in the presence of 0.5 µg/ml or 8 µg/ml mitomycin C was confirmed by TEM analysis. It was previously reported that 97% (38/39) of the published *G. vaginalis* genomes harbor up to 33 prophage sequences (59), suggesting a high probability of the presence of prophages in the three *G. vaginalis* isolates tested in this study; although, whole genome sequencing of these isolates is necessary to confirm this. Mitomycin C at the tested wide concentration range of 0.05-16 µg/ml was unable to induce prophages of up to 15 other *G. vaginalis* isolates (data not shown). This concentration range is well within the normal range of 0.5-3 µg/ml in which mitomycin C is a potent prophage inducer for other bacteria, including *Bifidobacterium* species (101, 105), the closest phylogenetic relatives of *G. vaginalis* (106). The reason behind the ability of mitomycin

C to inhibit growth but not induce prophages of *G. vaginalis* isolates is yet to be determined.

G. vaginalis isolates cultured in the presence of 2 µg/ml ciprofloxacin grew slower and to a lower stationary-phase cell density than isolates cultured without ciprofloxacin. Instead of the expected decrease in cell density following the addition of ciprofloxacin in the event of prophage induction (101), *G. vaginalis* isolates in this study maintained stationary phase until at least 36 h. Although, there have been reports of successful prophage induction in cells maintaining stationary phase or incomplete cell lysis of bacterial cultures following the addition of a prophage-inducing agent (104, 139). In this study, TEM analysis of at least one out of three *G. vaginalis* isolates (3G7) cultured in the presence of 2 µg/ml ciprofloxacin revealed the presence of phage-like particles. These particles are referred to as phage-like as they could not be confidently classified into any known phage families. Although, these phage-like particles present in head-tail structure, a defining characteristic of *Myoviridae*, *Siphoviridae* and *Podoviridae* phage families under the order of *Caudovirales* (140). These phage-like particles most resemble phages belonging to the family of *Podoviridae*, as they have a ~30 nm long tail that is shorter than the 80-455 nm *Myoviridae* and 65–570 nm *Siphoviridae* tail ranges, but is within the 10-46 nm *Podoviridae* tail range (108). However, their tails lack tail-spikes, which are a defining characteristic *Podoviridae* phage tails (107). Although, tail-spikes are not always clearly defined under TEM (109). The phage-like particles from this study generally displayed a ~50 nm wide, irregular-shaped head, and not the 60-70 nm wide, icosahedral-shaped head that characterizes *Podoviridae* phages (108), thus preventing these phage-like particles from being classified as *Podoviridae* phages. Eight cultures of *G. vaginalis* isolates were screened for their use as hosts to propagate the phage-like particles; however, none could be infected by the phage-like particles (data not shown). The *G. vaginalis* isolate from which the phage-like particles were obtained (3G7) cannot be used as a host to propagate them, as prophages cause superinfection exclusion, a process whereby prophages produce proteins that prevent infection of the same or similar phages into the same host cell (141). This experiment is

yet to be repeated using a wider panel of *G. vaginalis* isolates (or closely related species) that could be screened for their use as a host to propagate these phage-like particles in order to yield enough phage DNA to conduct whole genome sequencing, which is necessary to confirm the phage status of these phage-like particles.

4.6 Prospectives

In order to improve the current poor treatment outcomes of BV, research efforts should be directed towards strategies that aim to restore and maintain healthy vaginal microbiota as well as strategies that aim to prevent and eradicate the polymicrobial biofilm that characterizes BV. Alternative treatment strategies such as the use of probiotics, phage therapy and anti-biofilm agents have the potential to reduce BV recurrence, if used in combination with antibiotic therapy (142). Probiotics are live micro-organisms that are beneficial to the host when consumed at the right amounts (143). Studies evaluating long-term administration of probiotic lactobacilli, included in feminine hygiene products or food consumed regularly, such as yogurt, milk and ice cream, have shown controversial but overall promising results with regards to probiotics preventing, treating and reducing the recurrence of BV (143-145).

Phage therapy, which is the use of phages to treat bacterial conditions (146), has been suggested as an alternative BV treatment strategy (147). Unlike antibiotics, which tend to be broad-spectrum, phages tend to have a narrow host range, being species- or even strain-specific (148). Thus, phage cocktails targeting BV-associated bacteria but not commensal lactic acid-producing species could potentially restore dysbiotic vaginal microbiota to its healthy state. Another feature that makes phages ideal agents in preventing BV recurrence is their ability to eradicate biofilms. While this is usually credited to their ability to self-amplify at the target site to numbers high enough to destroy the entire biofilm (89), phages can encode depolymerases that degrade extracellular polymeric substances in the biofilm matrix, allowing phages to move around in the biofilm, in addition to degrading bacterial cell surface polymers, allowing phages to attach to their bacterial targets (149). The isolation of virulent phages against

vaginal bacteria has been an elusive task. However, depolymerases isolated from phages from other human compartments could be effective at disrupting BV biofilms and should thus be investigated.

Other anti-biofilm agents such as antibacterial hydrolases and antiseptics have been investigated for their ability to prevent and eradicate BV biofilms; however, the amphoteric tenside, cocoamphoacetate, was the only compound that showed great promise *in vitro*, where it completely prevented *G. vaginalis* biofilm formation and dissolved over of 50% of biofilm established over 20 h (114). However, in a follow up clinical trial, cocoamphoacetate-containing pessaries reduced biofilm formation but were unable to prevent BV recurrence when administered intra-vaginally to BV-positive women treated with metronidazole (150). The pessaries were only taken for three weeks after metronidazole treatment; it is possible that prolonging the treatment could have improved treatment outcomes. A different strategy to prevent BV biofilms could be to inhibit sialidase activity, as sialidase is thought to degrade vaginal mucus, allowing *G. vaginalis* to attach to the vaginal epithelial layer and form biofilms (81). The sialidase inhibitor, Zanamavir, decreased *G. vaginalis* sialidase activity by 30% *in vitro* (151). However, the functional association of sialidase activity and biofilm formation in the context of BV is yet to be confirmed *in vivo*.

4.7 Conclusion

Based on *cpn60* phylogenetic analysis, 90 *G. vaginalis* isolates used in this study clustered into four clades, referred to as subgroups A-D. Isolates from all subgroups produced similar amounts of biofilm; however, sialidase gene and activity was detected in subgroup B and C isolates only. As the presence of biofilm and sialidase activity are two hallmark traits of BV (26, 84), these data suggest *G. vaginalis* subgroups B and C as most likely to be involved in BV. *G. vaginalis* from subgroups B and C probably secrete sialidase during BV, an enzyme that is thought to degrade the protective mucus layer lining the vaginal tract, allowing *G. vaginalis* (from all four subgroups) and other

BV-associated bacteria to attach to the vaginal epithelial layer and form a polymicrobial biofilm. Metronidazole resistance was observed in the majority of *G. vaginalis* isolates (71%, 32/45) across all subgroups, providing a possible mechanism for how *G. vaginalis* biofilms can persist in the vaginal tracts of women treated for BV with metronidazole (87). In contrast, all *G. vaginalis* isolates were sensitive to clindamycin, which remains an acceptable alternative to metronidazole in the treatment of BV. Moxifloxacin and augmentin are also suggested as potential alternatives to metronidazole in the treatment of BV, as both antibiotics displayed microbiological efficacy against *G. vaginalis* isolates in this study, and have previously demonstrated clinical efficacy in the treatment of BV (90, 91). However, as with metronidazole, all these antibiotics are bound to suffer the same fate of being rendered ineffective by the emergence of antibiotic resistance. Moreover, antibiotics are not able to entirely destroy the polymicrobial biofilm that forms during BV, which contributes to the high BV recurrence rate (39, 87, 90). There is thus a great need to look into sustainable alternative treatment strategies in hopes of improving the current poor treatment outcomes of BV.

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