Bacteria against bacteria: Isolation, identification and characterization of bacteria with antimicrobial activities against *Gardnerella vaginalis*

by

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A thesis submitted in partial fulfillment

of the requirements for the degree of

Master of Science (MSc) in Biology

The Faculty of Graduate Studies

Laurentian University

Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis
Titre de la thèse: Bacteria against bacteria: Isolation, identification and characterization of bacteria with antimicrobial activities against Gardnerella vaginalis

Name of Candidate
Nom du candidat: Ross, Megan

Degree
Diplôme: Master of Science

Department/Program
Département/Programme: Biology

Date of Defence
Date de la soutenance: Tbd1

APPROVED/APPROUVÉ

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ACCESSIBILITY CLAUSE AND PERMISSION TO USE

Abstract

Gardnerella vaginalis is the etiological agent of bacterial vaginosis (BV), a chronic vaginal infection that affects women globally. Increasing antibiotic resistance and failing treatment options has resulted in chronic BV, demonstrating a need for novel alternative therapeutics. A total of 34 environmental bacterial isolates with antimicrobial properties against a *G. vaginalis* collection (n=17) were isolated and characterized for their antagonistic effects against *G. vaginalis* isolates and for their production of antimicrobial active agents. The *G. vaginalis* collection was shown to be diverse by genotyping and by their antibiotic susceptibility profiles. Anti-*G. vaginalis* (αGV) isolates were identified and phylogenetically clustered following full genome sequencing and their antiGV target range specificities were investigated. A subset of the αGV collection (n=20) were shown to produce and export active antimicrobial agents with molecular sizes of 4.6-35kD, which showed specific lethal effect on *G. vaginalis* isolates (target ranges of 5-100%). Cocktail formulations consisted of promising representatives from αGV clusters, with overlapping complimentary properties and specificity to *G. vaginalis* were identified for further study as the antimicrobial candidates for BV.

Keywords

Gardnerella vaginalis, Bacterial vaginosis, Antimicrobial peptides, Antibiotic Resistance, Host Range, Alternative Therapeutics, Genome, Clinical isolates, Bacteriocin

Acknowledgments

First and foremost, I thank my supervisor Dr. Reza Nokhbeh for selflessly adopting me into his lab family under unique circumstances. As a mentor, Reza always supported my strengths and challenged my weaknesses, which pushed me to develop greater confidence in my research capabilities. Without his knowledge and influence, this work would not have been as successful or fulfilling. This work was also furthered by the guidance and support of my co-supervisor Dr. Mazen Saleh, and fellow committee members Dr. Jeffrey Gagnon, and Dr. Amadeo Parissenti. Their unique backgrounds enhanced my focus and understanding of the project overall.

A large portion of this project hinged on results generated from bioinformatic data, and if Dr. Gustavo Ybazeta was not available to do this work all would be lost. When I first arrived at the lab, Jenna Graham and Cassandra Norton welcomed me to the Nohkbeh team and were essential in my training and providing emotional support. I thank them for everything that they have done for me. I also want to show appreciation to Sebastien, Kristy-Anne and Nya for enhancing my experience as a student at HSNRI. My gratitude also goes out to Ms. Kathy Bernard from NML for providing me with the clinical isolates crucial to my project and HSNRI for their financial, which supported my work.

Finally, I must thank my family and Jesse for providing the emotional support required for any graduate student. When I needed to practice a presentation a million times, or when I just wanted to hide away and read my books forever, they were there to listen and steer me back on track. I need to especially express my undying gratitude to my mom for always believing in me and being there for me every step of the way.

And to my dear Artemis, the worst therapy pet anyone could ask for, I still love you little monster.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

1KbP	1 Kb Plus DNA LadderαGV
AM	Ampicillin
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BAP	Biofilm Associated Protein
BHI	Brain Heart Infusion
BV	Bacterial vaginosis
BVAB	Bacterial vaginosis Associated Bacteria
CAms	Cationic Amphiphiles
CB	Columbia broth
CDC	Cholesterol-Dependent Cytolysin
CDS	Coding DNA sequence
CFU	Colony Forming Units
CHO	Chinese hamster ovary
CI	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CM	Clindamycin
dNTPs	Deoxyribonucleotide Triphosphstes
ETEST	Epsilometer
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FBS	Fetal Bovine Serum
gDNA	Genomic DNA
GV	Gardnerella vaginalis
hCD59	Human CD59
HIV-1	Human Immunodeficiency Virus Type 1
HSV-2	Herpes Simplex Virus Type 2
HPV	Human Papillomavirus
IFAT	Indirect Fluorescent Antibody Test
IL-1β	Interleukin -1β
IL-8	Interleukin -8
ILY	Intermedilysin
IUD	Intrauterine Device
LABs	Lactic acid bacteria
LZ	Linezolid
MAPK	Mitogen-Activated Protein Kinase
MH	Mueller-Hinton
MIC	Minimal Inhibitory Concentration
MRS	De Man, Rogosa and Sharpe agar

MZ	Metronidazole
NML	National Microbiology Laboratory
OD	Optic Density
ORF	Open reading frame
PID	Pelvic Inflammatory Disease
PLY	Pneumolysin
PMS	Premenstrual Syndrome
PROM	Premature Rupture of Membranes
RAPD	Random Amplified Polymorphic DNA
REA	Restriction Endonuclease Analysis
RFLP	Restriction Fragment Length Polymorphism
RI	Rifampicin
ROPEC	Robert O. Pickard Environmental Centre
rpoB	RNA polymerase subunit beta gen
SDS	Sodium Dodecyl Sulfate
SLD	Sialidase
STI	Sexually Transmitted Infection
TAE	Tris-acetate-ethylenediaminetetraacetic Acid
Taq pol	*Taq* DNA Polymerase
TC	Tetracycline
TMH1/2	Transmembrane Helix 1 and 2
tmRNA	Transfer-messenger RNA
tRNA	Transfer RNA
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
VA	Vancomycin
VLY	Vaginolysin
αGV	Anti *G. vaginalis*

1 Introduction

1.1 Gardnerella vaginalis

Gardnerella vaginalis, the etiological agent of bacterial vaginosis (BV), was first observed by Leopold in 1953 and later identified and characterized as *Haemophilus vaginalis* (Gardner and Dukes, 1954; Dukes and Gardner, 1961). *G. vaginalis* grows as circular, convex, small grey colonies on human and rabbit blood agar plates and produces β-hemolysis with diffused edges, with no effect on sheep blood (Leopold, 1953; Gardner and Dukes, 1954; Greenwood and Pickett, 1980; Piot *et al.*, 1982). *G. vaginalis* is a Gram-variable, coryneform, nonmotile, nonsporulating, facultative anaerobe of approximately 0.8-1.2 μm in size, containing a fibrillar nucleoid and volutin metaphosphate granular development in the cytoplasm in stationary and death phases of growth (Leopold, 1953; Gardner and Dukes, 1954; Dukes and Gardner, 1961; Criswell *et al.*, 1972; Wells and Goei, 1981; Sadhu *et al.*, 1989). Its pleomorphic properties have led to a great debate over the Gram classification and taxonomy of the bacterium. It was suggested that the bacterium be classified as part of the genus *Haemophilus* based on the organism being Gram-negative (Dukes and Gardner, 1961). This was supported by the low peptidoglycan content (about 20% of the cell wall), lack of teichoic acids, and an amino acid composition of cell wall preparations of 11-14 amino acids that resemble that of *Escherichia coli* but not Gram-positive *Bacillus megaterium.* Cell membrane/cell wall fine electron micrographs were also used to identify the microorganism (Criswell *et al.*, 1971, 1972). In 1966, Ryen *et al.* had produced electron microscopic data for *Haemophilus vaginalis* 594, the same strain that was used later in 1972 by Criswell *et al*., and compared it to other Gram-positive bacteria. It was shown that *G. vaginalis* had resemblance to low mucopeptide containing *Corynebacterium*

diphtheriae and *Butyribacterium retgerri*, but none of the Gram-positive *Lactobacillus acidophilus* and the Gram-negative *Haemophilus influenza* (Ryen *et al.*, 1966). This lead Ryen *et al.* (1966) to suggest that the *G. vaginalis* be classified under either of genera *Corynebacterium* or *Butyribacterium* but not *Haemophilus*. The taxonomic state of *G. vaginalis* claimed by Criswell *et al* (1971, 1972) was further called into question when several groups showed the resemblance of the amino acid composition of the peptide bridge of its cell wall to that of Gram-positive bacteria (Ryen *et al.*, 1966; Harper and Davis, 1982). In contrast to Criswell's findings it was shown that the cell wall amino acid composition of *G. vaginalis* isolates was limited in diversity, with the most common amino acids being alanine, lysine, glutamic acid and glycine, consistent with the majority of Gram-positive bacteria, having a range of 4 to 6 major amino acids in their peptidoglycans (Schleifer and Kandler, 1972; Harper and Davis, 1982; O'Donnell *et al.*, 1984). In addition, the cell division processes described by electron microscopy resembles Gram-positive bacteria with the development of a thick septa (Ryen *et al.*, 1966; Sadhu *et al.*, 1989). The lack of an outer membrane and LPS furthermore supported Gram-positive properties of *G. vaginalis* (Sadhu *et al.*, 1989). However, Criswell *et al.* reported that visualization of a layered cell wall was dependent on the angle at which the wall was dissected for imaging. An oblique angle was the culprit for this illusion of a Gram-negative cell wall structure for *G. vaginalis* (Criswell *et al.*, 1971, 1972; Greenwood and Pickett, 1980). Other components of the cell wall found in lesser amounts included glucose, galactose and 6-deoxytalose (Harper and Davis, 1982). It was then suggested that the bacterium was in fact Gram-positive but assembled a thinner cell wall than normal, resulting in growth dependent Gram-variable staining properties (Sadhu *et al.*, 1989).

Nevertheless, the taxonomic deliberation could not be put to rest due to finding that the bacterium lacked requirements for specific growth factors, X (hemin) and V (NAD), associated with the genus *Haeomophilus* and thus was reclassified as a member of genus *Corynebacterium*, more specifically *Corynebacterium vaginale* (Edmunds, 1960; Zinnemann and Turner, 1963; Dunkelberg and McVeigh, 1969; Greenwood and Pickett, 1980). Its microscopic morphology may present similarities to the genera *Haemophilus* and *Corynebacterium*, however, through DNA-DNA hybridization no genetic association between *G. vaginalis* to *Corynebacterium* or *Haemophilus* was observed resulting in the current classification of *G. vaginalis* as the only species of the genus *Gardnerella*, named after Gardner, and the species name as *vaginalis* referring to the vagina as the location of bacterial isolation (Leopold, 1953; Gardner and Dukes, 1954; Dukes and Gardner, 1961; Zinnemann and Turner, 1963; Greenwood and Pickett, 1980). Numerical taxonomy analysis additionally illustrated that 78 *G. vaginalis* isolates and several reference strains including ATCC 14018 had a relatedness exceeding 95% (Greenwood and Pickett, 1980).

1.2 Diversity among *G. vaginalis* isolates

Besides structural peculiarities, the *G. vaginalis* isolates show phenotypic and genotypic diversities. Discovery of biological markers and developing typing schemes are important in understanding the diversity among *G. vaginalis* isolates. Molecular typing methodologies have been developed based on biochemical/metabolic properties (Biotyping), serological properties (Serotyping), and genomic sequence properties (Genotyping and oligotyping). The biotyping scheme relies on three biochemical tests; hippurate hydrolysis, β-galactosidase activity and the presence of lipase (Piot *et al.*, 1984). At the present, there are eight biotypes to distinguish *G.*

vaginalis isolates (Piot *et al.*, 1984). Biotype 1 (hippurate +, β-galactosidase +, lipase +), biotype 2 (hippurate +, β-galactosidase -, lipase +) and biotype 5 (hippurate +, β-galactosidase -, lipase -) are the most commonly isolated biotypes from women who suffer from BV, while hippurate hydrolysis negative biotypes 3, 4, 7 and 8 having the lowest occurrence rates (Piot *et al.*, 1984; Briselden and Hillier, 1990; Pleckaityte *et al.*, 2012). A modified biotyping scheme was developed based on the addition of sugar fermentation tests for arabinose, galactose and xylose to the standard hippurate, β-galactosidase and lipase tests (Piot *et al.*, 1984; Benito *et al.*, 1986). This modified scheme resulted in 4 groups I, II, III and IV, which was further subdivided into a total of 17 biotypes (Benito *et al.*, 1986).

Serological typing is another method that has been used to characterize *G. vaginalis* isolates. This method relies on immunologic responses to bacterial antigenic determinants that aid in clustering bacteria to serotypes. Edmunds (1962) originally identified seven serotypes based on precipitin tests that used antisera produced against 13 strains of *G. vaginalis*. Thirty-six of the fifty (72%) *G. vaginalis* isolates where sorted into these serotypes (Edmunds, 1962). The method was deemed unsatisfactory and provided difficulty in standardization of antigenic determinants (Edmunds, 1962). Due to the weak responses observed in this time-extensive detection method, this system may lack sufficiency to be used as a standard method of *G. vaginalis* classification (Ison *et al.*, 1987). In addition, cross-reactivity of *G. vaginalis* antiserum against other bacteria including *Neisseria gonorhoeae* and *Haemophilus influenzae* has been observed in several other studies (Vice and Smaron, 1973; Boustouller *et al.*, 1986).

Genotyping methods aim at identifying and grouping the bacterial strains on the basis of their unique genomic sequence and genomic organization differences. Unlike serotyping and

biotyping methods, genotyping is independent from external variables such as the diversity of host immune responses and does not rely on phenotypic detection of enzymatic pathways that is limited by the number of tested traits and might be influenced environmentally. Therefore, genotyping methods offer uniquely strain-specific resolution. Southern blot-based classical typing (RFLP) were not successful, and PCR ribotyping using intergenic regions of the16S and 23S ribosomal RNA operons of *G. vaginalis* did not produce diverse banding patterns (Ingianni *et al.*, 1997). Genotyping based on amplified ribosomal DNA restriction analysis (ARDRA) and random amplified polymorphic DNA analysis (RAPD) of the 16S rRNA gene of *G. vaginalis* have been developed, which were able to resolve the genomic differences between subgroups of *G. vaginalis*. ARDRA was utilized to originally group *G. vaginalis* isolates into 3 to 4 genome types (Ingianni *et al.*, 1997). It is important to note that the current molecular taxonomic classification of bacteria is based on the 16S rRNA sequence. Digestion of the 16S rRNA gene amplicon with restriction enzyme *Taq*I produced distinct banding patterns resulting in genotypes A, B, and C, while digestion with *Hpa*II, categorized the *G. vaginali*s isolates into 4 genotypes (Ingianni *et al.*, 1997). No specific correlation was observed between the above genotypes and the symptomatic manifestation of bacterial vaginosis. However, distribution of prevalent genotypes was shown to be unique to the places that *G. vaginalis* isolates were originated from, validating ARDRA as a powerful epidemiologic study tool. RAPD uses a fluorescently labeled single primer to randomly amplify segments of the chromosomal DNA to generate amplicons of variable sizes resulting in a species-specific banding fingerprint (El Aila *et al.*, 2009; Lopes dos Santos Santiago *et al.*, 2011). An assessment of RAPD as a genotyping technique for *G. vaginalis* was completed for 134 *G. vaginalis* isolates. In this study one-hundred and fifteen

(86%) isolates were originated from 10 Kenyan women and the remaining 19 isolates (14%) were from 16 Belgian women (Lopes dos Santos Santiago *et al.*, 2011). Each of the 134 isolates had one of three DNA fingerprinting patterns, hence genotypes 1-3, where genotype 3 was the resultant of the combined patterns of genotypes 1 and 2 (Lopes dos Santos Santiago *et al.*, 2011). Confirmation of the diversity observed by DNA fingerprinting was further established by ARDRA *Taq*1 procedure, demonstrating that both techniques were reliable methods of genetic typing of *G. vaginalis* clinical isolates (Lopes dos Santos Santiago *et al.*, 2011). Pleckaityte *et al.* (2012) subsequently identified 17 *G. vaginalis* clinical isolates with the previously described *Taq*I ARDRA technique, as being either genotype 1 or 2, which furthermore corresponded to Ingianni et al (1997) genotypes B and A, respectively (Ingianni *et al.*, 1997; Lopes dos Santos Santiago *et al.*, 2011). Direct sequencing of the 16S rRNA gene corresponding to genotypes 1 and 2, followed by digestion with *Hind*III and *BamH*I, resulted in the subtyping of genotype 2 (Ingianni *et al.*, 1997). *G. vaginalis* isolates were consequently assigned into three genotypes; genotype 1, genotype 2 (subtypes 2A, 2B, 2C) and genotype 3 or B, A and C respectively (Ingianni *et al.*, 1997; Lopes dos Santos Santiago *et al.*, 2011; Pleckaityte *et al.*, 2012). Since a standard naming scheme is absent for *Taq*I *G. vaginalis* genotyping we propose a unified naming scheme consistent with Ingianni *et al.* (1997) original scheme along with a modification to the subtyping nomenclature of Pleckaityte *et al.* (2012). This unified scheme results in genotypes; A (A1, A2, A3), B and C. Additionally, reference to ARDRA genotypes by *HpaII* digestion of *G. vaginalis* isolates will be indicated as genotypes 1, 2, 3, and 4 as stated previously by Ingianni *et al.* (1997).

Genotype prevalence with geographical bias has been detected among diverse populations as seen in the lack of genotype 3 *G. vaginalis* isolates acquired from Lithuania (Ingianni *et al.*, 1997; Pleckaityte *et al.*, 2012). Additionally, no genotypic bias was observed among isolates acquired from Rome, Italy; however, in Cagliari, a municipality of Italy with a significantly lower population than Rome, genotype 2 was prevalent (Ingianni *et al.*, 1997). *Taq*I genotype 2 (i.e. genotype A) was also the most common genotype discovered in the Lopes dos Santos Santiago *et al.* study, making up to 49% of the isolates. Nevertheless, genotype 1 (26%; i.e. genotype B) and genotype 3 (25%; i.e. genotype C) were both represented in this study as well (Lopes dos Santos Santiago *et al.*, 2011). These observations suggested that *G. vaginalis* isolates may differ between geographic regions. In addition, *Hpa*II genotype 2 and *Taq*I genotype 2 (i.e. genotype A) may be more pathogenically relevant (Ingianni *et al.*, 1997; Lopes dos Santos Santiago *et al.*, 2011).

Oligotyping, is another genotyping scheme that was used to demonstrate diversity among *G. vaginalis* isolates. This high throughput technique was used to demonstrate low level taxonomic differences among 65,710 pyrosequencing reads of 16S rRNA gene segments in V4-V6 region of 16S rRNA gene from 53 monogamous women and their male partners (Eren *et al.*, 2011). The highly variable nucleotides within these segments were identified by Shannon entropy analysis that was translated to a total of 46 oligotype clusters. The oligotypes were demonstrated to be strongly correlated between monogamous couples providing evidence for sexual transmissibility of this pathogen (Eren *et al.*, 2011). Further exploration into the validity of oligotyping as a method of high resolution subtyping and as a reliable epidemiological tool has to be established.

However, oligotyping has been utilized in the typing of the human oral microbiome, as well as in ecological studies (Eren *et al.*, 2013, 2014; Kleindienst *et al.*, 2016).

In summary, typing methods have been invaluable in providing detailed insight into the biological diversity among *G. vaginalis* isolates, nevertheless, more important areas of future studies must focus on establishing link between the biological diversity and pathophysiology, prognosis of BV and antibiotic susceptibility. This will lead to effective preventative and treatment strategies, improved patient care and reduced demand on the healthcare system.

1.3 Pathogenesis

Bacterial vaginosis occurs when the balance of the vaginal microflora is disrupted resulting in a decline in natural vaginal *Lactobacilli*. Colonization of vagina by *G. vaginalis* leading to recruitment of other anaerobic bacteria by 100- to1000-folds displace the *Lactobacillus species* resulting in a polymicrobial establishment of bacterial vaginosis (reviewed in: Bautista *et al.*, 2016).

A healthy vaginal environment is predominantly comprised of *Lactobacilli* including *L. crispatus, L. gasseri, L. iners, L. plantarum, L. rhamnosus* and *L. suntoryeus* (Swidsinski *et al.*, 2005; Anukam *et al.*, 2006). *Lactobacillus spp* are known as a protective members of the vaginal microflora through competitive exclusion of pathogenic species by producing antimicrobials such as hydrogen peroxide (H_2O_2) and lactic acid (Patterson *et al.*, 2007). Moreover, their competitive exclusion of *G. vaginalis* is facilitated by producing surface ligands and adhesions (Ojala *et al.*, 2014). The type of lactobacillus species tested, however, shows variance in their protective nature against BV biofilm production (Castro *et al.*, 2013). *L. crispatus* was shown to

reduce the adhesion percentage of BV and non-BV associated strains to a lesser extent than *L. iners;* however, *L. iners* required a higher dosage to cause a significant decline in adhesion of the non-BV associated strain (Castro *et al.*, 2013).

G. vaginalis strains can also counteract *Lactobacillus spp* competitive exclusion capacities to differing degrees depending on the nature of the *G. vaginalis* isolates. For example, BV associated strains had a *L. crispatus* displacement rate of 63.8% while non-BV strains displacement rate was 19.0% (Castro *et al.*, 2015). *L. crispatus* was also equally displaced by the addition of non-BV and BV associated *G. vaginalis* strains by up to 88% whereas *L. iners* displacement range was 8.6-11.4% from the non-BV associated *G. vaginalis* and 30.6-81.0% from the BV associated strain; where 81% displacement was induced with a high dosage of *G. vaginalis* (Castro *et al.*, 2013).

G. vaginalis initiates a BV infection partially due to its capability of inducing cytotoxicity and adhering to vaginal epithelial cells. Of the 30 BV associated bacteria (BVAB), isolated from BV-positive women in Alves *et al.*'s (2014) *in vitro* study, it was discovered that *G. vaginalis* had the highest cytotoxicity level whereas 80% of remaining 29 isolates had low cytotoxicity (Alves *et al.*, 2014). It was determined to have the highest initial adhesion ability to Hela cells (Alves *et al.*, 2014). This was supported by *G. vaginalis* adherence to an inert glass surface, which was pre-coated with *L. crispatus,* more efficiently than other BVAB: *Fusobacterium nucleantum, Provetella bivia, Atopobium vaginae* and *Mobiluncus mulieris.* The other BVAB adherence capacity is indicative of which stage of BV biofilm colonization they partake in (Machado *et al.*,

2013). *F. nucleatum* was identified as an intermediate colonizer, while *P. bivia*, *A. vaginae* and *M. mulieris* as late colonizers (Machado *et al.*, 2013).

As well as displacing *Lactobacillus spp* and initiating biofilm, *G. vaginalis* has also been shown to enhance the growth of BVAB, *P. bivia* and *F. nucleatum* providing further support for *G. vaginalis* as the major etiological agent of BV (Machado *et al.*, 2013). Furthermore, it has been established that *G. vaginalis* biofilm grows when an indiscriminate secondary BV-associated bacterium is established within the biofilm (Machado *et al.*, 2013). Specifically, for *P. bivia* this symbiotic relationship occurs due to *G. vaginalis* production of amino acids that are utilized by *P. bivia* (Pybus and Onderdonk, 1997). The cycle continues as *P. bivia* produces ammonia that *G. vaginalis* utilizes further perpetuating an abundance of *G. vaginalis* and amino acid production (Pybus and Onderdonk, 1997).

Other known BVAB, *Brevibacterium mcbrellneri*, *Enterococcus faecalis*, *Mycoplasma hominis* and *Staphylococcus hominis* have been shown to be capable of producing a biofilm, which contributes to the polymicrobial BV infection (Alves *et al.*, 2014). *Atopobium spp* have additionally been observed to form biofilms and even generate up to 40% of a BV biofilm mass (Swidsinski *et al.*, 2005).

M. hominis is a BV-associated bacterium that has significant increased prevalence and elevated bacterial load in BV compared to non-BV afflicted women (Cox *et al.*, 2016). *M. hominis* also is positively correlated with *G. vaginalis* prevalence and bacterial load in co-infections indicating a symbiotic relationship conducive to the development of a BV infection (Cox *et al.*, 2016). *Ureaplasma parvum* prevalence rates were also increased in BV relative to intermediate and

non-BV subjects (Cox *et al.*, 2016). Additionally, co-infection with *G. vaginalis* in BV positive women was 78.6% suggesting a possible link between *U. parvum* and BV (Cox *et al.*, 2016). *Atopobium vaginae* and *G. vaginalis* may also have a symbiotic relationship as a higher Nugent score was more likely when both species were present in BV associated biofilms and through 95.5% of samples containing *A. vaginae* also contained *G. vaginalis* (Hardy *et al.*, 2016). Also increased bacterial loads of both *G. vaginalis* and *A. vaginae* are observed in the biofilms (Swidsinski *et al.*, 2005; Hardy *et al.*, 2015). However, *A. vaginae* has not been identified without the presence of *G. vaginalis* and has only a 14% predictive value for BV diagnosis via quantitative PCR, further supporting the hypothesis that *G. vaginalis* colonization initiates an environment change inducive to other anaerobic bacteria (Menard, 2008; Hardy *et al.*, 2015). Horizontal gene transfer between BV associated bacteria does transpire, possibly increasing virulence properties and contributing to disease state (Harwich *et al.*, 2010).

1.4 Virulence factors

Virulence factors of *G. vaginalis* strains associated with BV were compared to non-BV associated strains, and showed several differences with respect to adhesion, cytotoxicity and biofilm formation (Castro *et al.*, 2015). Overall, BV-associated *G. vaginalis* strains were found to be more virulent than non-BV associated *G. vaginalis* strains (Castro *et al.*, 2015). This was observed through findings of higher levels of cytotoxicity to HeLa cells, as well as greater adhesion to HeLa cells (Castro *et al.*, 2015). BV-associated strains can adhere at an average of 14.8 bacteria per HeLa cell, whereas non-BV *G. vaginalis* strains adhere at a rate of only 2.8 bacteria per HeLa Cell (Castro *et al.*, 2015). These results were also supported by a trend

towards a higher biofilm forming index among the BV-associated *G. vaginalis* strains (Castro *et al.*, 2015).

Harwich *et al.* (2010) identified biochemical and genetic variations between BV-associated *G. vaginalis* isolates and commensal *G. vaginalis* isolates. It was observed that pathogenetic *G. vaginalis* induced vaginolysin (VLY)-dependent cytotoxicity compared to its commensal counterpart even though the vaginolysin amino acid sequence, encoded by *vly* gene, was only altered by one amino acid while its expression level was equivalent between strains. It is noteworthy that VLY requires direct contact with vaginal epithelial cells to induce cytotoxicity. The pathogenic *G. vaginalis* strain was observed to adhere to epithelial cells better and form a thicker biofilm, illustrating its significantly enhanced biofilm-forming capacity. This difference could further be due to sequence disparity in the biofilm associated protein (BAP) family gene identified in both isolates (Harwich *et al.*, 2010).

Vaginolysin is a cholesterol-dependent cytolysin (CDC), most closely related to Intermedilysin (ILY) and Pneumolysin (PLY) produced in *Streptococcus spp*, which form membrane embedded pores of 250-300 Å (Gelber *et al.*, 2008; Dunstone and Tweten, 2012; Hotze and Tweten, 2012). CDCs are comprised of 4 domains; domains 1 and 3 form the head region that contains Transmembrane Helix 1 and 2 (TMH1/2), which unwind before insertion into the host bilayer membrane. Domain 2 links the head region to domain 4 through β-sheets (Rossjohn *et al.*, 1997; Shepard *et al.*, 1998; Shatursky *et al.*, 1999; Tilley *et al.*, 2005). Domain 4 is necessary for the completion of attachment to the host membrane, especially through binding of cholesterol (Soltani *et al.*, 2007). The accepted pore forming model is that domain 1 undergoes a vertical

collapse and significantly alters domain 2 conformation before prepore formation to facilitate pore formation (Dunstone and Tweten, 2012; Hotze and Tweten, 2012). However, a new model, illustrates a less extensive conformational change could be responsible for the pore formation (Reboul *et al.*, 2014). The monomer configurations alter the flexibility of the complex away from domain 4 and towards domains 1 and 3 (Reboul *et al.*, 2014). Domain 2 then becomes more paralleled with the host membrane surface therefore when the vertical collapse of domains 1-3 simultaneously occurs the TMH1/2 are pushed closer to the membrane surface allowing for insertion of their β-hairpins (Reboul *et al.*, 2014). There are 3 modes of receptor recognition of CDCs based on binding affinity to membrane-bound cholesterol and human CD59 (hCD59). VLY falls into GROUP III: affinity for both cholesterol and hCD59 (Tabata *et al.*, 2014). This cytolysin is species-specific, due to its dependence on the CD59, a complement regulatory molecule, which lyses human erythrocytes and human vaginal epithelial cells but is unable to lyse sheep, mouse, or horse erythrocytes (Gelber *et al.*, 2008; Zvirbliene *et al.*, 2010). However, Zilnyte *et al*. (2015) confirmed that high levels of VLY could lyse both wildtype Chinese hamster ovary (CHO) cells, and CD59-null CHO cells (CHO-hCD59) cells. However, CHO cells lacking hCD59 expression required 32-fold more VLY than CHO cells containing hCD59. Further demonstrating that there are 2 pathways for oligomerization of the VLY complex; cholesterol alone or in the presence of hCD59. Nevertheless, in humans attachment of VLY to CD59 may enhance VLY oligomerization resulting in increased lysis by the toxin (Budvytyte *et al.*, 2013; Zilnyte *et al.*, 2015). The presence of functional pores in vaginal epithelial cells, may be the basis of ultrastructural rearrangements, e.g. blebbing, of the epithelial cell membranes (Randis *et al.*, 2013). However, VLY is also known to induce phosphorylation of the p38

mitogen-activated protein kinase (MAPK), inducing the epithelial cells immune response (Ratner *et al.*, 2006; Gelber *et al.*, 2008). An upregulation of the interlukin-8 (IL-8) pathway in epithelial cells was also observed in the presence of VLY (Gelber *et al.*, 2008).

Sialidases are considered to be virulence factors in *G. vaginalis*. Sialidases cleave sialic acid from glycoproteins, glycolipids and oligosaccharides by hydrolyzing α-glycosidic linkages between sugar residues and sialic acids (Moncla *et al.*, 2015; Srinivasan *et al.*, 2015). The sialidase produced by *G. vaginalis* has a molecular weight of 75000 Daltons and an optimum pH of 5.5 (von Nicolai *et al.*, 1984). It was shown to contribute to enhanced pathogenesis and inhibition of host immune responses (von Nicolai *et al.*, 1984; Cauci *et al.*, 2008).

The host's protective mucosal layer is weakened as the result of desialylation of mucin, providing a growth advantage for *G. vaginalis* on the vaginal epithelium (Wiggins *et al.*, 2001; Cauci *et al.*, 2008). This provides two advantages to GV and to the progression of infection. Firstly, the cleaved sialic acid can be utilized by bacterial cells as a carbon source, which further promotes their growth (Moncla *et al.*, 2016); secondly, damage to the mucosal layer allows for other glycosidases, including the upregulated α-galactosidase, α- glucosidase and β-galactosidase to reach and cleave substrates that are exposed to the bacteria. This results in a significant decline in bound α-2,6 and α-2,3-linked sialic acid in BV-positive women (Lewis *et al.*, 2013; Moncla *et al.*, 2015, 2016). By destroying the mucosal membranes, as well as causing exfoliation of the epithelial cells, the bacterium can invade epithelial tissue further and establish the biofilm (Cauci *et al.*, 2008; Onderdonk *et al.*, 2016).

The adaptive immune system can also be affected by sialidase activity through the deglycosylation of immunoglobulins, IgA, Secretory IgA and IgM (Cauci *et al.*, 1998; Lewis *et al.*, 2012). This degradation is associated with increased sialidase levels, therefore increased susceptibility risks in BV positive women (Cauci *et al.*, 1998; Cauci, Hitti, *et al.*, 2002; Cauci and Culhane, 2011). Furthermore, high concentrations of sialidase and prolidase disrupt the adaptive immune system and increase induction of interleukin -1β (IL-1β) (Cauci *et al.*, 2008). However, despite the elevated concentrations of IL-1β, the IL-1β cascade activation is hindered by the possible degradation of cytokines following the IL-1β concentration increase (Cauci *et al.*, 2008). This degradation is hypothesized to be caused by sialidase and/or prolidase either in a direct or indirect manner (Cauci *et al.*, 2008). Therefore, resulting in low levels of neutrophils, therefore reducing localized inflammation (Cauci *et al.*, 2008). The IL-1β cascade inactivation also inhibits the induction of the proinflammatory chemokine interleukin-8, resulting in low levels of neutrophils and reduced localized inflammation (Cauci *et al.*, 2008). The IL-1β cascade inactivation also inhibits the induction of the proinflammatory chemokine interlukin-8, limiting the presence of leukocytes in the vaginal environment further induces a local immunosuppression (Cauci, Guaschino, *et al.*, 2002; Cauci *et al.*, 2008). Another possibly method of host immune evasion is that some strains of *G. vaginalis*, including ATCC 14019, have genes encoding cell-surface expressed Rib-proteins, which provide protection from the host immune system (Yeoman *et al.*, 2010).

Carrying the genes for either virulence factors vaginolysin (*vly*) or sialidase (*sld*) does not seem to be indicative of a *G. vaginalis* strains association with BV, as both BV and non-BV associated *G. vaginalis* strains in Castro et al, (2015) study were shown to carry at least one of the genes

mentioned above, expect for the BV associated strain, *G. vaginalis* UM224 which was found to be negative for both genes. This was supported by non-BV and BV positive *G. vaginalis* isolates containing the *vly* gene in 100% and 98.3% of isolates, respectively (Knupp de Souza *et al.*, 2016). In contrary, Hardy *et al.* (2017) noted a higher probability of an increased Nugent score when high loads of sialidase A was present in *G. vaginalis* isolates. However when looking at the expression of the *sld* gene between BV and non-BV isolates no significant difference was observed (Castro *et al.*, 2015). Sialidase activity in a murine model has been established by Gilbert et el (2013) when sialidase was present in 67% of vaginal washes from *G. vaginalis* infected mice whereas only 14% of the control groups had sialidase activity from non-*G. vaginalis* bacterium. The sialidase activity was positively correlated with *G. vaginalis* bacterial load indicating a potential role in BV infection regardless of its presence in non-BV *G. vaginalis* isolates (Gilbert *et al.*, 2013). As for VLY, however, there was a mean 2-fold difference in the expression of VLY by BV associated strains compared to non-BV associated strains. The expression of VLY, however, has been observed to be downregulated in BV associated *G. vaginalis* isolates within their biofilm (Castro *et al.*, 2017). It has been hypothesized that this significant reduction in *vly* gene expression may be a means of evading immune response in chronic and recurrent BV infections (Castro *et al.*, 2017).

The transcriptomic profiles of BV associated *G. vaginalis* isolates in biofilm and planktonic states were characterized by Castro et al (2017). It was discovered that 78% of 1045 transcribed genes had differing expression levels with an overall conclusion that biofilm cultures induced a protective phenotype. Antibiotic resistance genes were upregulated while genes involved in metabolism and translation were downregulated (Castro *et al.*, 2017). *G. vaginalis* infection

alone can induce the BV sign of vaginal epithelial cell exfoliation (Clue cells) in a murine model consistent with the host response observed in BV positive women (Gilbert *et al.*, 2013). As well, *G. vaginalis* biofilms are significantly less sensitive to hydrogen peroxide (H_2O_2) and lactic acid, produced by *Lactobacillus* species relative to planktonic cells by 5-folds and 4 to 8-folds, respectively, further illustrating the virulence factor nature of *G. vaginalis* biofilms in BV (Patterson *et al.*, 2007). Other possible candidate virulence factors were identified via genome analysis of *G. vaginalis* strains. Among these candidates are the proteases and peptidases, which via proteolysis of host proteins can potentially provide a source of nitrogen for BVAB (Yeoman *et al.*, 2010).

Understanding the genetic basis of *G. vaginalis* virulence has been an area of interest. It was determined that *G. vaginalis* isolates carried a sialidase gene, although sialidase activity has only been found to be present among 40% of strains (Moncla and Pryke, 2009; Lopes dos Santos Santiago *et al.*, 2011; Pleckaityte *et al.*, 2012). Genotype 1, found to be predominantly represented by biotype 1 and to a less extent by biotypes 2 and 4, which were found to have no or weak sialidase activity, whereas all genotype 2 strains, exclusively represented by biotype 5, had strong sialidase activities (Pleckaityte *et al.*, 2012). Contradictory results, demonstrated the genotype 1 and 3 were sialidase positive and genotype 2 was negative for sialidase activity (Lopes dos Santos Santiago *et al.*, 2011). Opposing results, are potentially associated to population or geographical limitations or methodology (Moncla and Pryke, 2009; Lopes dos Santos Santiago *et al.*, 2011; Pleckaityte *et al.*, 2012; Schellenberg *et al.*, 2016). No relationship among genotypes with vaginolysin production levels or HIV type 1 stimulatory activity were determined, however, this could be due to low sample sizes (Simoes *et al.*, 2001; Pleckaityte *et*

al., 2012). However, genomic differences of pathogenic compared to commensal *G. vaginalis* strains have demonstrated that there is a potential for further investigation into virulence factors' correlation with genotypes and biotypes (Harwich *et al.*, 2010; Yeoman *et al.*, 2010). Such pathogenic properties include bacterial toxins, antimicrobial resistance, strains ability to adhere to epithelial cells and evasion of immune detection (Harwich *et al.*, 2010; Yeoman *et al.*, 2010).

1.5 Health risks associated with bacterial vaginosis

BV has also been found to be a risk factor for other serious co-morbidities and health concerns. BV increases susceptibility to the acquisition of sexually transmitted diseases (STI). BV has been significantly linked to vaginal infections caused by *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* (Wiesenfeld *et al.*, 2003; Rathod *et al.*, 2011; Bautista, 2017; Tosheva-Daskalova *et al.*, 2017). It has been observed that BV-positive women can have a 1.5 to 2.7-fold increased risk of acquiring trichomonas, gonococcal and chlamydial infections (Brotman *et al.*, 2010; Allsworth and Peipert, 2011). Furthermore, these risks increase each time a women acquires a BV infection by 26% and 13% for gonococcal and chlamydial infection, respectively (Bautista, 2017). Additionally, BV has been linked to the increased rate of acquiring human Immunodeficiency Virus Type 1 (HIV-1) as well as being responsible for increased HIV-1 virus load within the genital tract (Simoes *et al.*, 2001; Msuya *et al.*, 2002; Myer *et al.*, 2005; Denslow *et al.*, 2011). BV alone or in conjugation with *T. vaginalis* has also been linked with vaginal shedding of HIV-1 RNA (Fastring *et al.*, 2014); further BV/HIV-1 co-infection leads to increased rate (>3 fold) of female-to-male HIV-1 transmission (Cohen *et al.*, 2012). *G. vaginalis* and other BVAB additionally have the capability to hinder HIV preventative treatment, by metabolizing the antiretroviral drugs, e.g. Tenofovir, at a rate faster than the target cells can

uptake the drug and convert it to its active agent (Klatt *et al.*, 2017). Herpes Simplex Virus Type 2 (HSV-2) is another STI agent whose risk of acquisition is increased when an underlying chronic BV infection is present in the patient (Cherpes *et al.*, 2003; Kirakoya-Samadoulougou *et al.*, 2008). BV has also been linked to increased duration and persistence of high-risk human papillomavirus infection (HPV) when compared to BV negative women (Gillet *et al.*, 2011; Caiyan *et al.*, 2012; Guo *et al.*, 2012; Magaña- Contreras *et al.*, 2015). Bacterial vaginosis is therefore, considered a risk factor for several STI concurrent infections. Such co-morbidities complicate patient health and treatment outcomes.

Prolonged BV infection is furthermore linked with several adverse health outcomes, which include pregnancy complications; induced spontaneous abortions, increased risk of preterm labour, late stage miscarriages and stillborn occurrences (Hay *et al.*, 1994; Donders *et al.*, 2000; Leitich *et al.*, 2003; Bretelle, 2015; Shashikala *et al.*, 2015). Infants born to mothers who suffer from BV are more likely to have low birth weights (Svare *et al.*, 2006). Premature rupture of membranes (PROM) and chorioamnionitis, along with endometritis, and Pelvic Inflammatory Disease (PID) that can result in infertility, are other pregnancy related sequalae of bacterial vaginosis (McGregor *et al.*, 1993; Haggerty *et al.*, 2004; Ness, 2005; Svare *et al.*, 2006). There is also a high occurrence of cervicitis in BV positive women (Marrazzo *et al.*, 2006). Additionally, *G. vaginalis* colonization, comparable to BV infections, has been determined to have the capability of damaging the oviducts and triggering salpingitis (Taylor-Robinson and Boustouller, 2011). BV is also significantly associated with cervical intraepithelial neoplasia, pre-cancerous lesions that are common in cervical cancer (Platz- Christensen *et al.*, 1994; Caiyan *et al.*, 2012;

Gillet *et al.*, 2012). Premenstrual syndrome (PMS)-associated nausea also has been linked to *G. vaginalis* infections (Doyle, 2015).

Moreover, non-reproductive organs can be adversely affected by chronic BV. There have been reported cases of *G. vaginalis* associated meningitis, retinal vasculitis, vertebral osteomyelitis, acute hip septic arthritis, balanoposthitis, acute encephalopathy and bacteremia (Kinghorn *et al.*, 1982; Berardi-Grassias *et al.*, 1988; Amaya *et al.*, 2002; Calvert *et al.*, 2005; Graham *et al.*, 2009; Neri *et al.*, 2009; Sivadon-Tardy *et al.*, 2009; Tankovic *et al.*, 2017). The mental health issues triggered or worsened by contraction of *G. vaginalis* infection are rarely discussed. Nevertheless, BV has negative psychological impact on women suffering especially from chronic infection (Bilardi *et al.*, 2013). The self-esteem of women with BV plummet due to feelings of embarrassment and shame, which can severely impact the quality of their daily lives (Bilardi *et al.*, 2013, 2016). Self-blaming for acquiring a BV infection has also been observed (Bilardi *et al.*, 2016). Confusion in understanding how BV is transmitted and risk factors of acquiring the infection induce distress on BV positive women (Bilardi *et al.*, 2017). The stressful state that BV positive women find themselves in may even increase the frequency of BV incidences, as it has been shown that there is a link between chronic stress and BV infection (Culhane *et al.*, 2002; Nansel *et al.*, 2006). Additionally, fear of judgement and safety has also been observed in female/female relationships in South Africa, which leads to women not seeking proper treatment for STIs thus worsening prognosis (Poteat, 2015).

1.6 Transmission and Risk factors

There are many risk factors for the acquisition of BV infection among diverse populations of women. Most commonly stated risk factors are the use of broad spectrum antibiotics, resulting in vaginal microflora disruption, conducive to BV infection, as well as a history of multiple sexual partners (Barcelos *et al.*, 2008; Caiyan *et al.*, 2012; Mayer *et al.*, 2015; Melkumyan *et al.*, 2015). *G. vaginalis* has been isolated from up to 86.8% of asymptomatic women, who are the carriers of *G. vaginalis* clade 4 that lack the coding gene for sialidase, a GV associated virulence factor (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017). In contrast, Clades 1, 2 and 3 have been positively correlated with BV and carry the sialidase coding gene (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017). There appears to be consistency that certain clades are associated with the acquisition of BV (Balashov *et al.*, 2014; Janulaitiene *et al.*, 2017; Vodstrcil *et al.*, 2017). Even if specific clades of *G. vaginalis* are commensal more evidence is suggestive of BV being a STI, as the transmission of BV through sexual partners is well documented (Fethers *et al.*, 2008; Eren *et al.*, 2011; Vodstrcil *et al.*, 2015). Eren *et al* (2011) supported STI status of BV through heterosexual, monogamous couples colonized with *G. vaginalis* isolates that were of the same oligotype. BV infection of sexually experienced women has been strongly associated with penile-vaginal intercourse (Fethers *et al.*, 2009). Since *G. vaginalis* has been isolated from semen consequently males can repeatedly transmit the infection to their female partners thus perpetuating a chronic infection or transmit the infection through multiple partners (Andrade-Rocha, 2009). However, it is interesting to note that male circumcision has been linked to reducing the risk of transmission of *G. vaginalis* to female partners (Gray *et al.*, 2009). A cross-sectional study with homosexual women also supported BV as a STI through strong correlation

between sexual partners' vaginal microflora (Vodstrcil *et al.*, 2015). When comparing heterosexual couples to same sex female partners, reported BV rates are increased due to exchange of vaginal fluids (Fethers *et al.*, 2000; Marrazzo, Thomas, Agnew, *et al.*, 2010; Marrazzo *et al.*, 2011), as well as a lower level of vaginal microbiome diversity when compared to heterosexual and or bisexual women (Muzny, Sunesara, Kumar, *et al.*, 2013). Nevertheless, in a monogamous relationship female/female BV risk is reduced (Bradshaw *et al.*, 2014; Vodstrcil *et al.*, 2015). In contrast, Verstaelen *et al.* (2010) have suggested that BV is not a STI but is instead a sexually enhanced disease as BV have occurred in non-sexually active females (Vaca *et al.*, 2010).

The greater number of sexual partners, lack of condom protected sex, infidelity, frequency of intercourse and young age at first sexual experience increase the risk of BV transmission (Barcelos *et al.*, 2008; Fethers *et al.*, 2008, 2009; Verstraelen *et al.*, 2010; Caiyan *et al.*, 2012; Bradshaw *et al.*, 2013; Durugbo *et al.*, 2015; Marconi *et al.*, 2015; Muzny *et al.*, 2017). Sharing vaginal sex toys, the use of vaginal lubricants, as well as participating in oral sex have also been associated with BV prevalence (Marrazzo, Thomas, Agnew, *et al.*, 2010; Marrazzo, Thomas, Fiedler, *et al.*, 2010). Also the presence of an already established urogenital infection increases the risk of BV infection; such infections include *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, trichomoniasis, urinary tract infections, HSV-2 and other reproductive tract infections (Thorsen *et al.*, 2006; Stoner, 2011; Li *et al.*, 2014, 2015; Marconi *et al.*, 2015). Also a previous extra-vaginal infection with BVAB increases the likelihood of acquiring BV infection (Marrazzo *et al.*, 2012). Additionally, women who were diagnosed with tubal factor infertility have a 19-fold increased risk of acquiring BV (Durugbo *et al.*, 2015). Other health related risk factors for BV

acquisition include abnormal cervical mucus, dysmenorrhea, 3 or more previous abortions, low levels of estradiol and vitamin D deficiency in pregnant women (Fang *et al.*, 2007; Wilson *et al.*, 2007; Barcelos *et al.*, 2008; Li *et al.*, 2014; Skowrońska-Jóźwiak *et al.*, 2014). However, the vitamin D deficiency has not been linked to BV prevalence in non-pregnant women (Turner *et al.*, 2016). Contraceptive methods, oral pill vs intrauterine device (IUD) have also not been proven to increase the risk of acquisitioning BV and may even be protective (Castro *et al.*, 1999; Alice *et al.*, 2012; Vodstrcil *et al.*, 2013; Marconi *et al.*, 2015). However, more research in how hormonal contraceptive methods may affect the immune system of BV positive women needs to be conducted (Wang *et al.*, 2015). Hormonal changes in *Lactobacillus spp* deprived women, during the first 2 weeks of menstruation may be partially responsible for microflora shifts that increase the risk of BV prevalence (Morison *et al.*, 2005; Chaban *et al.*, 2014).

Another risk factor associated with BV infections is hygenic practices. Specifically, the act of frequent genital washing and vaginal douching as these acts result in the disruption of the vaginal microflora (Ness *et al.*, 2002; Cottrell, 2006; Brotman *et al.*, 2010; Vaca *et al.*, 2010). Vaginal douching practice per month and per week increase the risk of BV by 2.5 and 2.75-folds, respectively (Cottrell, 2006). Additionally, douching is a significantly associated risk factor for preterm birth, between gestational weeks 32-34 (Luong *et al.*, 2010). Women's vaginal douching practices rates were reported as high as 76% (Cottrell, 2006). The main reasons provided for vaginal douching by participants was to "feel clean" including after intercourse and menstruation (50.5-87.7%). Additionally, 14% of women stated they preform vaginal douching as a treatment method against vaginal infections. Up to 49.9% of women have stated that they believed vaginal douching was beneficial to vaginal health (Ness *et al.*, 2003). Vaginal tightening and drying

products have also been associated with increased BV risk (17-fold) (Durugbo *et al.*, 2015). In contrast, the type of sanitary protection, sanitary pads vs traditional cloth pads, during menstruation were not found to be related to BV prevalence (Morison *et al.*, 2005). Regardless, this continued use of harmful hygiene practices seems to be reliant on female relatives' influences, media and even health workers. A lack of understanding of the health risks strongly associated with vaginal douching put women at risk for chronic BV infections. These practices may also have cultural basis, as African and Hispanic women have reported rates of douching higher than Caucasian women (Abma *et al.*, 1997; Cottrell, 2006; Durugbo *et al.*, 2015).

Other studies have indicated that specific ethnicities may be at increased risk of GV infection (Wenman *et al.*, 2004; Dai *et al.*, 2010; Li *et al.*, 2014). Additionally, Goldenberg *et al* (1996) discovered that there was a significant difference between the colonization of BV associated microorganisms between ethnic groups. African and Hispanic women had the highest rate of colonization with 22.7% and 15.9%, respectively, while white (8.8%) and Asian-pacific islander (6.1%) women had lower colonization rates.

Socioeconomic status has similarly been associated as a risk factor for BV (Kalinka *et al.*, 2002; Bahram *et al.*, 2009; Durugbo *et al.*, 2015). Lower educational background, single marital status for both pregnant and non-pregnant women, smoking and younger maternal age have all been linked to increased prevalence of BV (Kalinka *et al.*, 2002; Bahram *et al.*, 2009; Desseauve *et al.*, 2012; Bradshaw *et al.*, 2014; Li *et al.*, 2014; Durugbo *et al.*, 2015; Marconi *et al.*, 2015). However, increased age has also been indicated as a risk factor possibly due to increased likelihood of having multiple sexual partners and exposure to previously mentioned risk factors (Fang *et al.*, 2007; Dai *et al.*, 2010).

1.7 Epidemiology

Bacterial vaginosis affects women on a global scale, however, despite its association with severe physical and psychological backlash, it is not currently considered as a reported infection by CDC and other health organizations. Therefore, all prevalence rates are estimated based on literature reports. As seen in Appendix A, the rates of BV can range from 0.4% in asymptomatic and symptomatic women to as high as 93%, with an overall trend of BV rates increasing over time. The BV prevalence rates in North America range from 9% to 93% depending on the study population (Alice *et al.*, 2012; Schwebke *et al.*, 2016). The Canadian estimated BV rates are only for Edmonton, Alberta and Toronto, Ontario at 14% and 9%, respectively (Wenman *et al.*, 2002; Alice *et al.*, 2012). The United States reported average rates typically stay around 30%, however, the asymptomatic BV incidence has been observed at rates as high as 70.5% and 93% (Cottrell, 2006; Schwebke *et al.*, 2016). Other North American countries have been shown to have similar rates as the United States; Grenada with 21.4% and Jamaica with 44.1% (Kamara *et al.*, 2000; Brooks-Smith-Lowe and Rodrigo, 2013). In South America, most of the studies have occurred in Brazil. Between 1997 and 2013, BV rates in Brazil have doubled from 15.3% to 30.1% (De Lima Soares *et al.*, 2003; Marconi *et al.*, 2015). Chile, Ecuador, and Peru have similar reported rates, 32%, 31.5% and 26.6%, respectively (Jones *et al.*, 2007; Lillo G *et al.*, 2010; Vaca *et al.*, 2010). Table A.1 (Appendix A) also summarizes the bacterial vaginosis prevalence rates for 14 countries, and their multiple cities, in Asia. Reported rates range from 5.9% in Shandong Province of China to 50% in Fars Province and 70% in Qom city of Iran (Keshavarz *et al.*, 2001; Fang *et al.*, 2007; Ghiasi *et al.*, 2014). The literature from Africa reported BV rates ranging from 6.4% to 58.4% with most of the studies reporting BV rates of 25% and higher (Frohlich *et al.*,

2007; Kirakoya-Samadoulougou *et al.*, 2008). South Africa's rates are continually higher than the other African countries, illustrated with its lowest BV rating at 33.7% and the highest at 58.4% (Frohlich *et al.*, 2007; Dols *et al.*, 2011).

Across Europe BV prevalence is relatively low, averaging around 14.5%, ranging from 3.5% for asymptomatic BV positive women to 44.7% for symptomatic patients (Appendix A, Table A.1). However, few longitudinal studies have taken place in Europe, so a trend could not be established. Additionally, the most recent studies are from 2013, with the majority of data coming from 1990s, therefore, demonstrating the need for BV infection reporting. The Oceania Countries, Australia, Papua New Guinea and New Zealand, additionally have some of the lowest reported BV rates, all under 12% (Passey *et al.*, 1998; Fethers *et al.*, 2009; Lim *et al.*, 2010; Bradshaw *et al.*, 2013) (Table A.1). Nevertheless, it can be seen through Australian studies that the frequency of BV infection has increased over the time (Fethers *et al.*, 2009; Bradshaw *et al.*, 2013). The rates reported in Table A.1 demonstrate that BV is a serious global health issue. This vaginal infection is not tracked by world health organizations and to stress the importance of the issue, many records kept in private clinics remain inaccessible, leading to an underestimation of incidences. Even the studies shown in Table A.1 cannot provide accurate prevalence rates due to the specific nature of research reports. Limiting factors include inadequate population sizes, limited geographic locations of studies as well as criteria of studies based on socioeconomic status, pregnancy, antibiotic use and presence of sequelae, which results in data that cannot be generalized to the entire countries. The rates provided also do not include intermediate vaginal infections, where a women's diagnosis score is not high enough to be considered BV positive, but is also not deemed to have a normal vaginal flora. Age is another limitation in acquiring

accurate BV rates since the presence of BV infection in postmenopausal women, with increased prevalence with age illustrate a misconception in the epidemiology of this infection when considering BV has been thought to affect only women of childbearing ages (Hoffmann *et al.*, 2014). This concept has also been a challenge through the isolation of *G. vaginalis* in premenarcheal females, demonstrating that *G. vaginalis* may be commensal as well as suggesting that BV infection could occur in prepubescent females (Myhre *et al.*, 2010; Hickey *et al.*, 2015). Additionally, in rare instances *G. vaginalis* has been isolated from rectum of preschool aged children of both male and females again establishing *G. vaginalis* as part of the normal microflora (Myhre *et al.*, 2002). These studies are also limited due to reduced interest in participation and commitment to longitudinal studies (Forcey *et al.*, 2014). In particular, *Forcey et al.* (2014) noted a link to attrition among women 30 years and under, as well as difficultly in recruiting participants without post-secondary education. Consequently, based on the reported BV rates and numerous limitations it is likely that bacterial vaginosis infection rates are being grossly underestimated.

In summary, Bacterial vaginosis is a worldwide, womens' health issue that has remained unreported and is significantly associated with many several health complications. Could the lack of attention placed upon BV be due to its taboo subject matter?

1.8 Current therapies

In the past other antibiotics e.g. Secnidazole (5-nitroimidazole derivative) and Garenoxacin (a quinolone antibiotic) were used to treat BV, however, the current standard clinical treatment for BV rely on metronidazole and clindamycin (Goldstein *et al.*, 2002; Nunez, 2005). The primary antibiotic of choice is metronidazole that is delivered orally at 500mg/twice a day for 7 days. In

case of treatment failure with metronidazole, clindamycin is indicated at 100mg in 5g of cream for 7 days (Skidmore-Roth, 2015). Metronidazole is thought to be a cost-effective antibiotic used to target anaerobic bacteria, however, the effectiveness of this antibiotic has been called into question (Löfmark *et al.*, 2010). Clindamycin, a broad-spectrum member of lincomycin class of antibiotics, can negatively affect the vaginal microflora leading to selection of resistant bacteria and outgrowth of yeast. Within 24-hours of treatment with metronidazole, a rapid decline in abundance of BVAB including *G. vaginalis* occurs at a slow rate; however, re-emergence of BV-associated bacteria often occurs in 1 week post treatment (Mayer *et al.*, 2015). Before *G. vaginalis* re-emergence an increase in lactobacilli is observed; however, their abundance declines again as BVAB re-emerge, leading to a recurring infection (Mayer *et al.*, 2015; Swidsinski *et al.*, 2005; Gottschick *et al.*, 2016). Recurrence of BV is considered to be due to development of metronidazole resistance at high rates (68.7%) in a short duration of time, which might potentially be due to its mutagenic capacity (Tomusiak *et al.*, 2011; Chaudhary, 2014; Mayer *et al.*, 2015; Schuyler *et al.*, 2016; Dingsdag and Hunter, 2018).

The route of metronidazole administration only plays a minor role in effectiveness against BV treatment as no significant differences were observed between oral versus intra-vaginal administration (Mitchell *et al.*, 2009). Furthermore, delivery of metronidazole via vaginal route only showed weak evidence (7.6%) that metronidazole can reduce the risk of upper genital tract infections following a dilation and curettage (Crowley *et al.*, 2001). However, metronidazole administration by the intra-vaginal rings provided persisting drug availability over a longer period than systemic delivery via oral administration. This is due to the gradual release of antibiotic over 12 days after an initial release of 35-55% of the antibiotic over the first 24 hours

(Pathak *et al.*, 2014).

In addition to low efficacy of metronidazole treatment, patients treated with this antibiotic might suffer from side effects, e.g. dizziness, epigastric pain, nausea and dyspnea (Decena *et al.*, 2006). Combination therapy with metronidazole and azithromycin was shown to be linked with increased adverse pregnancy outcomes (Tita, 2007).

1.9 Alternative therapies

1.9.1 Probiotics

The most common alternative treatment strategy for BV relies on delivery of probiotics. Specifically *Lactobacillus* species are considered since they are often native to the vaginal environment, produce lactic acid and/or hydrogen peroxide as antimicrobials, and may competitively displace *G. vaginalis* from epithelial surfaces (Kaewsrichan *et al.*, 2006; Saunders *et al.*, 2007; Pessoa *et al.*, 2017). Lactic acid and H_2O_2 production by *Lactobacillus helvetiuc* MTCC5463 and *L. crispatus* respectively, are examples of the members of lactobacilli that can control *G. vaginalis*. However, some *Lactobacilli* such as *Lactobacillus plantarum* do not kill *G. vaginalis* (Pessoa *et al.*, 2017). Lactic acid is produced by the members of lactic acid bacteria (LABs) as a result of sugar fermentation process reduces the vaginal pH to acidic ranges as low as pH, 4.5 where G. vaginalis cannot thrive. This leads to inhibition of its colonization (Kaewsrichan *et al.*, 2006; Mogha and Prajapati, 2017). However, for *Lactobacillus* species to increase the vaginal acidity they must be able to survive and replicate within a vaginal environment that already has alkaline pH due to the growth of *G. vaginalis*. Consequently, since *Lactobacillus* species are sensitive to alkaline pH, and a pH 6.3 can inhibit Lactobacilli survival by greater than 50%, this demonstrates a notable drawback to probiotic therapy (Pessoa *et al.*,

2017). The limited inhibitory capacity of probiotics was supported in an *ex vivo* study where *L. crispatus* colonization prevented *G. vaginalis* colonization to different degrees depending on the environmental pH; i.e. within an alkaline environment *L. crispatus* was less effective as an inhibitor of *G. vaginalis* colonization (Breshears *et al.*, 2015). However, when women are diagnosed with BV the initial colonization has already occurred, therefore it is important to determine how effective *Lactobacillus* probiotics are at destructing an established biofilm. It has been shown that *G. vaginalis* biofilm is 5-fold and 4.8-fold more tolerant to hydrogen peroxide and lactic acid, respectively, compared to *G. vaginalis* planktonic cells illustrating that the probiotics may not be effective treatment for BV with a persistent biofilm infection (Patterson *et al.*, 2007). To lower the pH level within the vaginal environment, therefore allowing *Lactobacillus* species to thrive, bioadhesive polycarbophil gels and acidic douches were compared (Milani, 2000). Although polycarbophil gels were superior in lowering the vaginal pH to physiologic level (pH 4.5) as compared to acidic douches, it remains to be determined if such treatment could systematically restore the lactobacilli rich vaginal microflora and eliminate the BVAB (Milani, 2000).

Probiotics have been considered as adjuvants for existing antibiotic treatments and assessed for the duration of recovery. Delivery of EcoVag probiotic vaginal capsules containing *L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869 in conjunction with metronidazole and clindamycin and/or fluconazole significantly prolonged the time before infection relapsed (Larsson *et al.*, 2008, 2011; Pendharkar *et al.*, 2015). The health benefit of the using *L. rhamnosus* DSM 14870 and *L. gasseri* DSM 14869 prompted further studies of these natural inhabitants of vagina by complete genome sequencing. Analysis of their genomes identified the

gene clusters coding for multiple adhesions, formation of thick exopolysaccharide capsule (EPS) that provided them with advanced competitive colonization properties leading to formation of thick biofilms (Marcotte *et al.*, 2017).

Nevertheless, different combinations of *Lactobacillus* probiotics have produced promising candidates for treatment of BV. Ingestion of yogurt containing the bacterial species such as *L. crispatus, L. garrerii, L. jensenii* and *L. rhamnosus* was shown to reduce symptoms of BV as well as improve the recovery rate (Laue *et al.*, 2017). As well as the use of a slow release vaginal tablets containing probiotics *L. plantarum* LPO1 and *L. fermentum* LF15 was determined to be a promising form of treatment for acute cases of *Gardnerella* infections (Vicariotto *et al.*, 2014). Another promising candidate is *L. helvetiuc* MTCC5463 that was incorporated into a vaginal cream and shown to produce inhibition zones (8-24mm) against *G. vaginalis in vitro* (Mogha and Prajapati, 2017). Additionally, *L. fermentum* L23, utilized in a mouse model, was determined to be a promising candidate as a probiotic against *G. vaginalis* although without evidence for recurrence rates following treatment, therefore, reservations for long term effectiveness should be considered (Daniele *et al.*, 2014). There are also potential probiotics that do not directly affect the colonization of *G. vaginalis,* rather they lead to modulation of immune system. For example, the oral and intra-vaginal administration of *L. rhamnosus* HN001 and *L. acidophilus* La-14 together with lactoferrin to a mouse model attenuated GV-induced vaginosis via up-regulation of the vaginal and gastrointestinal innate and adaptive immune responses (Jang *et al.*, 2017).

1.9.2 Pessary products

Pessaries may be utilized to deliver therapeutics for BV treatment. Gottschick *et al.* (2016) showed that the pessary containing amphoteric tenside (WO3191) was more effective in

reducing *G. vaginalis* biofilms than the pessary containing lactic acid, however, three months post-treatment follow up showed that there was a 28% recurrence rate with the amphoteric tenside pessary. Another study using lactic acid gel in combination with metronidazole showed long-term outcome in treating BV, reducing recurrence, and promoting colonization of lactobacilli (Decena *et al.*, 2006). Although use of pessaries seems not to be effective in preventing infection recurrence, the health benefit and safety of using amphoteric tenside (WO3191) was tested in a controlled randomized clinical trial following metronidazole treatment, which showed promising restorative shift in microbiome composition among the test group (Gottschick *et al.*, 2017). Interestingly, on the contrary, pessary products themselves have been blamed to increase the risk of developing BV (Alnaif and Drutz, 2000). Therefore, to treat the pessary associated-BV among pessary users, application of antimicrobial TrimsoSan gel (hydroxyquinoline based) was evaluated during standard pessary care. However, no significant difference in BV prevalence between patients treated with and without TrimsoSan gel was noted (Meriwether *et al.*, 2015).

1.9.3 Antiseptics

Broad spectrum, local antiseptic, octenidine dihydrochloride in a spray application was tested for its cure rates for BV (Swidsinski *et al.*, 2015). High initial cure rates were observed (87.5%), however, recurrence of infection was seen in 66.6% of women within 6 months post treatment. After an additional application of octenidine dihydrochloride, the cure rate was reduced to 75%, and then reduced further to 62.5% after a third treatment. Additionally, a subset of women was found to have 100% resistant bacteria to the antiseptic. This high resistance rate deterred Swidsinski *et al.* (2015) from further studying the antiseptic. Another side effect with this

antiseptic was the side effect of vaginal burning and dryness associated with the application of the octenidine dihydrochloride product.

1.9.4 Plant-derivatives

Vaginal gels with metronidazole containing plant components, *Myrutus communis* L (2%) and *Berberis vulgeris* (5%) have been studied as possible therapeutics against BV (Masoudi *et al.*, 2016). These gels were shown to be more effective than a metronidazole (0.75%) vaginal gel treatment, however, they are still not promising candidates since after the trial ended 57.5-95% of women were not cured of their BV (Masoudi *et al.*, 2016). Brazilian pepper tree, *Schinus terebinthifolius Raddi*, extract (7.4%) gel was also compared to metronidazole (0.75%) vaginal gel treatment. The cure rate using the Brazilian pepper tree extract gel was significantly lower than the metronidazole with only 12.4% cure rate further supporting evidence that vaginal gels are not effective treatments for BV (Leite *et al.*, 2011). The side effects associated with the Brazilian pepper tree extract gel (vaginal burning, rash, heat and abdominal pain) were noteworthy limitation to this treatment since the treatment should not exacerbate discomfort in the patient (Leite *et al.*, 2011).

Thymol, a molecule in thyme essential oil has also been studied for its antimicrobial effect for *G. vaginalis* (Braga *et al.*, 2010). It was determined that thymol could inhibit formation of *G. vaginalis* biofilm by up to 32.8%, but could not eliminate the biofilm (Braga *et al.*, 2010). Likewise, another essential oil from *Thymbra capitata* had anti-biofilm activity significantly more specific to *G. vaginalis* biofilm than native vaginal *lactobacillus spp* exemplifying a potential candidate for further evaluation against *G. vaginalis* infection (Machado, Gaspar, *et al.*, 2017).

1.9.5 Antimicrobial peptides

Subtilosin, a cyclic bacteriocin that is produced by *Bacillus subtilis* KATMIRA1933 was shown to prevent formation of *G. vaginalis* biofilm by targeting and inhibiting bacterial quorum sensing (Algburi, Zehm, *et al.*, 2017). Additionally, *Bacillus amyloliquefaciens* produces subtilosin A, a 35 amino acid circular bacteriocin, which targets *G. vaginalis* cytoplasmic membrane causing pore formation (Noll *et al.*, 2011). Subtilosin A in combination with clinically relevant antibiotics exerted synergistic effect on planktonic *G. vaginalis* cultures, while antagonistic effect on vaginal *lactobacillus* species was shown (Cavera *et al.*, 2015). Similarly, subtilosin synergized with both metronidazole and clindamycin against *G. vaginalis*, while not affecting *Lactobacillu*s biofilm (Algburi *et al.*, 2015). Algburi *et al.* (2015) also studied the antimicrobial, lauramide arginine ethyl ester, which additionally synergized with both metronidazole and clindamycin against *G. vaginalis*, without disrupting *Lactobacillu*s spp biofilms. Both studies indicate that subtilosin has a potential as a therapeutic agent for BV and can potentially be used in combination with other antimicrobials without significant detrimental effect on vaginal natural microflora.

The bacteriocin lactocin 160, produced by *Lactobacillus rhamnousus* has a narrow spectrum affecting *G. vaginalis* and *Prevotella bivia*, but not bacteria native to the vaginal microflora (Turovskiy *et al.*, 2009). Similar to subtilosin A, lactocin 160 targets the cytoplasmic membranes of bacterial cells resulting in cell death (Turovskiy *et al.*, 2009; Noll *et al.*, 2011). Lactosporin has also been isolated from *Bacillus coagulans* and was also identified as a potential antimicrobial against BV (Riazi, 2012). Additionally, human lysozyme has been suggested as a therapeutic agent for BV since alone or in combination with metronidazole the human lysozyme

inhibited the growth of *G. vaginalis* biofilm and degraded the already established biofilm (Thellin *et al.*, 2016; Hukić *et al.*, 2017).

Some alternative therapies target specific aspects of a BV infection. The following studies use compounds specific against *G. vaginalis* biofilm formation. The use of inhibitors has been suggested to interfere with establishment of BV-associated biofilms. Retrocyclin RC-101, a synthetic cyclic peptide has been shown to have a broad antimicrobial activity and inhibitory effect on *G. vaginalis'* vaginolysin cytotoxicity that reduces biofilm formation, however, it does not have a direct effect on planktonic cell growth. Therefore, its use as a therapeutic candidate for BV needs further *in vivo* studies (Hooven, 2012). Recombinant single-chain antibodies and polyclonal rabbit immune serum were also suggested to control the cytotoxicity of VLY (Randis *et al.*, 2009; Pleckaityte *et al.*, 2011). Although VLY antiserum could reduce VLY-mediated lysis of CD59 epithelial cells, the use of antibodies would only be considered a preventative approach instead of a treatment modality for BV (Randis *et al.*, 2009). However, overall diminishing vaginolysin activity would result in limiting the virulence of *G. vaginalis*.

G. vaginalis has been observed to release extracellular DNA during its early stationary growth phase that is essential for its structural integrity and might assist in colonization. It has been hypothesized that removal of extracellular DNA with DNases might have a potential to interfere with colonization of GV (Hymes *et al.*, 2013). DNase was demonstrated by Hymes *et al.* (2013) to decrease *G. vaginalis* biofilm density by >10 fold *in vivo* using a murine model. It was shown that DNase treatment liberated the GV cells from the biofilm and increased the efficacy of antibiotics. Other anti-biofilm agents such as amphoteric tenside sodium cocoamphoacetate also

has been shown to decrease the GV biofilm by 51% (Gottschick *et al.*, 2016), while, cationic amphiphiles (CAms) were shown to prevent and eliminate *G. vaginalis* ATCC 14018 biofilms by disrupting the bacterial cell membrane due to their membrane-lytic properties (Algburi, Zhang, *et al.*, 2017). Additionally, CAms seem to have a narrower spectrum, needing a much higher concentration to affect *Lactobacillus* species (Algburi, Zhang, *et al.*, 2017). They were also shown to be promising in combination with metronidazole in the treatment of BV (Algburi, Zhang, *et al.*, 2017).

In summary, the difficulty in treatment of BV is in part due to the polymicrobial nature of the BV biofilm to which *G. vaginalis* has the primary contribution as the progenitor of biofilm initiator and establishment, leading to progressive detrimental shift in healthy vaginal microflora. The problem is further potentiated by biodiversity inherent to *G. vaginalis* that might have been the basis for its plasticity with respect to its virulence capacity and response to treatment. Limitation in availabilities of treatment options and the menace of rapidly growing rates of antibiotic resistance has inevitably contributed to the chronic nature of infection and treatment failure leading to increased epidemiological prevalence of disease globally. Therefore, progressively increasing rates of treatment failure of BV using antibiotics has prompted the research toward discovery of alternative therapeutics and testing novel modalities *in vitro* and *in vivo* in the recent years. Consequently, targeting *G. vaginalis* is fundamental for developing alternative therapies.

A good alternative therapeutic candidate for treatment of GV-related BV must satisfy the following conditions:

i) it must have a narrow spectrum and specifically target *G. vaginalis* as the initiator of BV biofilm production preferably through bactericidal function.

ii) it must not have a deteriorating effect on the vaginal healthy microflora;

iii) its bactericidal effect must not be discriminatory with respect to biological diversity among *G. vaginalis*;

iv) it must show superiority to current antibiotics with respect to having novel mode of action, lack of toxicity and resistance development.

1.10 Scope of the study

We hypothesize that natural antimicrobial agents are promising candidates as future alternative therapeutics for the etiological agent of bacterial vaginosis. Proof of concept will be demonstrated by acquiring a collection of naturally occurring bacteria with potential for producing antimicrobials against a diverse population of *G. vaginalis* isolates resulting in a proposed minimal cocktail. We aim to accomplish this by first obtaining a diverse collection of *G. vaginalis* isolates and further characterizing the isolates based on their genotypic and antibiotic susceptibility profiles. Secondly, a collection of bacteria with antimicrobial activities against our *G. vaginalis* collection will be isolated and characterized. The nature of the antimicrobial active agents will be further studied to be able to propose a minimal cocktail formulation consisting of diverse antimicrobials with overlapping complimentary properties against the *G. vaginalis* collection.

2 Materials and Methods

2.1 Materials

2.1.1 Bacterial strains

A total of fourteen *G. vaginalis* clinical isolates, collected from across Canada and confirmed to be *G. vaginlais* by 16S rRNA sequence, were obtained from the bacterial collection of National Microbiology Laboratory (NML), Manitoba, Canada (Table 2.1). The *Gardnerella vaginalis* ATCC 14018, 14019 and 45149 together with non-GV bacteria that were used for target spectra analysis were also acquired from ATCC and are listed in (Table 2.).

2.1.2 Culture media, supplements, antibiotics, reagents, enzymes and kits

Dehydrated nutrient media Brain-heart infusion broth (BHI) (Nutri-Bact, Terrebonne, Quebec, CA, Cat: QB-48-0305) , Columbia broth (CB) (Quelab, Montreal, Quebec, CA, Cat: QB-48-1106), Mueller-Hinton (MH) broth (Oxoid Ltd., Basingstoke, Hampshire, England, Cat: CM0405), Difco Lactobacilli MRS broth (Becton, Dickson and Company, Le Pont de Claix, France, Cat: 288130), Tryptic Soy Broth (TSB) (Becton, Dickson and Company, Le Pont de Claix, France, Cat: 211825) were utilized to prepare broth culture media. Bacteriological Agar (Quelab, Montreal, Quebec, CA, Cat: QB-46-0221) was added to broth media at 1.5% and 0.7% to prepare solid culture plates and top agar, respectively. The following supplements were added to culture media as needed: 10% (v/v) fetal bovine serum (FBS) (Life Technologies Corporation, Grand Island, NY, USA, Cat:12483-020), soluble starch (Sigma-Aldrich, St. Louis, MO, USA, Cat: SLBP7458V), Hemin from bovine with ≥ 90% purity,

Table 2.1 Clinical *G. vaginalis* isolates given names, NML identification number and source of isolation.

Name	NML #	Source
cGV1	02-0045	Amniotic Fluid
cGV2	02-0183	Urine
cGV3	60420	Blood
cGV4	100244	Uterine cavity
cGV5	100570	Urine
cGV6	110582	Blood
cGV7	110707	Urine
cGV8	120040	Urine
cGV9	120093	Urine
cGV10	120820	Tracheal aspirate
cGV11	130068	Tracheal aspirate
cGV12	130180	Deep Wound
cGV13	130503	Urine
cGV14	130786	Urine

Table 2.2 List of standard G. vaginalis and Non-*G. vaginalis* bacteria used for target spectra analysis of αGV isolates.

Organism	Strain Number (ATCC)
G. vaginalis	14018
G. vaginalis	14019
G. vaginalis	45149
Bifidobacterium adolescentis	15703
Bifidobacterium animalis	700541
Bifidobacterium longum	15707
Clostridium difficile	9689
Enterococcus durans	6056
Enterococcus durans	11576
Enterococcus durans	19432
Enterococcus hirae	8043
Enterococcus hirae	51686
Lactobacillus acidophilus	4356
Lactobacillus acidophilus	53544
Lactobacillus casei	393
Lactobacillus delbrueckii	11842
Lactobacillus reuteri	23272
Lactobacillus rhamnosus	53103
Listeria monocytogenes	13932
Listeria monocytogenes	19115
Staphylococcus warneri	49454

(Sigma-Aldrich, Saint Louis, MO, USA, Cat:H9039-1G) and vitamin K_1 (Sigma-Aldrich, Saint Louis, MO, USA, Cat: V3501-1G), Single Donor Human Whole Blood Unit CPD (Cedarlane, Burlington, NC, USA, Cat: IPLA-WB1-UNIT-CPD) or laked, defibrated sheep blood (Nutri-Bact, Terrebonne, QC, CA). Preparation and final concentrations of supplements are listed in Appendix B. Selective *G. vaginalis* Human Blood Bilayer Plates were prepared in BHI agar with 10% human blood supplement and by the addition of antibiotics, Nalidixic acid sodium salt (Sigma-Aldrich, St. Louis, MO, USA, Cat: N4382) and Amphotericin B (Sigma-Aldrich, St. Louis, MO, USA, Cat: A9528) as per Appendix B.

Sterile plastic 1µL inoculation loops (Sartedt, Germany, Cat: 86.1562.010) were used to inoculate broth and plates with bacteria. Cell resuspension solution was from wizard Plus Midipreps DNA Purification kit that was used for preparation of bacterial DNA libraries (Promega, Madison WI, USA, Cat: A711F).

The Epsilometer test (Etest) was applied for antibiotic susceptibility testing and the E-strips were purchased from bioMérieux Canada Inc (St. Laurent, QC, CA). The E-strips included: metronidazole MZ 256 US S30 (Cat: 412403), clindamycin CM 256 WW S30 (Cat: 412315, ampicillin AM 256 US (Cat: 501558), ciprofloxacin CI 32 WW S30 (Cat: 412311), linezolid LZ 256 WW S30 (Cat: 412450), rifampicin RI 32 WW S30 (Cat: 412450), tetracycline TC 256 WW S30 (Cat: 412471), and vancomycin VA 256 US S30 (Cat: 412486).

The following stock enzymes, chemicals and solutions were used for isolation of *G. vaginalis* genomic DNA (gDNA): pronase (20µg/µL) (Boehringer Mannheim GmbH, Germany), Ribonuclease (RNase) A/T1 (2mg/mL RNase A and 5000 U/mL RNase T1) (Thermo scientific,

Rochester, NY, USA, Cat: EN0551), EDTA disodium salt dihydrate biotechnology grade (Bioshop, Burlington, ON, CA, Cat: EDT001.500). Bacterial Genomic DNA Isolation Kit (Norgen biotek Corp. Cat: 17900) was used to isolated αGV bacteria gDNA.

Taq DNA polymerase (*Taq* Pol) (50U/µl), 10mM deoxyribonucleotide triphosphates (dNTPs), polymerase chain reaction (PCR) buffer without Mg^{+2} (10X) and 50mM $MgCl_2$ were used along with PCR primers (Section 2.1.3) were used in PCR reactions (Invitrogen, Carlsbad, USA). Restriction enzymes FastDigest HpaII (Thermo Scientific, Rochester, NY, USA Cat: FD0514) and *Taq*I (Thermo Scientific, Rochester, NY, USA, Cat: FD0674) were used in genotyping experiments.

Agarose gels (0.7%–2%) were prepared in 1X tris-acetate-ethylenediaminetetraacetic acid (TAE) running buffer and were used for DNA gel electrophoresis (Appendix C). The DNA ladder 1 kb plus ladder (Invitrogen, Carlsbad, USA, Cat: 10787018) was used as marker in DNA electrophoresis experiments. RedSafe Nucleic Acid Staining Solution (20,000x) (FroggaBio scientific solutions, Toronto, Ontario, CA, Cat: 21141,) was used to direct visualization of DNA bands in agarose gels (BioShop, Burlington, ON, CA, Cat: 9012-36-6).

The concentration or extraction of anti-GV active agent protein from the culture supernatants were completed with either chloroform extraction ACS, reagent grade (BioShop, Burlington, ON, CA, Cat: CCL402.1) or by precipitation in 100% methanol (Fisher Scientific (Fair Lawn, NJ, USA, Cat: BP1105-4).

SDS-PAGE gels (12% or 15% separating; 4% stacking) were made with the following components (Appendix C): acrylamide, electrophoresis Grade, min 99.5% (BioShop, Burlington,

ON, CA, Cat: ACR002.500), bis-acrylamide (Sigma Chemical Company, St. Louis, MO, USA, Cat: M-2022), Tris base (BioShop, Burlington, ON, CA, Cat: TRS001.1), Sodium dodecyl sulfate (SDS) BioUltraPure electrophoresis grade (BioShop, Burlington, ON, CA, Cat: SDS001.500), ammonium persulfate electrophoresis grade (BioShop, Burlington, ON, CA, Cat: AMP001.10), β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA, Cat: M3148-25ML, bromophenol blue, glycerol certified ACS (Fisher Scientific, Fair Lawn, NJ, USA Cat: G33-500), and TMED electrophoresis purity reagent (Bio-Rad Laboratories, Hercules, CA, USA, Cat: 48411A) (Appendix C). High range, BLUeye prestained protein ladder (FroggaBio scientific solutions, Toronto, Ontario, CA Cat: PM007-0500) and Spectra Multicolor low range protein ladder (Thermo Scientific, Rochester, NY, USA Cat: 26628) were used in protein analysis in SDS-PAGE experiments.

The Silver Staining Plus kit (Bio-Rad Laboratories, Hercules, California, USA Cat: 161-0449) was used to stain and visualize the protein bands in SDS-PAGE slabs. Glacial acetic acid ACS, reagent grade (BioShop, Burlington, ON, CA, Cat: ACE222.4) was used to preparation of protein gel distaining solution.

2.1.3 PCR primers

G. vaginalis specific oligonucleotide primers were synthesized by Sigma-Aldrich (Oakville, Canada). Primers were utilized in identification and genomic typing of *G. vaginalis* collection (Table 2.).

Table 2.3 *G. vaginalis* specific primers based on *G. vaginalis* ATCC 14018 reference strain (Genbank reference no. AP012332.1).

Primer ID	**Direction**	**Primer sequence (5'→3')**	**Nucleotide Target**	**Amplified Regions (5'→3')**	**Fragment size (bp)**	**Reference**
Ingia-F	Forward	TTCGATTCTGGCTCAGG	16S rDNA	96,453…97,886	1,434	Ingianni *et al.*, 1997
Ingia-R	Reverse	CCATCCCAAAAGGGTTAGGC		219,594…221,027	1,434	

2.1.4 Equipment and other tools

Bacterial cultures were incubated in the following incubators depending on atmospheric acquirements of bacteria: An 855-AC Controlled Atmosphere Chamber was used for anaerobic incubation (Plas Labs, Lansing, USA). Aerobic and 10% CO_2 incubation was carried out in VWR 1545 Incubator (VWR) and Air-jacketed DHD Autoflow Automatic CO_2 Incubator NU-5510/E (NUAIRE), respectively. Candle jars (Oxoid, Basingstoke, UK) were used for storage of *G. vaginalis* culture plates in 5% CO_2. Bacteria were visualized in Axio Scope.A1 fluorescent microscope (Carl Zeiss Microscopy, Thornwood, USA).

Beckman Coulter Allegra X-12 Benchtop Centrifuge with SX4750A rotor (Palo Alto, USA) was used for centrifugation of volumes larger than 2mL. While for volumes of $\leq$ 2mL, the Sorvall Legend Micro 21 Microcentrifuge with the 24 x 1.5/2.0 mL Rotor was used (Thermo Scientific, Germany). Centrifugation of supernatants during methanol extraction was completed in Beckman J2-21M with JA-20 rotor.

CLASSIQswab swabs were used to create bacterial lawns for antibiotic susceptibility testing (Copan Flock Technologies, Brescia, Italy, Cat:138CS01). Target ranges of the αGV collection was determined on Nunc OmniTrays (Thermo Scientific, Rochester, NY, USA, Cat: 12565296) with the use of 96 Pin Long Replicators (Scinomix, Earth City, USA, Cat: SCI-5010-05).

Bacterial gDNA purity and concentrations was determined using NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Overnight incubation for DNA

extractions were performed in Fisher Scientific Mini-Tube Rotator (Cat: 05-450-127). Optical densities (OD) of bacterial cultures were read on a computerized plate reader TECAN spectra.

SnapGene software was used to construct the *Taq*I and *Hpa*II restriction maps on an *in silico* generated PCR amplicon using the GV-specific 16S rDNA primers on *G. vaginalis* ATCC 14018 genome (Genbank reference no. AP012332.1).

A PTC-100 Programmable Thermal cycler (MJ Research Inc, Watertown, USA) was used for conducting PCR and genotyping of *G. vaginalis* isolates. Gel visualization and imaging were completed by the FluorChem FC3 imaging system using the AlphaView Software.

Samples of gDNA of αGV isolates were prepared and contained in skirted/colourless Eppendorf twin Tec PCR Plate 96, (Fisher Scientific, Fair Lawn, NJ, USA, Cat: 951020401) and Pierceable Adhesive Foil, as per instructions by Genome Quebec Innovation centre, prior to shipment and sequencing by Illumia HiSeq platform.

2.2 Methods

2.2.1 Culture conditions, cryopreservation and preparation of bacterial gDNA libraries

All bacterial strains and clinical isolates were grown at 37°C. *G. vaginalis* isolates were cultured on BHI human blood bilayer plates and/or grown in BHI broth supplemented with 10% FBS and 1% soluble starch and incubated at 37°C and 10% CO_2. Anti-GV isolates, *Enterococcus faecalis* ATCC 29212, and *S. aureus* ATCC 29213 were incubated anaerobically on CB plates. For antibiotic susceptibility testing, *G. vaginalis* isolates were grown on TSA plates (Tryptic Soy

Agar) plates supplemented with 5% laked, defibrated sheep blood and *Enterococcus faecalis* ATCC 29212 was cultured on Mueller Hinton (MH) agar plates. The non-*G. vaginalis* bacterial collection were cultured in an anaerobic chamber. *Bifidobacterium* ATCC strains were incubated in BHI supplemented with Hemin (5mg/L) and Vitamin K_1 (1mg/L). The following bacteria were cultured on CB medium: *Clostridium difficile* ATCC 9689, *Listeria monocytogenes* (ATCC 13932 and 19115), *Enterococcus durans* (ATCC 6056, 11576, and 19432), *Enterococcus hirae* (ATCC 8043 and 51686) and *Staphylococcus warneri* ATCC 49454. *Lactobacillus spp.* ATCC strains were cultured on MRS medium. Details of preparation of media and supplements are shown in Appendix B.

Cryopreservation of bacterial strains was completed by growing 5mL broth cultures for 1-3 days under appropriate atmospheric conditions until a visible turbidity was reached. Cultures were pelleted by centrifugation at 3200xg for 30 minutes. Supernatants were removed, and the bacterial pellets were resuspended in 1mL 10% sterile glycerol. Concentrated cell suspensions were transferred to 2 cryovials, 500µL in each. The glycerol preserved cell stocks were stored in -80°C for long-term storage.

For preparation of DNA libraries, bacterial cultures were established via inoculation of broth media (20mL for *G. vaginalis* collection, and 10mL for αGV collection). Cultures were incubated for 1-3 days under appropriate atmospheric conditions. Once turbid, broth cultures (full volume of *G. vaginalis* cultures and 2mL of 10mL αGV cultures) were centrifuged at 3200xg for 30 minutes. Supernatants were removed, and pellets were resuspended and washed with 500µL of cell resuspension solution. Cells were transferred to 1.5mL sterile epitubes and

centrifuged at 14000xg for 5 minutes at 4°C. Supernatants were decanted and pellets were resuspended in 400µL of cell resuspension solution. Cell suspensions were stored in -80°C until DNA extraction was completed. Detailed procedures for preparation of buffers and reagents are shown shown in Appendix C.

2.2.2 Morphological and biochemical testing

2.2.2.1 Microscopy

A volume of 7µL of turbid culture was pipetted onto a glass microscope slide and covered with a cover slip. The Axio Scope.A1 at 400X magnification was used to observe cell morphology and images were captured using the phase contrast setting of the microscope.

2.2.2.2 Hemolysis

G. vaginalis isolates were plated onto BHI bilayer plates and incubated under 10% CO_2 at 37°C for 2 days. Hemolysis pattern was observed as clear zone around individual colonies.

2.2.2.3 Catalase Test

A volume of 5µL 30% H_2O_2 was pipetted onto a glass microscope slide. A sterile inoculation loop was used to select a colony from the *G. vaginalis* or αGV culture plates and placed into the H_2O_2. Catalase positive colonies showed bubbling pattern upon contacting hydrogen peroxide. *S. aureus* 29213 and *G. vaginalis* ATCC 14018 were used a negative and positive controls, respectively.

2.2.3 Identification and genetic characterization of *G. vaginalis* collection

2.2.3.1 Extraction of Genomic DNA from *G. vaginalis* isolates

The suspension of *G. vaginalis* bacterial cells prepared in section 2.2.1 for DNA library were thawed. Cells were pelleted from the suspension following centrifugation 14000xg for 2 minutes at 4°C. Pelleted cells where then washed with 1mL sterile ddH_20 and resuspended in 240µL lysis cocktail. Lysis cocktail consisted of 208µL cell lysis buffer (Appendix C), 6µL pronase (20µg/µL), 24µL 10% SDS and 2µL RNase A/T1 (2 µg/µL, 500U/mL). Cells/lysis cocktail mixture was mixed by pipetting up and down and then incubated overnight at 37°C in Mini-Tube Rotator (2 rpm). The lysed cells were incubated in 70°C water bath for 10 minutes. Subsequently, 120µL of 3 M potassium acetate (pH 5.5) was added and mixed well by inversions. The cleared supernatant was collected by centrifugation at 17000xg for 15 minutes at 4°C. The collected supernatant (300µL) was then transferred to a sterile 1.5mL epitube, and 1200uL of ethanol/0.16 M sodium acetate was added. Gentle inversion was used to mix the contents and to provide visualization of condensing chromosomal DNA. If chromosomal DNA was not visible, 2µL of glycogen (20mg/mL) was added, and the contents were mixed by inversion and stored in -20°C for 1-2 hours followed by centrifugation at 17000xg for 15 minutes at 4°C. The supernatant was discarded, and the remaining DNA pellet was washed in 700µL of 70% ethanol chilled to -20°C. The epitube was then inverted onto a clean Kimwipe absorbent surface for 2 minutes before fully dried in a vacuum evaporation chamber for 10 minutes. The DNA pellet was then resuspended in 200µL sterile ddH_20.

Purity and concentration of the extracted gDNA was verified by Nanodrop spectrophotometry, where 2µL of gDNA was read in triplicate and an average value was calculated. A 2µL sample of the gDNA with 5µL of 1X DNA loading dye was subjected to electrophoresis in a 0.7%

agarose gel containing RedSafe (2.5ul/25 ml of gel). Three microliters (0.25ug) of 1 KbP DNA ladder was also loaded and the gel electrophoresis ran for 40 minutes at 80V. The gDNA bands were visualized and imaged with the use of FluorChem FC3 imaging system with AlphaView Software. The prepared gDNA library was stored in -20°C until use.

2.2.3.2 PCR based identification of *G. vaginalis* collection

G. vaginalis specific 16S rDNA primers (section 2.1.3 and Table 2.) were used to amplify and genetically type the *G. vaginalis* collection. The concentrations of gDNA extracted in section 2.2.3.1 were adjusted to 0.05µg/mL in each PCR reaction. A PCR mixture (50µL) containing: 38.5µL of sterile ddH_2O, 5µL of 10X *Taq* pol buffer without magnesium, 1.5µL magnesium chloride (50mM), 1µL dNTPs (10mM), 1µL *Taq* Pol (5U/µL), 1µL Ingia-F primer (10µm), 1µL Ingia-R primer (10µm) and 1µL of gDNA (0.05µg). The PCR mixture was mixed via pipetting up and down and then ran on PTC-100 Programmable Thermal Cycler under optimized PCR conditions modified from Ingianni *et al*. (1997). Conditions are as follows: predwell at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and extension at 72°C for 1 minute, followed by postdwell at 72°C for 5 minutes. A 4°C temperature was held until tubes were removed from PCR machine and stored in -20°C.

The amplified single PCR product (Figure 3.4) was resolved by gel electrophoresis on 0.7% agarose gel with RedSafe and 1X TAE buffer as follows: Two microliters of PCR reaction mixture combined with 5µL 1X loading dye was loaded to the agarose gel. Three microliters of 1 Kb DNA ladder was also included. Gel electrophoresis was run at 80V for 1 hour and the gel was photographed by FluorChem FC3 imaging system with AlphaView Software.

2.2.3.3 Genotyping of *G. vaginalis* collection

Genotyping of the *G. vaginalis* collection was completed by two different restriction enzymes, FastDigest *Taq*I and *Hpa*II (Thermo Scientific, Inc.), which provided two different genotyping patterns. One microliter of each of the restriction enzymes *Taq*I and *Hpa*II were added to 5µL of PCR reaction mixture in separate tubes and mixed by pipetting followed by incubation for 15 minutes in PTC-100 Programmable Thermal Cycler at 65°C and 37°C for *Taq*I and *Hpa*II reactions, respectively.

Entire volume of each of the restriction reaction was mixed with 1µL 6X loading dye and loaded into 2% agarose gel with RedSafe and ran against 3µL of 1 KbP DNA ladder. Gel electrophoresis was run at 80V for 1.5 hours. The gel was photographed in the FluorChem FC3 imaging system with AlphaView Software. Images were analyzed, and genotypes were assigned for each isolated depending on the number and sizes of visible bands in comparison to known controls.

2.2.4 Antibiotic susceptibility testing

Antibiotic susceptibility testing was completed with E-tests based on bioMérieux and Clinical and Laboratory Standards Institute (CLSI) protocols (Clinical and Laboratory Standards Institute, 2004; bioMérieux, 2012b). *G. vaginalis* E-testing was completed on 150mm plates containing 4mm thick TSA agar plates supplemented with 5% laked sheep blood. To make sure of the accuracy of the experimental conditions, the *E. faecalis* ATCC 29212 was used as the quality control strain that was plated on MH agar plates as per standard protocols (De Backer *et*

al., 2006; bioMérieux, 2012b; Schuyler *et al.*, 2016). Expected and observed MIC values for quality control strain, *E. faecalis* ATCC 29212, is summarized in Appendix D.

Cryopreserved *E. faecalis* ATCC 29212 was subcultured twice onto MH plates prior to E-testing. *G. vaginalis* isolates and quality control strain were grown in appropriate broth media and atmosphere. The turbidity of the *G. vaginalis* and *E. faecalis* ATCC 29212 cultures were adjusted to 1.0 and 0.5 McFarland turbidity index, respectively, as per standard protocols prior to plating (De Backer *et al.*, 2006; bioMérieux, 2012b). The standardized bacteria suspensions were streaked onto the surface of the agar plates with sterile swabs three times. The swabs samples were taken three time from the bacterial suspension before each set of streaking, rotating the plate 90 degrees each time thus creating a uniform bacterial lawn. Lawns were allowed to dry before E-test strips were laid down on the plates. E-test strips were equilibrated to room temperature for 30 minutes before applying onto bacterial lawns according to recommendation by the manufacturer (bioMérieux, 2012b). Plates were then inverted and incubated under 10% CO_2 or O_2, no higher than 5 stacked plates for approximately 72 hours for *G. vaginalis* isolates and 16-20 hours for *E. faecalis* ATCC 29212 (bioMérieux, 2012b).

Minimal inhibitory concentrations (MIC) for quality control from European Committee on Antimicrobial Susceptibility Testing (EUCAST) (LZ, VA, RI, AM). CLSI for MIC ranges (CM, CI, TC) were considered for evaluation of the results for *E. faecalis* ATCC 29212 quality control strain (bioMérieux, 2012b, 2014; Clinical and Laboratory Standards Institute, 2015; EUCAST, 2016). MIC ranges for *G. vaginalis* isolates were extracted from the literature (De Backer *et al.*, 2006; Nagaraja, 2008; Clinical and Laboratory Standards Institute, 2015).

Colonies present within the zone of inhibition ellipse of cGV2 after exposure to antibiotics during E-testing were isolated from both sides of the E-test strip, starting from the lowest antibiotic concentration to the highest. Colonies isolated from the left-hand side of the E-test strip were labelled as presumptive mutants “a” while the colonies isolated from the righthand side of the strip were presumptive mutants “b”, additionally with identification by numbers increasing from lowest to highest based on the level of antibiotic concentration the colonies were exposed to. Ten colonies from either side of the E-strip were isolated and colony purified for further testing by genotyping and antibiotic susceptibility as previously described.

2.2.5 Isolation of αGV bacterial collection

A co-culture utilizing agar overlay method was employed in the isolation of the αGV collection. About 1-2 litre of raw sewage sample was collected on the April 25th, 2016 from Robert O. Pickard Environmental Centre (ROPEC) in Ottawa and stored at 4°C prior to usage.

ROPEC samples were diluted by 100-folds in BHI broth. A volume of 200µL of diluted ROPEC sample was added, along with 500µL of turbid *G. vaginalis* ATCC 14018 or 14019 broth cultures, to 5mL of Columbia broth (CB) top agar (0.7%). Contents were mixed by inversion and overlaid onto BHI plates. Once top agar had solidified, plates were inverted and incubated under anaerobic conditions at 37°C. This was repeated for each of the *G. vaginalis* ATCC strain (14018 and 14019) on 20 plates.

Colonies which produced inhibition zones on the *G. vaginalis* lawns were isolated by a sterile inoculating needle and streaked on a BHI plate to isolate single colonies. A single colony from

each plate was picked and subcultured three times to make sure of the purity of the culture. Total of 34 pure isolates formed the αGV collection. αGV inhibition zones diameters (mm) on the primary plates were also noted. The αGVs were assigned alphanumerical identities on the basis of the *G. vaginalis* ATCC strain they were isolated on. For example, αGV18-12 denotes the isolate number 12 on G. vaginalis ATCC 14018, etc.

2.2.6 Identification and characterization of αGV collection

2.2.6.1 Genomic DNA extraction and sequencing

αGV gDNA extraction was completed with Bacterial Genomic DNA isolation Kit (Norgen Bioteck Corp, ON, Canada). Alterations to protocol are as followed: RNase A/T1 (2 μg/μL, 500 U/mL) instead of 10 Kunitz of RNase A, Pronase (20μg/μL) instead of Proteinase K and incubation during lysate preparation was extended from 2 hours to overnight at 37°C in Mini-Tube Rotator (2 rpm). Sequencing of αGV gDNA was accomplished by 2 x 125 paired end reads in Illumina HiSeq platform (McGill University and Génome Québec Innovation Centre). The technical control for clustering reactions, sequence accuracy and sequencing diversity in low complexity libraries was phage ΦX174 genomic DNA. Samples were demultiplexed and fastq files were provided for each strand (R1-R2).

2.2.6.2 Genome Assembly of αGV collection

Contig assembly and further genomic analysis was completed by Dr. Gustavo Ybazeta. The provided sequences were checked for contamination from ΦX174 with the program BBsplit from the BBMap package through BBtools (Bestus Bioinformaticus, Inc.) (Mukherjee *et al.*, 2015; Bushnell). The code used was as follows:

bbsplit in1 =foo_R1.fastq in2=foo_R2.fastq ref=phix.fa basename=out_%.fq outu1=foo_R1_phi.fastq outu2=foo_R2_phi.fastq

Quality control of the sequences was completed using the program FastQC High Throughput Sequence QC report (version 0.11.5) (Andrews, 2010). Sickle sequence trimming tool was further utilized to complete a quality trimming of the 3'and 5' ends of the sequence reads based on the quality scores and length parameters, which resulted in single sequences to be removed from the R1 and R2 paired trimmed sequences (Joshi and Fass, 2011). Parameters for considering the reads for trimming included, i) paired end sequences reads with an average quality score less than 20, ii) their lengths had to be greater than 10bp.

Code used is as follows: Sickle pe –f R1.fastq –r R2.fastq –o trim.R1.fastq –p trim.R2.fastq –s singlets.fastq –q 20 –l 10 –t "sanger"

De novo assembly of the paired reads was produced using the program Spades v. 3.9.0. Error corrections (k 21, 33, 55,77, 99) were used to improve alignment of the contigs (Bankevich *et al.*, 2012; Nurk *et al.*, 2013). Prokka, an annotation pipeline software, was used to annotate the assembled genomes with a parallel approach and multiple cores when available (Seemann, 2014). The pipeline produced multiple outputs and outputs with the extension "txt" that were utilized in the production of the phylogenetic trees.

Code used for annotation is as follows: for i in `cat list.txt`; do prokka –outdir $i/prokka –cpus 40 –force –rnammer $i/prokka.fasta; done

2.2.6.3 Phylogenetic analysis of αGV collection

To identify the taxonomic standing of each genome sequence, 16S rRNA and RNA polymerase subunit beta genes (*rpoB*) were extracted with the use of BLAST 2.6.0+ (Camacho *et al.*, 2009).

The code used is as follows: for i in `cat list.txt`; do blastn –query 16.fasta –subject PROKKA*.fasta –out $i/16S_tmp –evalue 0.0001 –perc_identity 70 –outfmt “6 sseqid sseq”; done

The sequences used as general queries of the 16S rRNA and *rpoB* genes were extracted from the complete genomes of *Enterococcus faecalis* 62 (gb CP002491.1) and of *Enterococcus faecium* 1352 (gb LN99984). Once sequences were identified for each αGV genome the files were formatted to fasta and an alignment for each gene was completed. Furthermore, the extracted 16S rRNA sequences were ran against the SILVA database (https://www.arb-silva.de/) to determine taxonomic similarity (Quast *et al.*, 2013; Silva). Blast was performed locally and then upgraded to January 18, 2017.

The code used is as follows: nice –n 40 update_blastdb.pl –passive –force –verbose –decompress nr nt taxdb >> nr_nt.updatedb.log

Once the nucleotide and protein local database of blast were upgraded, blastn was used to extract the closest sequences to each one of the unidentified sequences and produced a phylogenetic analysis for the 16SrRNA and *rpoB* genes. Files were then changed from taxa ID to taxonomical identification using NCBI database. The files were again changed to fasta format and Seaview version 4 was used to align the sequences with the use of Clustal-omega and Muscle algorithms, with default settings (Edgar, 2004; Gouy *et al.*, 2010; Sievers *et al.*, 2011). For *rpoB* evaluation

of each αGV, sequences were translated to amino acid sequence view and aligned as proteins, and then converted into nucleotide view in Seaview version 4 (Gouy *et al.*, 2010). Phylogenetic trees were produced by phyml software with αGV identifiers as the taxa description in NCBI database (Guindon *et al.*, 2010).

2.2.6.4 Pangenome analysis of a subset of αGVs

The genome pipeline Roary v. 3.10.2 and Prokka were used to produce GFF3 files, which further used to generate the pangenome matrix of the *Lactococcus* genera (Seemann, 2014; Page *et al.*, 2015). Alignment of core genes was used to produce phylogenetic tree and the matrix showing the absence or presence of core and accessory genes.

The pipeline code used is as follows: Roary –p 40 –f Lactococcus_pangenome –e –n –i 70 –r –s –z –v

2.2.6.5 Identification of bacteriocin genomic cassettes

Genomes were analysed in the BAGEL3 program (http://bagel.molgenrug.nl/) to identify the potential bacteriocin cassettes (van Heel *et al.*, 2013).

2.2.7 Target Range of αGV bacterial isolates

2.2.7.1 High throughput target range assay

Preparation of αGVs for high throughput target range assay was completed by inoculating 20mL of BHI with each of αGV isolates and α523. Cultures were incubated anaerobically at 37°C until they became turbid. Five hundred microliters sample was taken from each culture to determine the turbidity via A_{405} readings in a TECAN computerized plate reader at the same time to verify

the cultures microscopically for expected cellular morphology and lack of contamination. The turbidity of cultures was determined as follows: in a 96 well plate, 100µL of each culture, as well as a blank control (BHI) were pipetted in triplicate. The OD was read using a 405nm filter and the average and standard deviation values were calculated thereafter in excel. Remaining cultures underwent centrifugation at 3200xg for 20 minutes at 4°C. Supernatants were poured off and pelleted cell were resuspended in a volume of sterile 10% glycerol in order to attain $5x10^6$ cfu/mL. To estimate the volume of the glycerol to be used, a calibration curve originally developed for α523 by the former technician, Laura Douglas, was used. A volume of 100µL of each standardized culture, along with BHI as a negative control, was pipetted into a 96 well plate based on the layout (Appendix E). Nine plates in total were prepared with the same cultures. The 96 well plates containing αGV cells were sealed with sterile adhesive foils and stored in -80°C until use.

For the high throughput target range assay experiment, the prepared plate #1 of the nine 96 well plates containing αGV cells were removed from -80°C to thaw and then placed in ice. Depending on the indicator strain a volume of 28-500µL (approximately 10^7 cells) was added to 5mL of 0.7% top agar (BHI) and seeded on OmniTray plates containing reduced BHI agar (ie oxygen free). Once agar overlay solidified a 96-pin standard replicator was placed into the 96 well plate containing the αGV cells. The 96-pin standard replicator was then used to inoculate the OmniTray bacterial lawn with approximately 2µL of each αGV. Inoculated OmniTrays were then incubated under anaerobic conditions at 37°C for 2-4 days. Control OmniTrays of each media type, with no bacterial lawn were also inoculated with the αGV cells. The 96 well plate containing αGV cells were again sealed with adhesive foils and stored in -80°C.

2.2.8 Target range analysis of αGV active agents

2.2.8.1 Supernatant extraction

Chloroform extraction of αGV isolates culture supernatants was performed using 25mL of supernatant and 17mL of chloroform (v/v ration of 1:0.7). The mixture was shaken for 45 minutes at 300 rpm on a rotary shaker at 37°C. Centrifugation at 3200xg for 30 minutes at 20°C was performed to separate the organic and aqueous phases. The aqueous layer was pipetted off while the organic layer was removed by pouring it off without disturbing the accumulated whitish interphase matter that stayed stuck to the sides of the tube. The separated interphase matter was collected and transferred to a 1.5mL epitube and then centrifuged at 14000xg for 10 minutes. Remaining aqueous and organic layers were removed via pipetting and the pellet was dried in vacuum drier chamber. The collected pellet was resuspended in 250µL of BHI broth and stored in a -20°C freezer.

The protein content of the αGV isolates culture supernatants were precipitated with methanol by adding 21mL 100% pure methanol to 8.5mL supernatant (i.e v/v ratio of 1 supernatant: 2.5 methanol). The solution was mixed by inversion and incubated at room temperature for 30-40 minutes. Centrifugation at 12000 rpm for 20 minutes at 4°C was used to precipitate the protein content of the supernatants. The pellet was recovered by discarding the supernatant and the tube was inverted onto an absorbent surface to drain residuals of the supernatant. The pellet was dried under vacuum in a vacuum condenser chamber and resuspended in 100µL of BHI broth. The wall of the tube was rinsed with additional 50µL of BHI broth to make sure of resuspending any residual precipitate. Finally, the whole volume was collected at the bottom of the tube by second

centrifugation at 12000 rpm for 5 minutes at 4°C. The total volume 150µL of extracted supernatant was transferred to a 1.5mL epitube and stored in -20°C until use.

2.2.9 Drop on the lawn testing

One milliliter of turbid *G. vaginalis* culture was added to 7mL of 0.7% BHI top agar that was supplemented with 1% soluble starch. The bacterial suspension was inverted to mix and was evenly overlaid onto a BHI agar plate supplemented with 10% FBS and 1% soluble starch to form a bacterial lawn. Once top agar was solidified, the concentrated αGV supernatants (100x chloroform extract, 57x methanol extract) were pipetted onto the lawn (Appendix E). For the chloroform extracted supernatants 5µL drops were pipetted onto the lawns whereas 8µL of the methanol precipitated supernatants were applied to the lawns.

2.2.10 αGV active agent protein analysis

2.2.10.1 Silver staining and gel diffusion

A volume equivalent to 10 µg, determined by Bradford protein assay, of each αGV methanol or chloroform concentrated supernatants was added to an equal volume of 2x SDS-PAGE loading buffer. Samples were heated in a water bath at 70°C for 5 minutes. Protein samples were then loaded into wells (5µg/well) of a 1.5mm thick, 12% or 15% SDS-PAGE gel; first in lanes 2-5 and again in the same order in lanes 7-10. A high range BLUeye prestrained protein ladder (10 µL) and Spectra Multicolour low range protein ladder (10 µL) were also loaded into lanes 1 and 6, respectively. Electrophoresis of SDS gel at 100V for 1 hour and 45 minutes was completed. The gel was cut in half, through the low range ladder lane (lane 6). Silver staining of gel half

containing both protein ladder lanes was completed with Silver Staining Plus kit according to manufacturer's protocol.

The second half of the gel was washed three times in 200mL of sterile ddH_2O for 1.5 hours with intermittent change of water. The washed gel half was transferred to a BHI agar plate (7mL total) supplemented with 10% FBS and 1% starch. Agar overlay technique was used to cover the gel with 10-15 mL of 0.7% BHI top agar with 1% soluble starch, mixed with 500µL of the *G. vaginalis* indicator strain. Incubation of plate was completed under 10% CO_2 at 37°C for several days until a turbid lawn formed and cleared areas were visible.

3 Results

3.1 Identification and characterization of *G. vaginalis* collection

3.1.1 Morphological and biochemical properties

The *G. vaginalis* clinical isolates exhibited expected morphological and biochemical properties. All *G. vaginalis* isolates were observed to have a coryneform cell morphology by microscopy (Figure 3.1). They were found also to be catalase negative and produced a representative clear β-hemolysis clearing zone on the agar plates containing human blood (Figure 3.2).

3.1.2 PCR identification and genotyping

The correct identity of *G. vaginalis* clinical isolates was further and strongly supported through PCR experiments with the use of *G. vaginalis*-specific primers for 16S rDNA operons. PCR amplification of a portion of the16S rRNA gene of *G. vaginalis* produced a product of expected size (1434 bp Figure 3.3; Figure 3.4). Digestion of *G. vaginalis* 16S rRNA gene PCR products with *Taq*I restriction enzyme produced two distinct banding patterns representing genotypes A and B (Figure 3.5). The majority of *G. vaginalis* isolates were typed as B (82.4%) while 17.6% were determined to be of genotype A. Another restriction enzyme (*Hpa*II) was used to characterize the *G. vaginalis* collection. Three genotypes were observed, indicated as genotypes 1, 3 and 4 (Figure 3.6). Genotype 1 was the most prevalent (82.4%), followed by genotype 4 (11.8%). Only 1 *G. vaginalis* isolate, cGV7, was determined to be genotype 3 (5.8% of the collection).

3.1.3 Antibiotic susceptibility testing of *G. vaginalis* collection

Antibiotic susceptibility profiles were determined for the *G. vaginalis* collection and the reference strain *E. faecalis* ATCC 29212 using E-test strips for the following antibiotics: CM, LZ, VA, RI, MZ, CI, AM and TC (see section 2.1.2 and abbreviation list for the full names of the antibiotics). Metronidazole and clindamycin are clinically relevant as they are the primary and secondary treatments for BV, respectively.

The application of E-strips onto a lawn of *G. vaginalis* or *E. faecalis* ATCC 29212 was as described in Section 2.2.4 and the minimal inhibitory concentrations were defined by the presence of an ellipse of an inhibition zone surrounding each E-strip (Figure 3.7). E-test procedural guidelines were utilized to read MIC values, where the MIC is read at the point of intersection of the E-strip with the bacterial lawn at the edge of the zone of inhibition (bioMérieux, 2011, 2012a, 2012c). MIC values for bactericidal antibiotics were read at 100% bacterial growth inhibition edge, while bacteriostatic antibiotics were read at 80% inhibition edge except for LZ, which was read at 90% of inhibition edge (bioMérieux, 2012b).

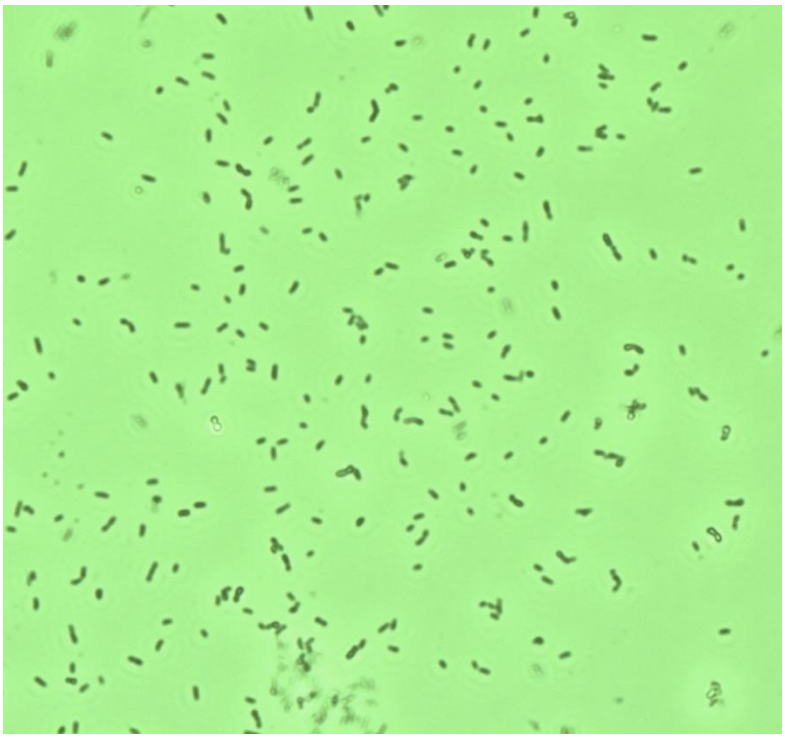

Figure 3.1 An example of coryneform cellular morphology of clinical G. vaginalis isolates observed at 400x magnification and photographed using phase contrast setting of the Axio Scope.A1 microscope. Here, the image of the cGV4 isolate is shown.

Figure 3.2 An example of the β-hemolysis pattern of *G. vaginalis* isolates on human whole blood agar plates. The hemolysis pattern of cGV1 is shown here.

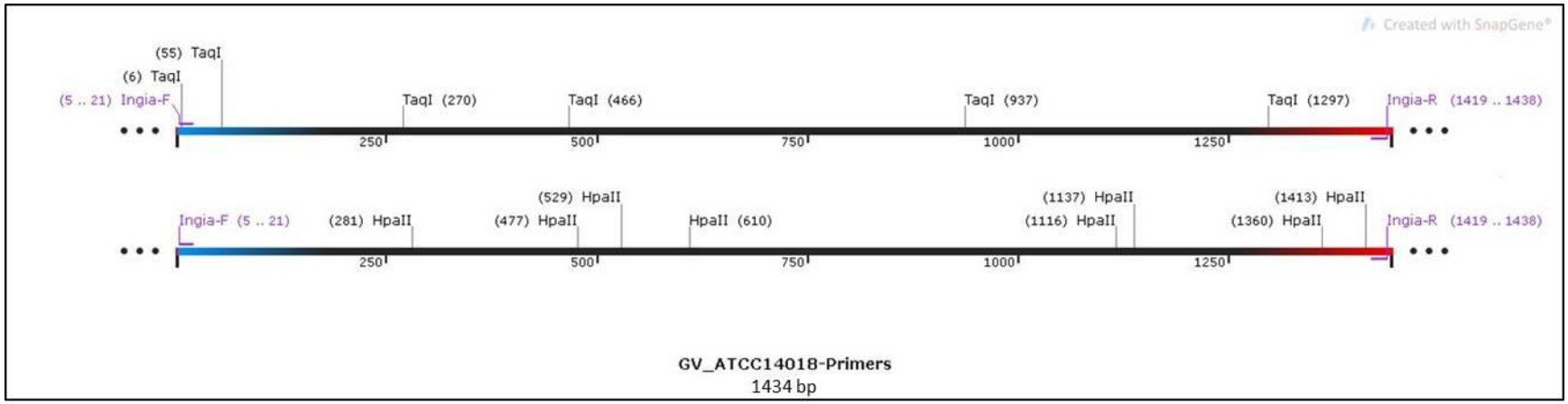

Figure 3.3 Restriction map of 16S rRNA gene locus of *G. vaginalis* ATCC 14018. *Taq*1 and *Hpa*II restriction sites are shown. The position of the primers Ingia-F and Ingia-R used in PCR amplification of 16S rDNA target are also shown. These maps are produced to provide a visual perception for the expected bands in genotyping experiments.

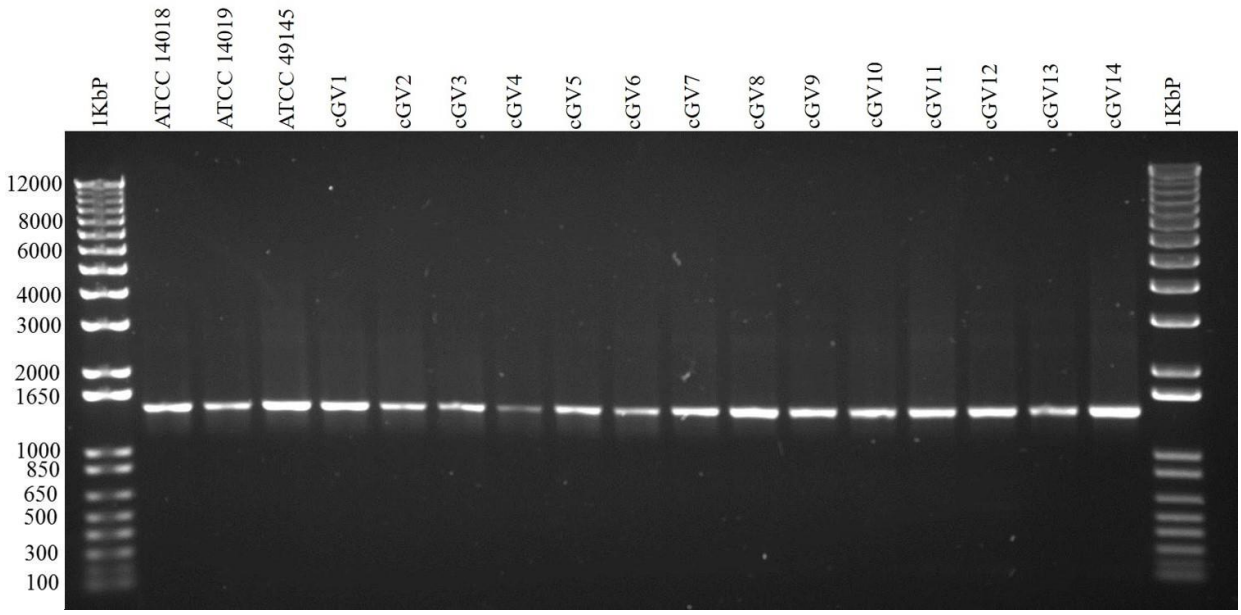

Figure 3.4 Agarose gel showing the PCR product of amplified 16S rRNA gene using GV specific primers. The expected amplicon size is 1434 bp for all isolates. *G. vaginalis* ATCC strains are included as controls. 1KbP: 1 Kb plus DNA ladder.

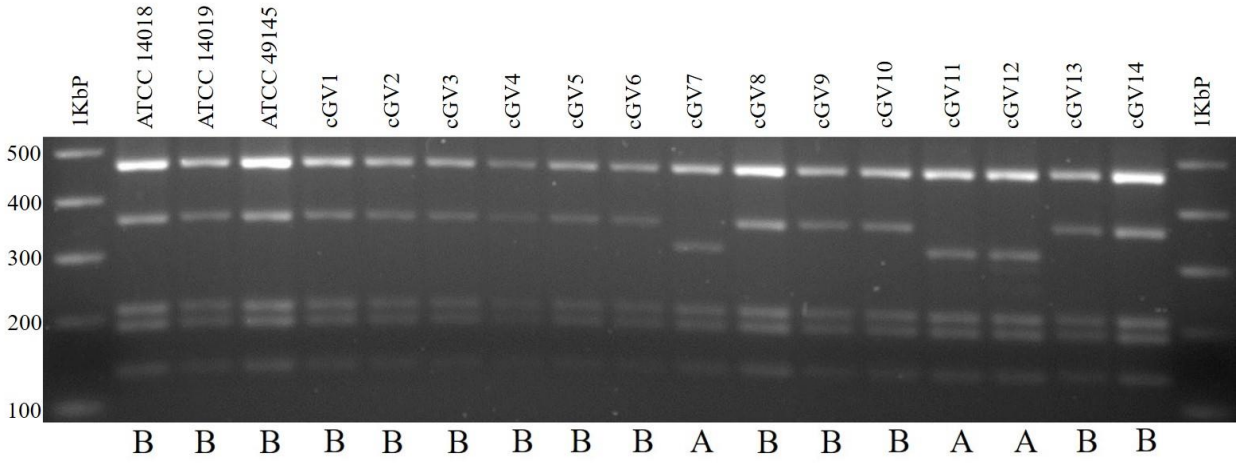

Figure 3.5 Electrophoretic restriction banding patterns of the 16S rRNA gene PCR products of *G. vaginalis* isolates digested with *Taq*I. *G. vaginalis* ATCC strains are included as controls. Two genotypes were noted: (A) Type A genotype. (B) Type B genotype. 1KbP: 1 Kb plus DNA ladder.

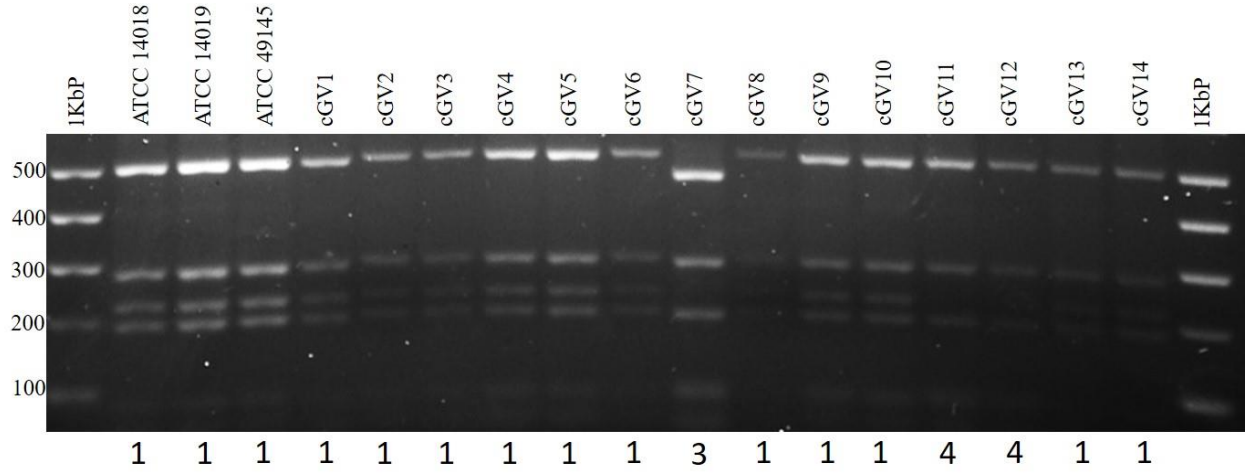

Figure 3.6 Electrophoretic restriction banding patterns of the 16S rRNA gene PCR products of *G. vaginalis* isolates digested with *Hpa*II. *G. vaginalis* ATCC strains are included as controls. Three genotypes were noted: 1, 3 and 4 represent genotypes 1, 3 and 4, respectively. 1KbP: 1 Kb plus DNA ladder.

E. faecalis ATCC 29212 MIC values within the appropriate ranges defined by EUCAST and CLSI were used as internal quality controls in this study (bioMérieux, 2012b, 2014; Clinical and Laboratory Standards Institute, 2015; EUCAST, 2016). The MIC values for the quality control strain were determined to be within the acceptable ranges for the antibiotics used in this study, supporting the reliability of the applied procedures (Appendix D). Standard antibiotic susceptibility breakpoints for *G. vaginalis* have not been described by EUCAST or CLSI; therefore, our antibiotic susceptibility profiles are based on published breakpoints in the literature specifically for E-testing (De Backer *et al.*, 2006; Nagaraja, 2008; Schuyler *et al.*, 2016).

Metronidazole and Clindamycin

For metronidazole activity against *G. vaginalis*, sensitivity has been defined as sensitive (<8µg/mL), intermediate as 8-16µg/mL, or resistant at either >16µg/mL or ≥32µg/mL, depending on the literature source (De Backer *et al.*, 2006; Nagaraja, 2008; Schuyler *et al.*, 2016). Based on these proposed breakpoint values, none of the isolates in our *G. vaginalis* collection were sensitive to metronidazole; 35.3% of isolates were intermediately susceptible and 64.7% of the isolates were resistant (Figure 3.8). Additionally, the MIC50 and MIC90 values (median and 90th percentile MIC values, respectively) were determined to be 32µg/mL and 256µg/mL, respectively. These values suggested a high propensity for the *G. vaginalis* population to develop resistance to metronidazole (see the sections below). We also noted that there was a relatively broad distribution range for metronidazole MIC values even in this small collection. Compared to the lowest MIC value it was noted that the MIC values were different

from each other within the range of 1 to 16-fold (Table 3.1). This observation is consistent with the genotypic diversity noted above in this collection.

All *G. vaginalis* isolates were determined to be susceptible to the clinically relevant drug clindamycin, since the MIC values were all well below 2µg/mL (Table 3.1) with a MIC50 and MIC90 of 0.016µg/mL and 0.032µg/mL, respectfully (Nagaraja, 2008). Additionally, the susceptibility profiles of the *G. vaginalis* isolates showed a 1 to 4-fold difference.

Non-clinically relevant antibiotics

Similar to metronidazole and clindamycin the *G. vaginalis* isolates showed diversity with respect to the MIC values for antibiotics not typically used against GV clinically (Table 3.1). The MIC values for VA ranged from 0.25µg/mL to 0.5µg/mL, having only a1 to 2-fold difference. While the MIC values for LZ, RI and CI varied within the range of 1-4-fold. The antibiotics with the broadest MIC ranges were AM (1 to 30-fold) and TC (1 to 256-fold), respectively. The MIC50 and MIC90, respectively, were as follows: LZ (0.25 and 0.5µg/mL), VA (0.5 µg/mL for both), RI (1 and 2 µg/mL), CI (2 and 4µg/mL), AM (0.064 and 0.35 µg/mL) and TC (0.5 and 26.2µg/mL).

3.1.4 Development of metronidazole resistance by *G. vaginalis*

Following exposure of *G. vaginalis* isolates to metronidazole E-strips, within the ellipsoidal

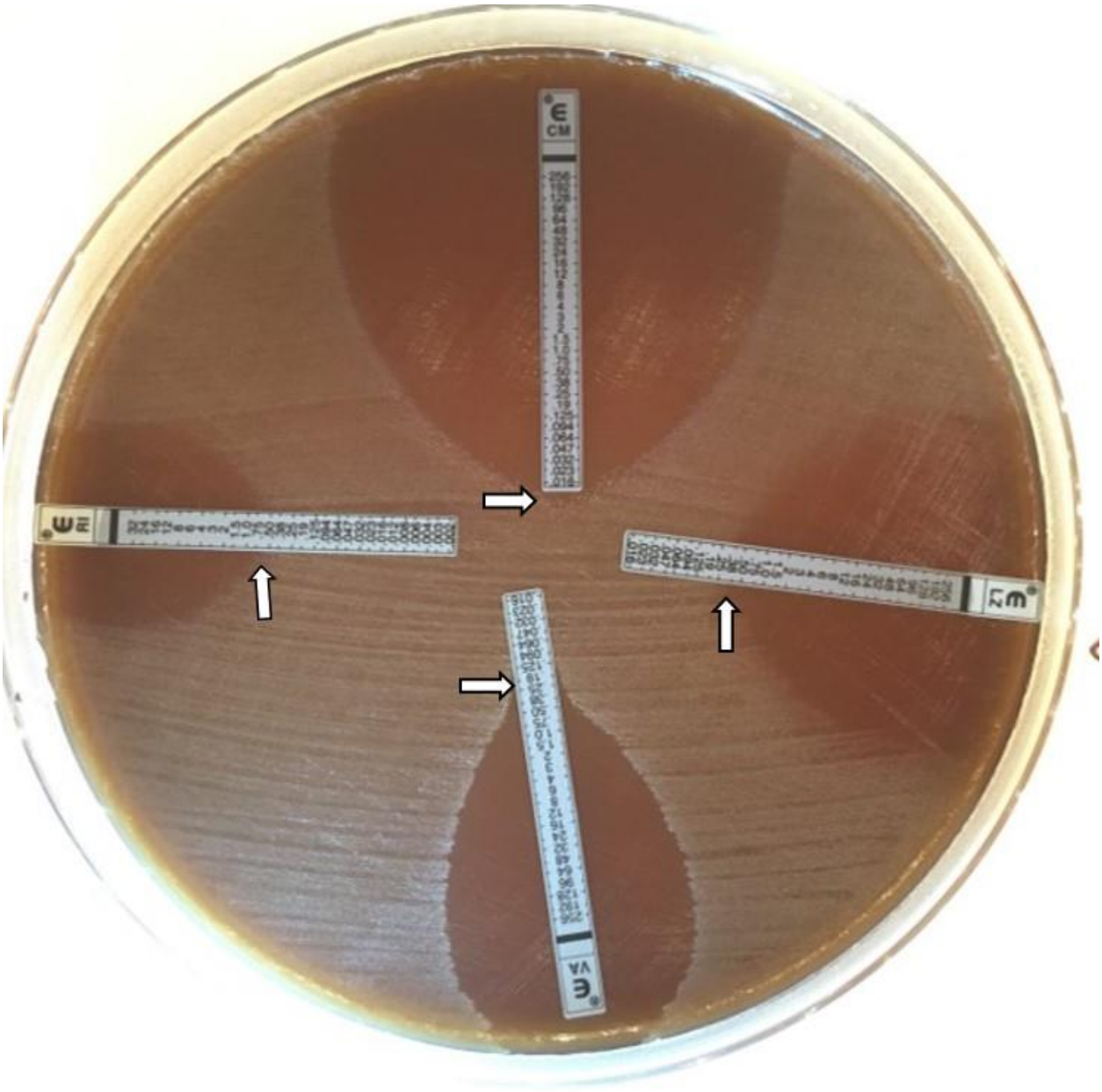

Figure 3.7 Antibiotic susceptibility results of antibiotics CM, LZ, VA and RI on a lawn of cGV11. E-tests were performed as described in Section 2.2.4. MIC (µg/mL) values are indicated by white arrows.

Table 3.1 Antibiotic susceptibility profiles and MIC fold ranges for *G. vaginalis* collection.

Bacteria Tested	Etest: MIC values (ug/mL)							
	CM	**LZ**	**VA**	**RI**	**MZ**	**CI**	**AM**	**TC**
G. vaginalis ATCC 14018	<0.016	0.25	0.25	2	>256	2	<0.016	0.50
G. vaginalis ATCC 14019	<0.016	0.25	0.50	0.50	>256	4	0.016	0.50
G. vaginalis ATCC 49145	<0.016	0.25	0.50	2	16	2	<0.016	64
cGV1	0.016	0.25	0.50	1	64	2	<0.016	1
cGV2	0.032	0.25	0.50	1	8	2	0.125	0.50
cGV3	<0.016	0.125	0.50	1	32	2	0.064	0.50
cGV4	<0.016	0.50	0.25	1	64	2	0.50	0.50
cGV5	<0.016	0.25	0.50	1	128	1	0.032	1
cGV6	0.064	0.25	0.50	1	32	2	<0.016	1
cGV7	<0.016	0.50	0.25	1	32	2	0.064	0.50
cGV8	<0.016	0.25	0.50	2	32	1	0.016	0.50
cGV9	<0.016	0.25	0.50	1	16	2	0.032	0.50
cGV10	0.032	0.25	0.50	2	16	4	0.064	128
cGV11	<0.016	0.25	0.25	1	>256	1	2	0.50
cGV12	0.032	0.50	0.50	1	>256	4	0.25	0.50
cGV13	<0.016	0.125	0.50	2	16	2	0.125	0.50
cGV14	<0.016	0.125	0.50	1	16	2	0.125	0.50
Fold Range	**1-4 fold**	**1-4 fold**	**1-2 fold**	**1-4 fold**	**1-6 fold**	**1-4 fold**	**1-30 fold**	**1-256 fold**

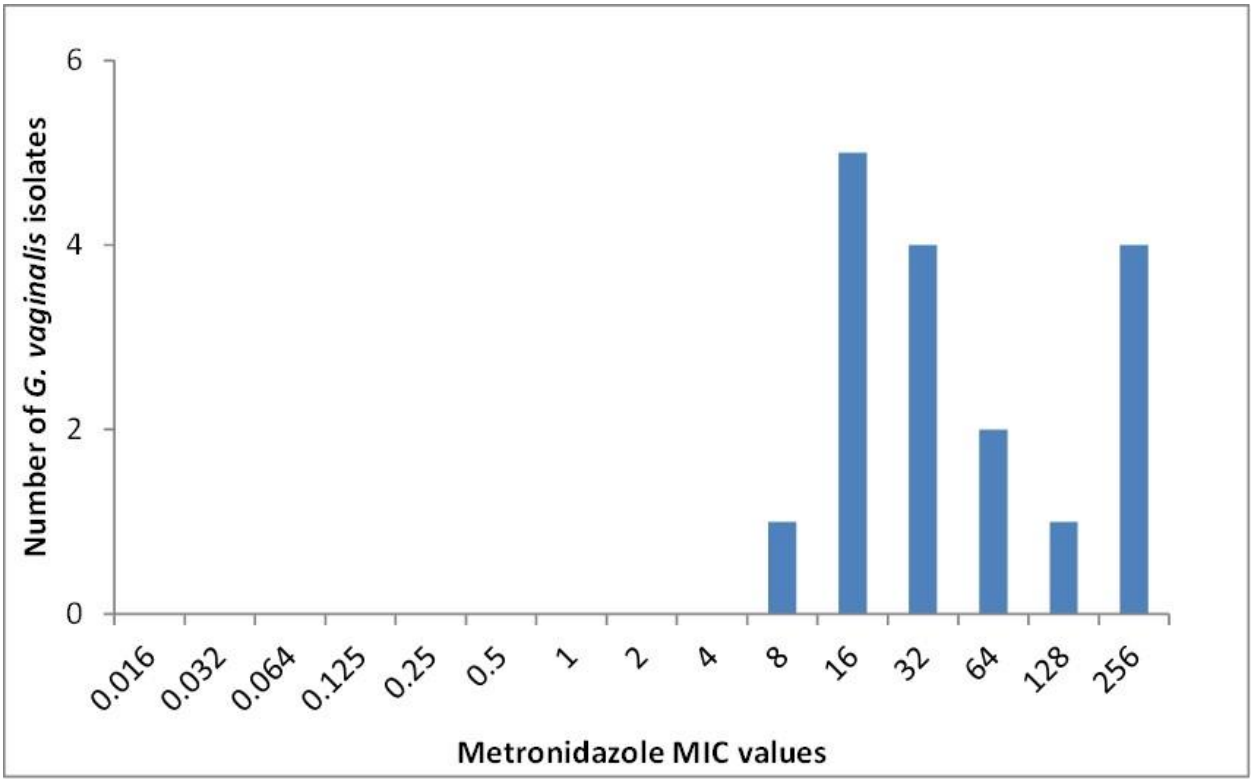

Figure 3.8 Distribution and of Metronidazole MIC values (µg/mL) among *G. vaginalis* collection.

inhibition zones numerous growing colonies were observed (Figure 3.9). To determine whether these colonies were metronidazole-resistant, 10 were picked from either side of the E-strips on plates of the cGV2 isolate (section 2.2.4). The genomic DNA was isolated from these 20 isolates along with the parental strain cGV2 and *G. vaginalis* ATCC 45149. The genomic DNA was used to amplify the 16S rRNA gene using *G. vaginalis* specific primers. The PCR products were further analyzed for their genotypes with restriction enzyme *Taq*I. All isolates were observed to have the same genotype as the parental strain (Figure 3.10). The MZ MIC values of the mutant cGV2 isolates were determined together with their sensitivities to the killing effect by the αGV bacterial isolates. These results indicated that the 20 cGV2 mutant isolates were fully resistant to metronidazole with MIC values >256µg/mL (Figure 3.11). In addition, the isolates' sensitivity profiles to the αGV bacterial collection did not differ from the parental cGV2 isolate.

Similar to metronidazole strips, resistant isolates within the inhibition zones were noted for other antibiotics such as rifampicin and ciprofloxacin. Rifampicin resistant isolates were observed among 23.5% of the *G. vaginalis* collection, 53% of the collection when exposed to metronidazole and 11.8% when exposed to ciprofloxacin E-strips (Table 3.2).

3.2 Isolation and characterization of αGV bacterial collection

3.2.1 Isolation of αGV collection

An αGV collection was isolated by using an agar overlay method containing ROPEC sewage samples and *G. vaginalis* ATCC 14018 and/or 14019 as indicator strains (as described in section 2.2.5).

Figure 3.9 Metronidazole resistant colonies within ellipse of cGV2 inhibition zone.

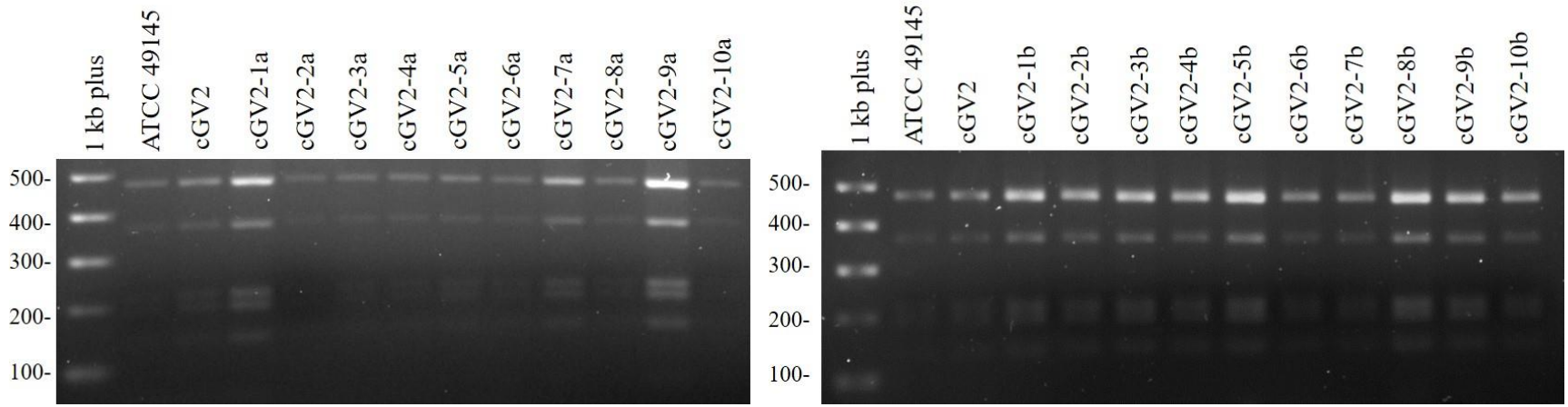

Figure 3.10 *Taq*I digestion pattern of parental strain cGV2 and cGV2 metronidazole resistant derivatives. G. vaginalis ATCC 49145 has been included as external control. All cGV2 metronidazole resistant derivative isolates showed *Taq*I genotypes similar to the parental cGV2 isolate.

Figure 3.11 An example of E-test results of metronidazole on cGV2 mutant isolates. The cGV2-10b did not produce inhibition zone even at around the highest concentration of antibiotic on the E-strip.

Table 3.2 Antibiotics that resistant *G. vaginalis* colonies were observed within their inhibition zones. "+" indicates the presence of resistant colonies; RI: rifampicin, MZ: metronidazole and CI: ciprofloxacin.

	Etesting Antibiotics		
***G. vaginalis* Isolates**	**RI**	**MZ**	**CI**
GV ATCC 14018	-	-	-
GV ATCC 14019	+	-	-
GV ATCC 49145	+	+	-
cGV1	+	-	-
cGV2	-	+	-
cGV3	-	-	-
cGV4	-	+	-
cGV5	-	-	+
cGV6	-	+	-
cGV7	-	+	-
cGV8	-	+	+
cGV9	-	+	-
cGV10	-	+	-
cGV11	-	-	-
cGV12	-	-	-
cGV13	-	-	-
cGV14	+	+	-

Colonies that produced inhibition zones on a lawn of *G.* vaginalis, as observed in Figure 3.12, were isolated and purified. A total of 33 bacterial isolates were recovered, 19 against *G. vaginalis* ATCC 14018 while 14 were isolated against *G. vaginalis* ATCC 14019. The αGV isolate identifiers are listed in Table 3.3. An additional bacterial isolate previously isolated in Dr. Nokhbeh's lab (α523) was determined to be affective against *G. vaginalis* and therefore was added to the αGV collection (Ybazeta *et al.*, 2017) (Figure 3.13).

3.2.2 Characterization of αGV collection

3.2.2.1 Morphological and biochemical testing

Visual inspection of αGV collection members by phase contract microscopy revealed differing bacterial morphological properties. Except for two rod-shaped isolates (αGV18-14S and αGV18-4L), the rest of the isolates were arranged as streptococci in short or long chains with varying cell sizes (Figure 3.14; Table 3.3). A catalase test of αGV collection members indicated that all αGV isolates were catalase negative. Gram staining of a subset of the αGV collection and α523 was completed. Of the subset, αGV18-1S, αGV19-4S, αGV18-8, αGV18-7aW, αGV18-11, αGV18-14S were Gram-positive, while αGV18-4L was Gram-negative/variable.

3.2.2.2 Genomics

The genomes of αGV collection members were sequenced using an illumina HiSeq platform and assembled into 16-520 contigs as described in section 2.2.6.2. None of the genomes could be closed. The genomes ranged in size from 2.1 Mbp to 3.1 Mbp with GC contents of 34.7% to

Figure 3.12 Inhibition of *G. vaginalis* ATCC 14019 by an unknown bacterium (αGV) isolated from raw sewage.

Figure 3.13 Inhibition of the lawn of *G. vaginalis* ATCC 14019 by α523 bacterial colonies. α523 was originally isolated from sewage and co-cultured on the G. vaginalis ATCC 14019 lawn.

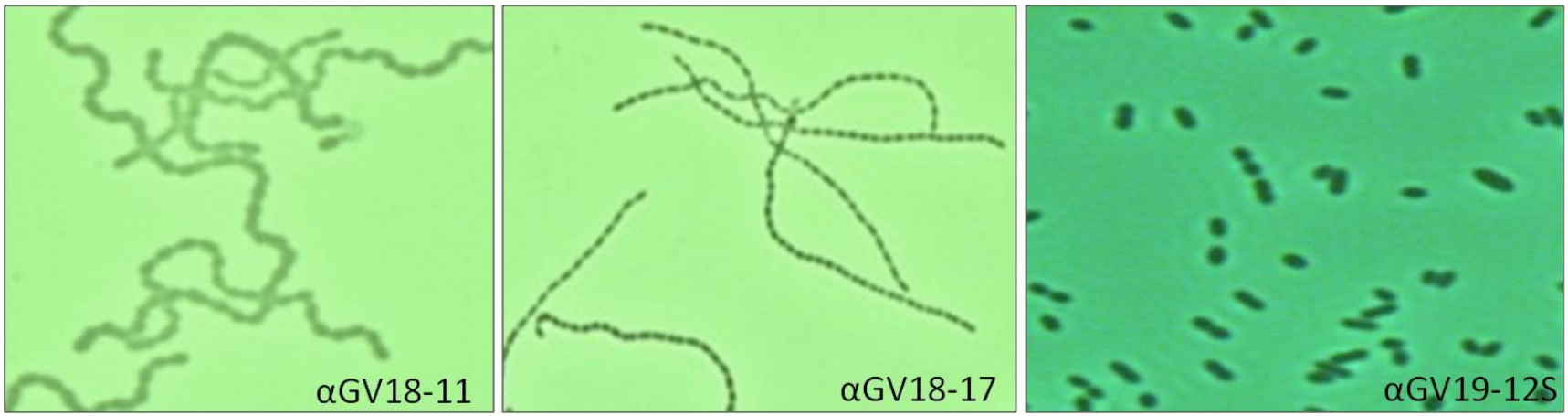

Figure 3.14 Phase contrast microscopic images of αGV18-11, αGV18-17 and αGV19-12S isolates. Differences in morphological properties (short and long chain streptococci) among anti-GV collection indicate the diversity among this collection. Photographs were taken using 400x magnification and zoomed digitally to show the detailed morphological properties of each isolate

Table 3.3 List of anti-GV isolates and their properties. Strep L: long chain streptococcus; Strep S: short chain streptococcus; rRNA: number of rRNA operons; tmRNA: number of transfer-messengerRNA, CDS: number of coding segments in genome; nv: not verified

Indicator strain	αGV	Morphology	Catalase	Genome Seq.(bp)	GC%	Genome coding%	CDS	no. rRNA operons	no. tRNA	no. tmRNA	contigs
G. vaginalis ATCC 14018	αGV18-11	Strep L	-	2130140	38.67	85.87	2044	5	45	1	56
	αGV18-12S	Strep L	-	2186826	38.92	84.15	2154	6	46	1	141
	αGV18-13	Strep S	-	2331233	38.7	85.92	2238	5	45	1	77
	αGV18-14S	Rod	-	2560328	35	86.36	2499	9	59	1	51
	αGV18-15	Strep L	-	2264417	38.54	85.56	2206	6	47	1	77
	αGV18-16	Strep S	-	2533569	34.99	86.77	2466	8	54	1	33
	αGV18-17	Strep L	-	2303060	34.85	86.82	2224	7	53	1	16
	αGV18-18	Strep S	-	2297125	38.73	86.54	2221	4	47	1	56
	αGV18-19W	Strep S	-	2325981	38.56	83.48	2269	6	44	1	175
	αGV18-19YW	Strep S	-	2849104	35.36	82.44	2742	7	53	1	240
	αGV18-1S	Strep S	-	2323323	39.77	86.81	2265	5	47	1	72
	αGV18-20	Strep L	-	2283381	38.45	84.02	2199	6	44	1	116
	αGV18-2S	Strep S	-	2181522	38.55	85.29	2101	6	45	1	87
	αGV18-3	Strep S	-	2307178	39.74	86.07	2230	7	46	1	119
	αGV18-5	Strep S	-	2210287	38.46	83.86	2112	6	44	1	138
	αGV18-6L	Strep S	-	2350635	38.67	85.72	2256	5	45	1	79
	αGV18-7aW	Strep S	-	2186171	39.21	84.81	2133	5	40	1	108
	αGV18-7b	Strep S	-	nv	47.47	82.2	nv	11	nv	2	nv
	αGV18-8	Strep S	-	2413314	39.33	84.19	2283	5	45	1	194
	αGV18-9	Strep S	-	3100738	35.62	80.22	2990	9	57	1	400
G. vaginalis ATCC 14019	αGV19-1	Strep S	-	2354650	38.8	85.18	2274	4	44	1	108
	αGV19-10L	Strep S	-	2566365	34.7	86.27	2436	8	54	1	37
	αGV19-11	Strep S	-	2621397	36.72	86.02	2420	8	57	1	20
	αGV19-12S	Strep S	-	2459756	39.32	85.77	2350	5	37	1	112
	αGV19-13	Strep S	-	2327210	39.79	87.31	2223	6	45	1	56
	αGV19-14	Strep S	-	2790778	38.18	85.95	2671	8	63	1	40
	αGV19-2S	Strep S	-	2291593	38.54	85.3	2203	5	43	1	63
	αGV19-3	Strep S	-	2276450	38.45	84.85	2188	6	47	1	105
	αGV19-4S	Strep S	-	2134436	38.65	85.43	2038	5	43	1	63
	αGV19-5	Strep S	-	2301248	38.79	85.94	2206	5	45	1	75
	αGV19-6	Strep S	-	2261830	38.73	83.75	2195	8	51	1	128
	αGV19-7	Strep S	-	2672568	37.71	77.94	2608	7	56	1	520
	αGV19-9	Strep S	-	2317193	38.65	85.92	2252	5	46	1	67
CD523	α523	Strep S	-	2779809	38.16	85.48	2680	8	62	1	81

39.8%. The nucleotide sequences with potential protein coding capacities accounted for 78-87% of the genomes corresponding to 2038-2990 CDS. They also contained 4-9 rRNA operons (*rrn*), a single tmRNA each, and 37 to 63 tRNAs (Table 3.3). A phylogenetic tree based on 16S rRNA gene sequences of the αGV collection and NCBI reference strains was constructed as detailed in section 2.2.6.3. The two rod shaped isolates were grouped with endospore forming Gram-variable *Paenibacillus spp*., however, they also erroneously clustered with lactococci probably due to partial sequence similarities and relatively low homology cutoff threshold or contamination of the DNA sources. The remainder of isolates were grouped in three main clusters with similarities to either of genera *Enterococcus*, *Streptococcus* and *Lactococcus* (Figure 3.15). However, the majority of isolates were in lactococcus group with the closest resemblance to *L. raffinolactis* followed by *L. lactis*. Only two isolates (αGV18-2S and αGV18-16) fell within Enterococci with the closest match with *E. faecium*. Members of these genera are lactose fermenters (commonly referred to as lactic acid bacteria, LABs) and are known for production of short antimicrobial peptides. Two isolates (αGV18-19 and αGV18-3) were clustered closely with *Streptococcus suis* or *Streptococcus lutetiensis* that are considered newly emerging human pathogens. We excluded six isolates from further analysis due to their potential pathogenic status in addition to their weak αGV activities. These isolates that were excluded from analysis included two isolates in an *Enterococci* cluster, two rod shaped isolates in a *Paenibacillus* cluster and two isolates in a *Streptococcus suis* cluster.

Bacterial isolates of the genus *Lactococcus* accounted for the majority of collection members (28 of 34 isolates) with antiGV activities. They mostly contained potential cassettes for coding bacteriocins and were thus further analyzed in a pangenome comparison matrix detailed in

section 2.2.6.4 (Figure 3.16). Following pangenomic analysis, the isolates were grouped into 3 clusters with clusters colored in blue and green having similarities to *Lactococcus raffinolactis sp.*, while the red cluster was represented best by *Lactococcus lactis sp*. All clusters share 312 core proteins (labeled in orange vertical lines). In addition to core proteins, a large number of proteins (2037-2990 proteins per αGV isolate) were considered in homology comparisons with an exclusion cutoff filter of 70% amino acid sequence similarity. The presence of a protein in any isolate was denoted by a vertical red line resulting in clustered patterns of isolates.

Additionally, bacteriocin cassettes were identified for 27 out of 28 lactococcal αGV isolates in addition to the previously identified α523 isolate (Table 3.4). Seven cassettes were found, two of which were class I, ie. Lanthipeptide class I and Lanthipeptide class I orf013. The following 5 cassettes were determined to be class II: orf10 HTCP (Head to Tail Cyclized Peptides), orf003 HTCP, small ORF1 HTCP, Lactococcin putative class II and orf007 class II. It was also discovered that bacteriocin cassettes orf003 HTCP and small ORF1 HTCP were always found in tandem. The frequency of the presence of each cassette among the αGV isolates' genome was 64% for orf10 HTCP; 25% for orf003 HTCP and small orf1 HTCP; 21% for Lactococcin putative class II, orf007 class II and Lanthipeptide class I; 17.9% for Lanthipeptide class I orf013. The orf10 HTCP cassette was identified in 88% of blue cluster αGV, 40% of green cluster isolates and 16.7% of red cluster isolates. The tandem cassettes of orf003 HTCP and small orf1 HTCP were identified in 41% of blue cluster isolates but not within the αGV isolates of the other clusters. The Lactococcin putative class II was only identified in the blue (5.9%) and green (100%) clusters. While the remaining three cassettes, Lanthipeptide class I, Lanthipeptide class I orf013 and orf007 class II were only identified in the αGV isolates that are a part of the

red cluster. The prevalence of these cassettes within the red cluster ranged from 83-100% of αGV isolates. The molecular weights of the identified cassettes ranged from 2.32kD to 9.84kD (Table 3.4). Additionally, different molecular weights of cassettes (Lactococcin putative class II, Lanthipeptide class I, Lanthipeptide class I orf013 and orf007 classII) were observed among αGV isolates. However, αGV18-15 was not shown to carry any of the above-mentioned cassettes. This might be due to the incompleteness of the genome sequencing data or the lack of reference in the databases.

3.3 Antibacterial activities of αGV isolates against *G. vaginalis* collection

3.3.1 Target range of αGV bacteria against *G. vaginalis* collection

A high throughput target range assay of αGV isolates (n=34) against the entire *G. vaginalis* collection (n=17) as well as an assortment of non-G. vaginalis bacteria (n=18) was completed (Figure 3.17) as described in section 2.2.7.1. Positive inhibition by αGV isolates against a *G. vaginalis* indicator strain was determined by the presence of a fully cleared inhibition zone around the αGV isolate in question. An example of the high throughput inhibition testing of αGV collection against *G. vaginalis* ATCC 4145 is shown in Figure 3.17. Differing inhibition zone diameters were observed for different αGV isolates against a given GV isolate, as well as differences between different GV isolates for a given αGV isolate. For the effect of αGV isolates against non-*G. vaginalis* bacteria, a positive result was considered if there was either full or hazy clearance zones of inhibition. Based on the zones of inhibition the target range of each αGV isolate against *G. vaginalis* and non-*G. vaginalis* collections were determined (Figure 3.18). The target range observed for the *G. vaginalis* collection was from 0% to 100%, with the majority of

αGV isolates (n=18) having the capability of killing at least 50% of the *G. vaginalis* isolates. While the αGV target range against the non-*G. vaginalis* collection ranged from 0% to 60%. Only six αGV isolates (17.6%) could inhibit the growth of greater than 40% of the non-*G. vaginalis* isolates.

Sensitivity of the *G. vaginalis* collection to each of the αGV isolates was also determined based on the high throughput target range assay (Figure 3.19). Among the *G. vaginalis* collection 76.5% were sensitive to more than 40% (n=12-24) of the αGV collection, with all isolates sensitive to at least 32% of the αGV collection. For the non-*G. vaginalis* collection, the growth of 33% of isolates was not affected by the presence of αGV bacteria. Of the isolates affected 67% were fully sensitive to less than 30% of αGV isolates (Figure 3.20). The αGV isolates had limited inhibition of vaginal microbiome surrogates, *Lactobacillus sp.* (n=6). Contrarily, one of the non-*G. vaginalis* isolates, *B. animalis* ATCC 700541, was exceptionally inhibited by 91% of αGV isolates. It is noteworthy that *B. animalis* is not part of vaginal microflora.

3.3.2 Target range of αGV concentrated supernatants and the sensitivity of *G. vaginalis* isolates

A total of 29 αGV culture supernatants including α523 were concentrated using either chloroform extraction or methanol precipitation followed by "drop on the lawn" experiments as described in section 2.2.9. Twenty out of 29 αGV isolates produced concentrated supernatants with active agents verified by "drop on the lawn" experiments as detailed in section 2.2.8. Examples of "drop on the lawn" results for *G. vaginalis* ATCC 14018 and cGV7 are shown in Figure 3.21 and Figure 3.22, which correspond to chloroform extraction and methanol

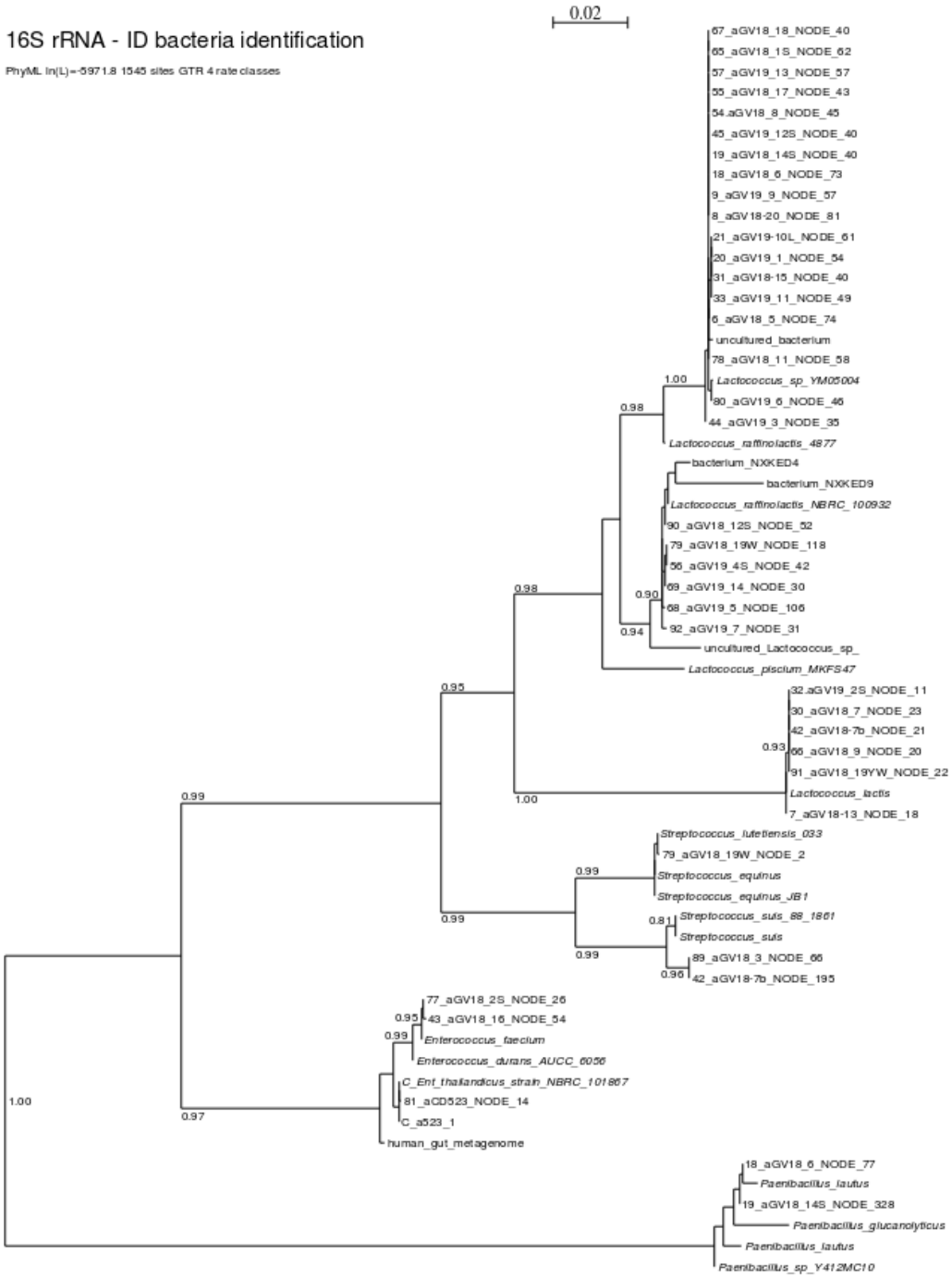

Figure 3.15 Phylogenetic tree analysis of 16S rRNA of entire αGV collection.

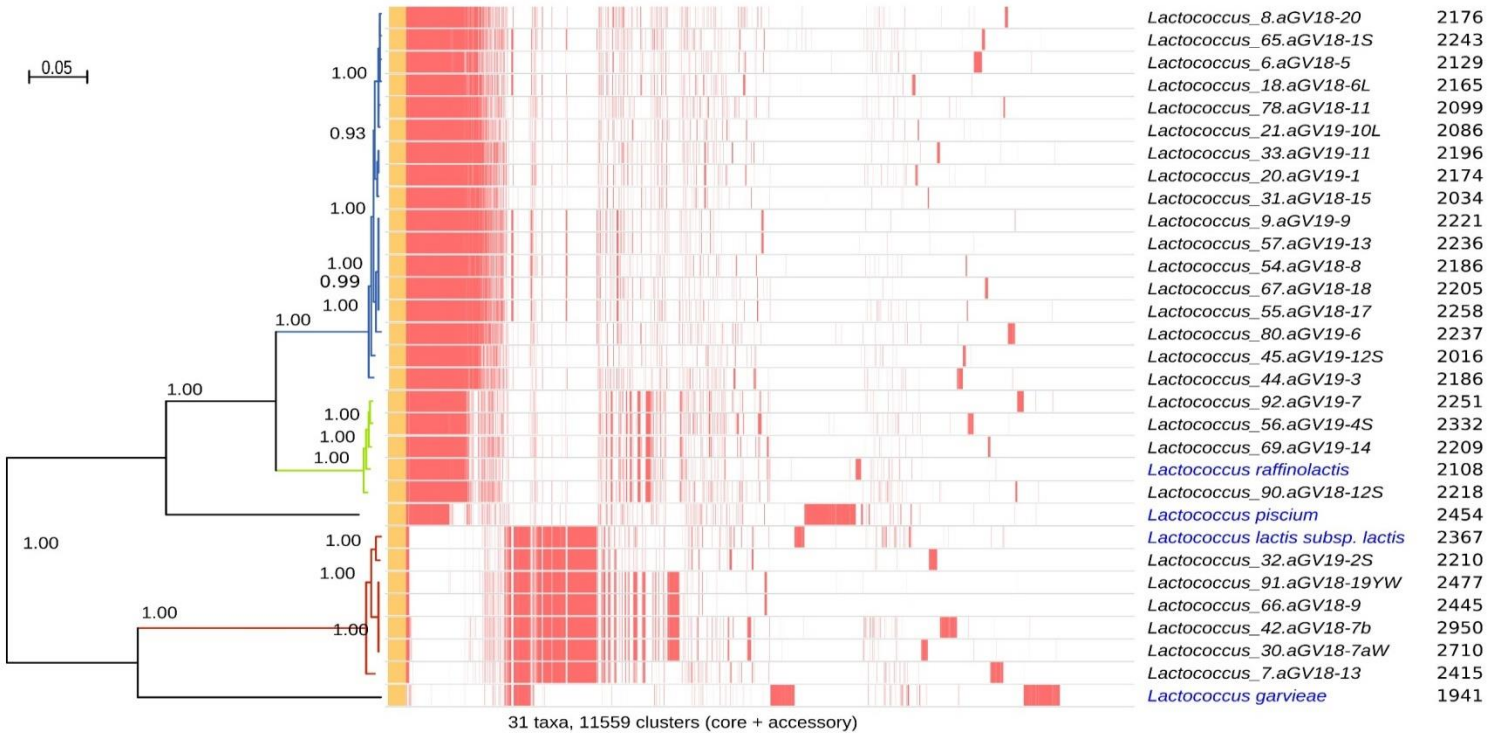

Figure 3.16 Phylogenetic tree and pangenome matrix of a selected cluster of the αGV collection and 4 NCBI reference strains. The blue, green and red sections of the phylogenetic tree indicate the clusters within the pangenome matrix. Each vertical line represents the presence of a protein with at least 70% amino acid similarity. The orange vertical bars denote the 312 core proteins. The red vertical bars indicate accessory proteins. The last column refers to the number of ORFs analyzed.

Table 3.4 Bacteriocin cassette types with their respective molecular weights (kD) identified in αGV isolates. Blue, green and red is indicative of clusters identified in pangenome matrix.

αGV isolates	orf10 HTCP (Class II)	orf003 HTCP (Class II)	Small orf1 HTCP (Class II)	Lactococcin Putative classII	Lanthipeptide classI	Lanthipeptide Class I orf013	orf007 (Class II)
aGV18-20	6.5 kD						
aGV18-1S	6.5 kD	8.61 kD	8.95 kD				
aGV18-5	6.5 kD						
aGV18-6L	6.5 kD						
aGV18-11	6.5 kD						
aGV19-10L	6.5 kD						
aGV19-11	6.5 kD						
aGV19-1	6.5 kD						
aGV18-15							
aGV19-9	6.5 kD	8.61 kD	8.95 kD				
aGV19-13	6.5 kD	8.61 kD	8.95 kD				
aGV18-8		8.61 kD	8.95 kD				
aGV18-18	6.5 kD	8.61 kD	8.95 kD	9.84 kD			
aGV18-17	6.5 kD	8.61 kD	8.95 kD				
aGV19-6	6.5 kD	8.61 kD	8.95 kD				
aGV19-12S	6.5 kD						
aGV19-3	6.5 kD						
aGV19-7				7.89 kD			
aGV19-4S	6.5 kD			7.89 kD			
aGV19-5				9.84 kD			
aGV19-14				7.89 kD			
aGV18-12S	6.5 kD			7.89 kD			
aGV19-2S					2.32 kD		2.32 kD
aGV18-19YW					2.34 kD	5.82 kD	2.34 kD
aGV18-9					2.34 kD	5.82 kD	2.34 kD
aGV18-7b	6.5 kD				7.86 kD	5.82 kD	5.66 kD
aGV18-7aW					7.86 kD	5.82 kD	5.66 kD
aGV18-13					7.86 kD	5.89 kD	5.66 kD

precipitation methods, respectively. Differences in clearance zone sizes and full versus partial clearance zones are illustrated in Figure 3.21. Target ranges of αGV concentrated supernatants against the *G. vaginalis* collection were determined (Figure 3.23). The minimum target range was 5% while the maximum target range observed was 100% for 7 of the 20 isolates (35%).

Overall, 60% of the αGV agents had target ranges greater than 45% indicating their inhibitory nature against 8 or more *G. vaginalis* isolates. Additionally, all *G. vaginalis* isolates (n=17) were shown to be sensitive to >30% of the 20 αGV concentrated supernatants, with a maximal sensitivity of 58% (Figure 3.24). It was concluded that the present collection of anti-GV isolates has the poteintail for formulation of minimal cocktails agaist GV collection; however, it would need to be expanded for larger collection of GV isolates.

3.3.3 αGV active agent SDS-PAGE analysis

Duplicate samples of the antiGV concentrated supernatants were fractionated in SDS-PAGE gels. Silver staining to observe protein banding patterns and diffusion of the bands from gel into indicator bacterial lawn are standard methods in screening for antimicrobial peptides, which are routinely applied to studying bacteriocins. Diffusion of peptide bands into the bacterial lawn results in localized clearance of bacteria corresponding to a band with antimicrobial activity as described in section 2.2.10. In practice, one half of the gel is cut and stained, and the other half is vigorously washed to remove the buffer and SDS and subsequently used in gel diffusion experiments. Alignment of the images of the gel halves using the low range protein ladder was used to identify the bands responsible for clearance of bacterial lawns. Several inhibitory bands were observed on the diffusion gel plates and matched to banding patterns on the silver stained

gel half (Figure 3.25, Figure 3.26, and Figure 3.27). The bands immediately corresponding to cleared areas in bacterial lawns were below 4.6 kD for αGV18-9 on the indicator strain *G. vaginalis* ATCC 14018 (Figure 3.25). On the *G. vaginalis* ATCC 14019 two clearance bands of different molecular weights, both between 4.6-10 kD, were observed for αGV19-2S and αGV19-7 (Figure 3.26). A band was also observed at approximately 35 kD for αGV19-7 on a lawn of cGV14 (Figure 3.27). It must be noted that the supernatant extract from αGV19-7 with clearance band at ~35 kD was active against 95% of GV isolates in drop on lawn experiments. This highlights the need for further thorough examination of the extracts in diffusion assays.

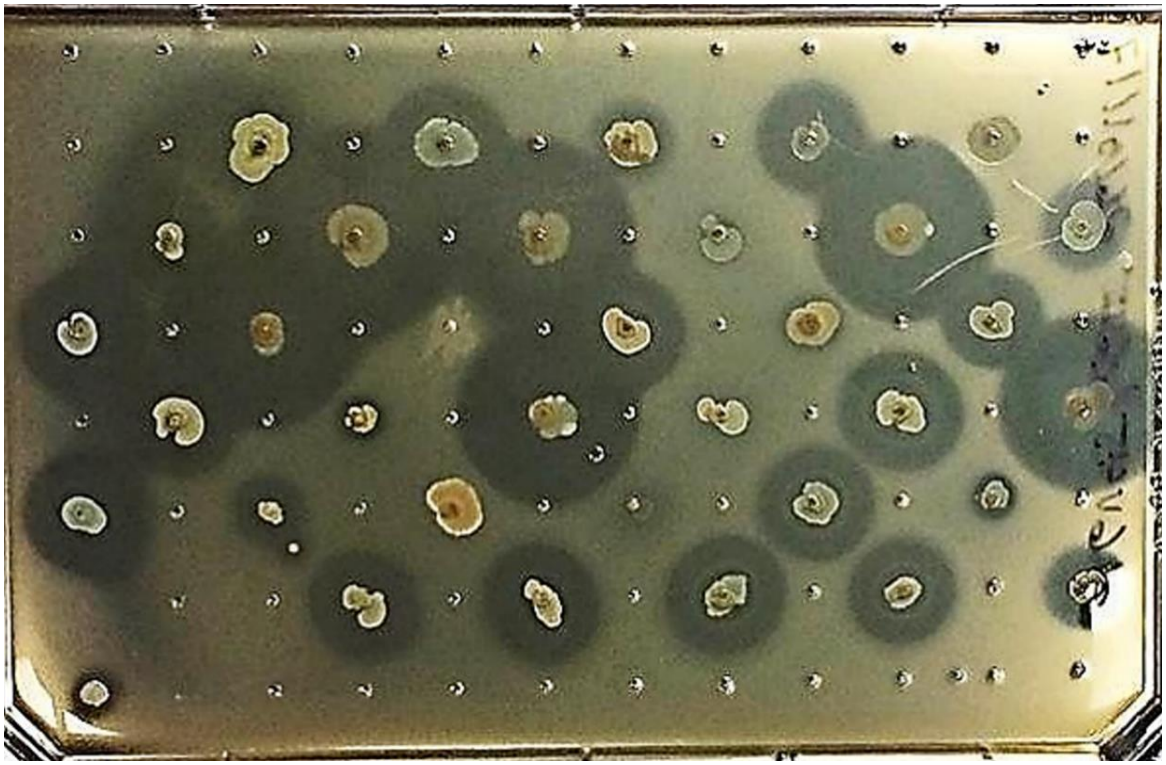

Figure 3.17 An example of high throughput target range assay of αGV bacterial collection against *G. vaginalis* ATCC 49145.

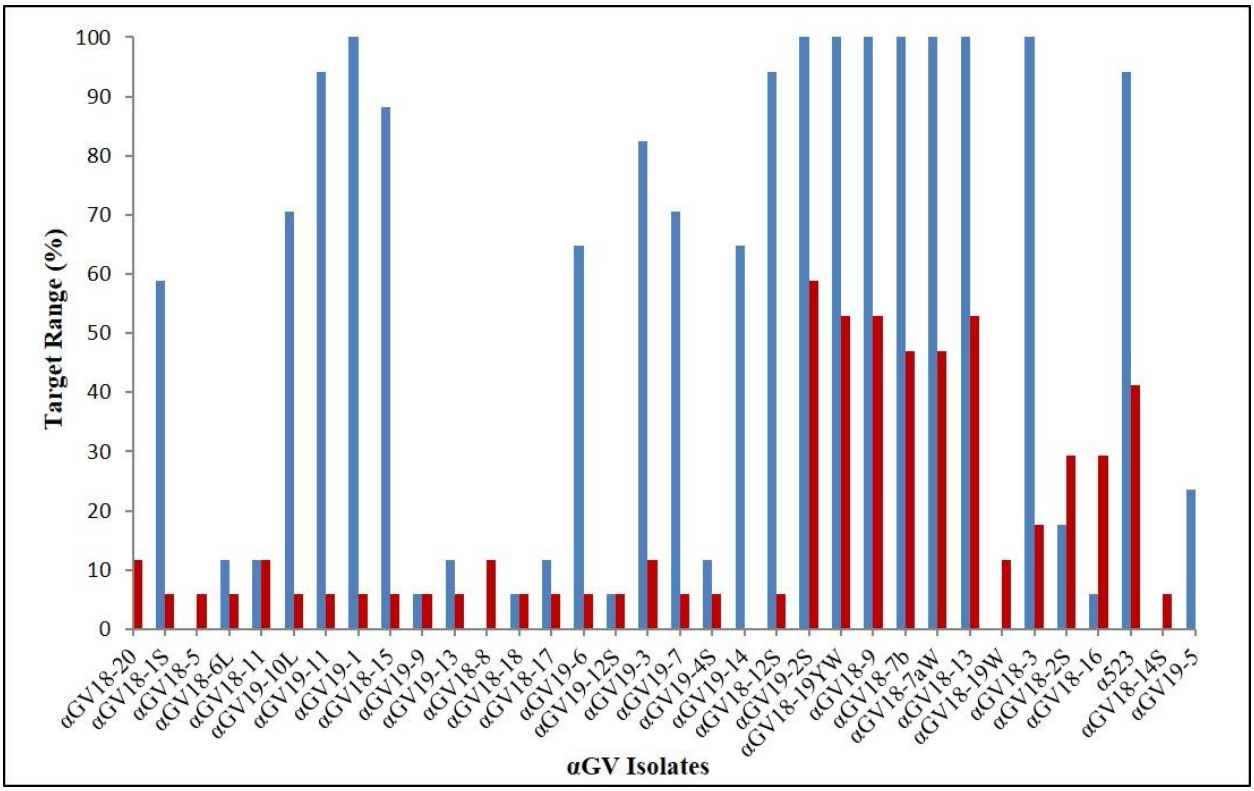

Figure 3.18 Target range of αGV bacteria against the *G. vaginalis* collection (n=17; blue bars) and non-*G. vaginalis* collection (n=19; red bars).

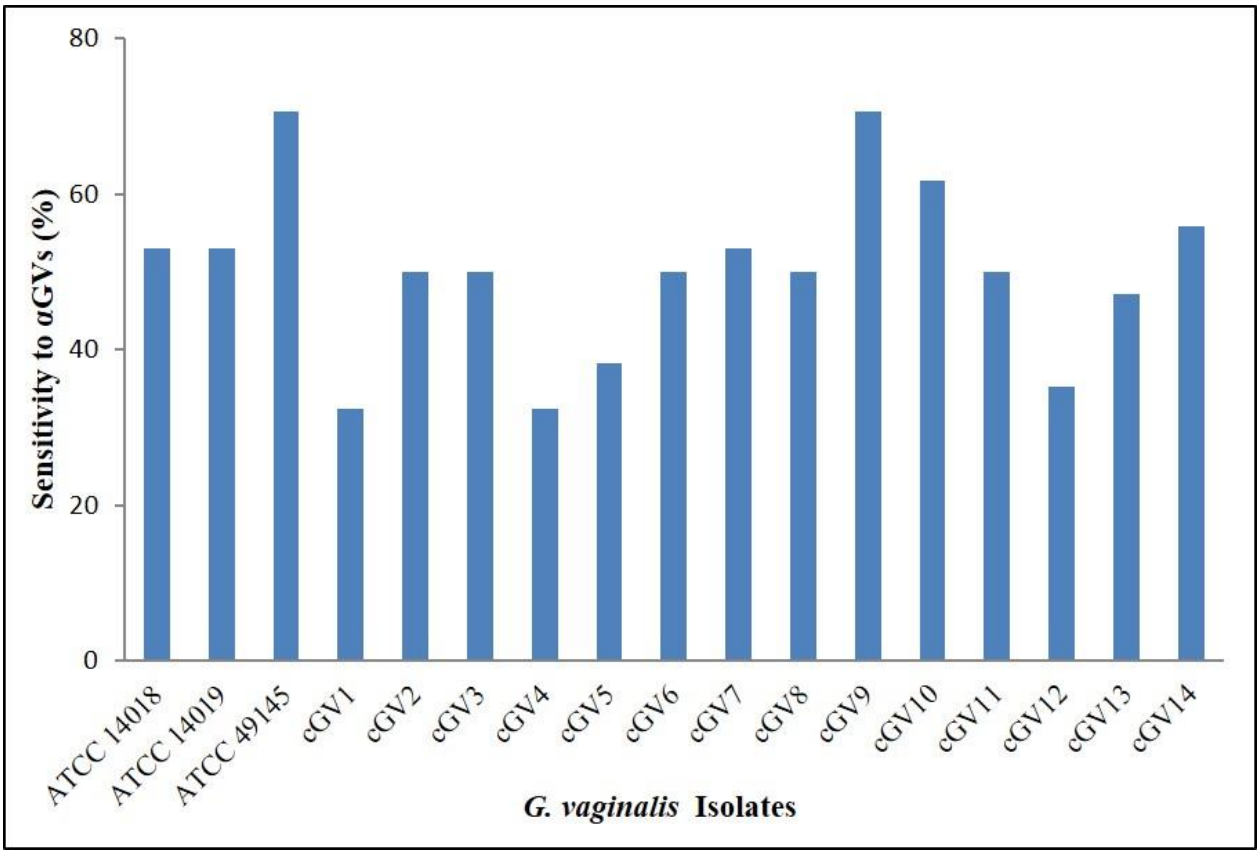

Figure 3.19 Sensitivity of *G. vaginalis* collection (n=17) to the αGV isolates (n=34).

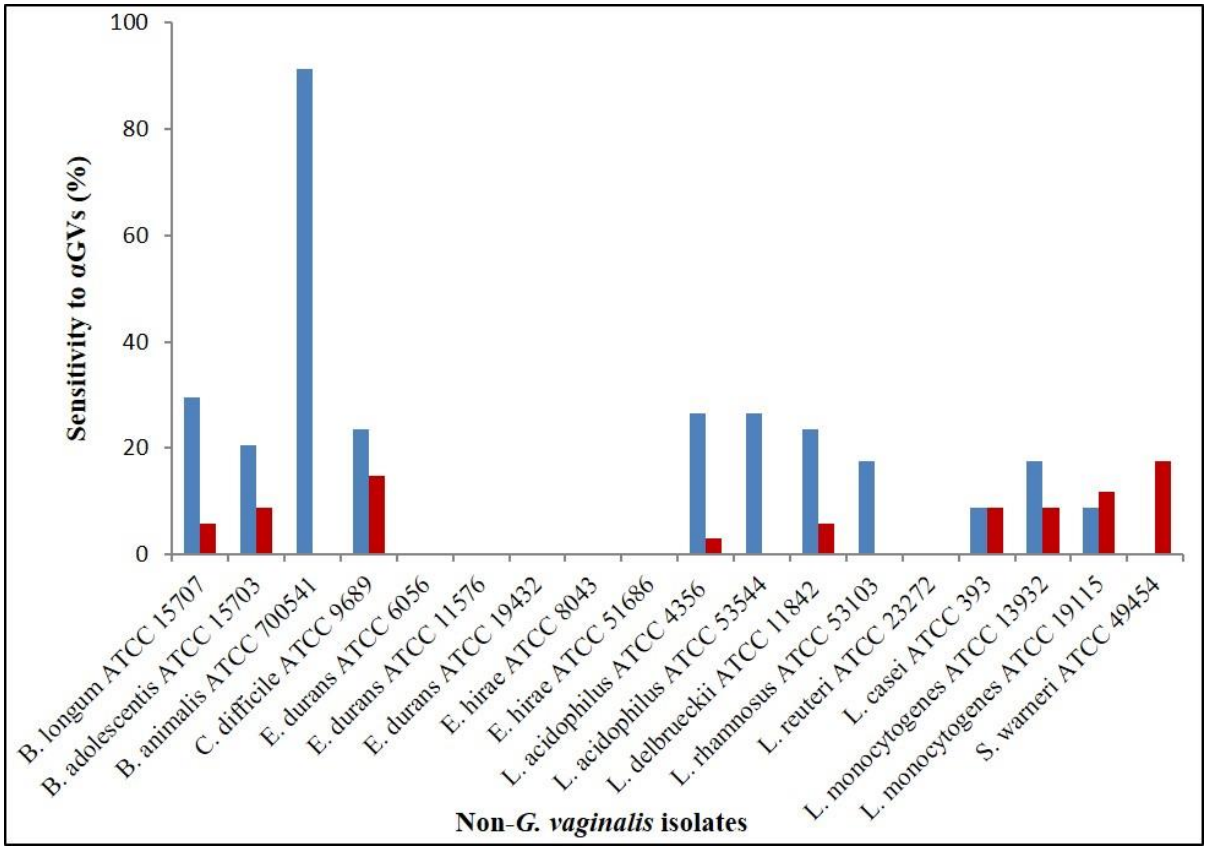

Figure 3.20 Sensitivity of the non-*G. vaginalis* collection (n=18) to the αGV collection (n=34). Blue and red bars indicate full (clear zone of inhibition) and partial sensitivities (turbid zone of inhibition), respectively.

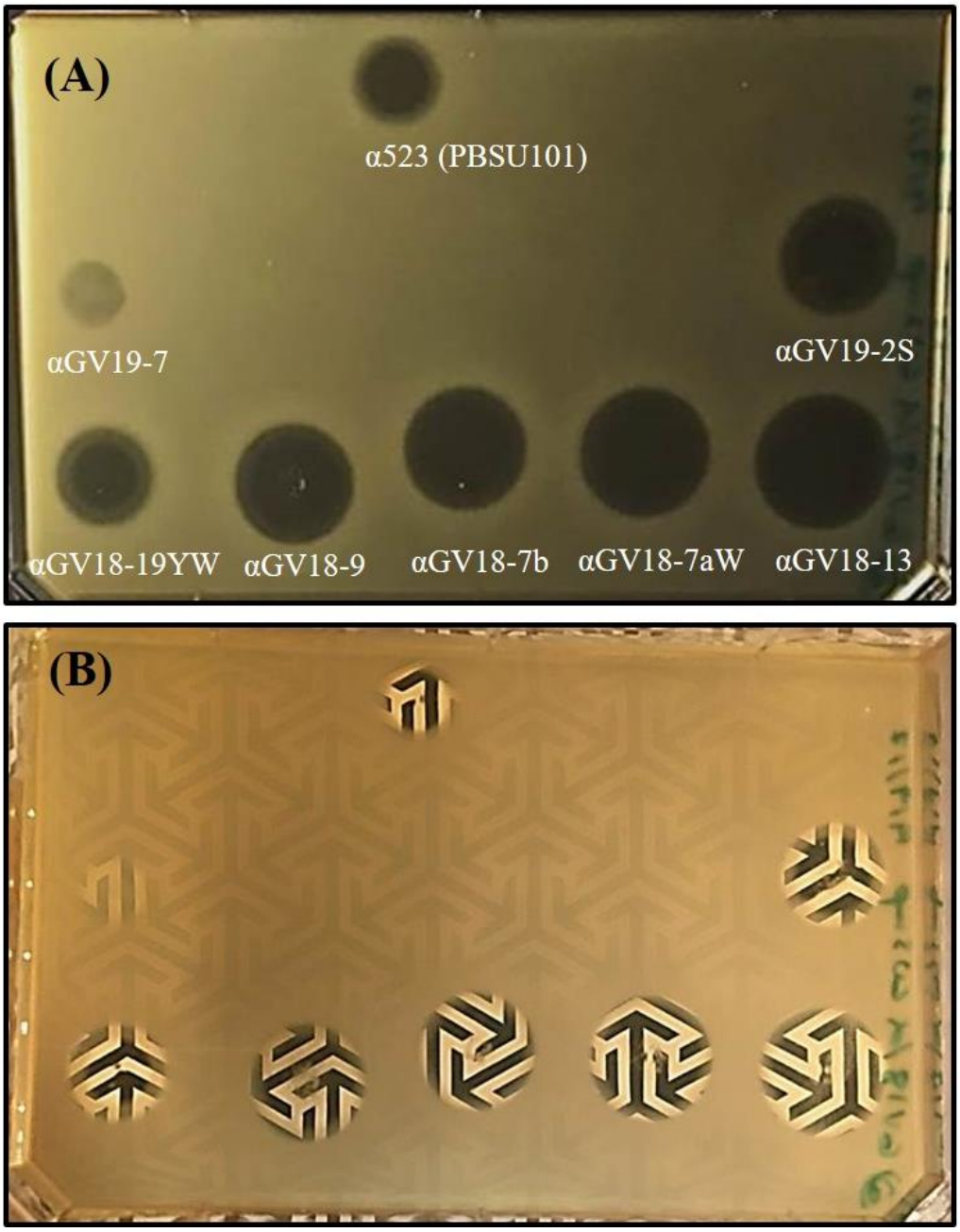

Figure 3.21 (A) An example of the "drop on lawn" target range assay results using αGV chloroform extracted supernatants against *G. vaginalis* ATCC 14018. (B) Transparency of clearing zone is illustrated by overlaying the plate on a paper with line patterns.

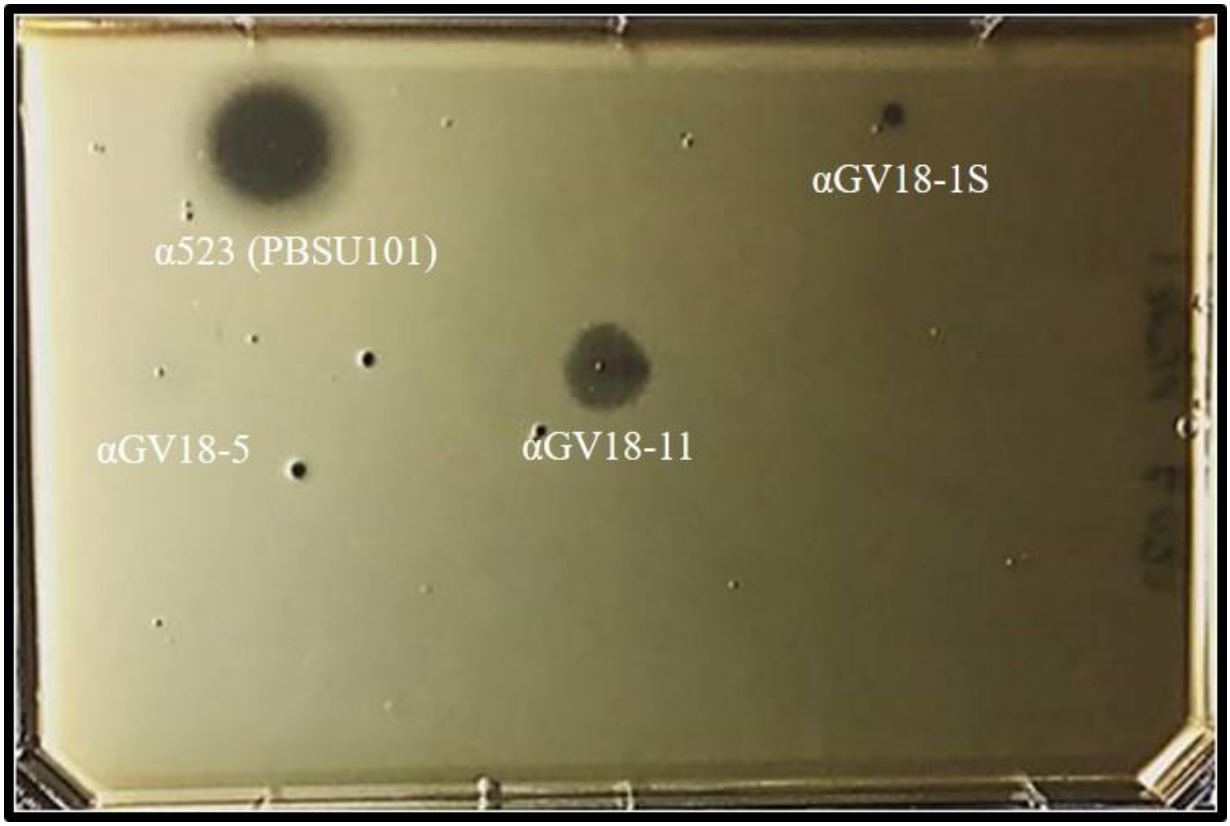

Figure 3.22 An example of the "drop on lawn" target range assay results using αGV methanol precipitated supernatants against cGV7.

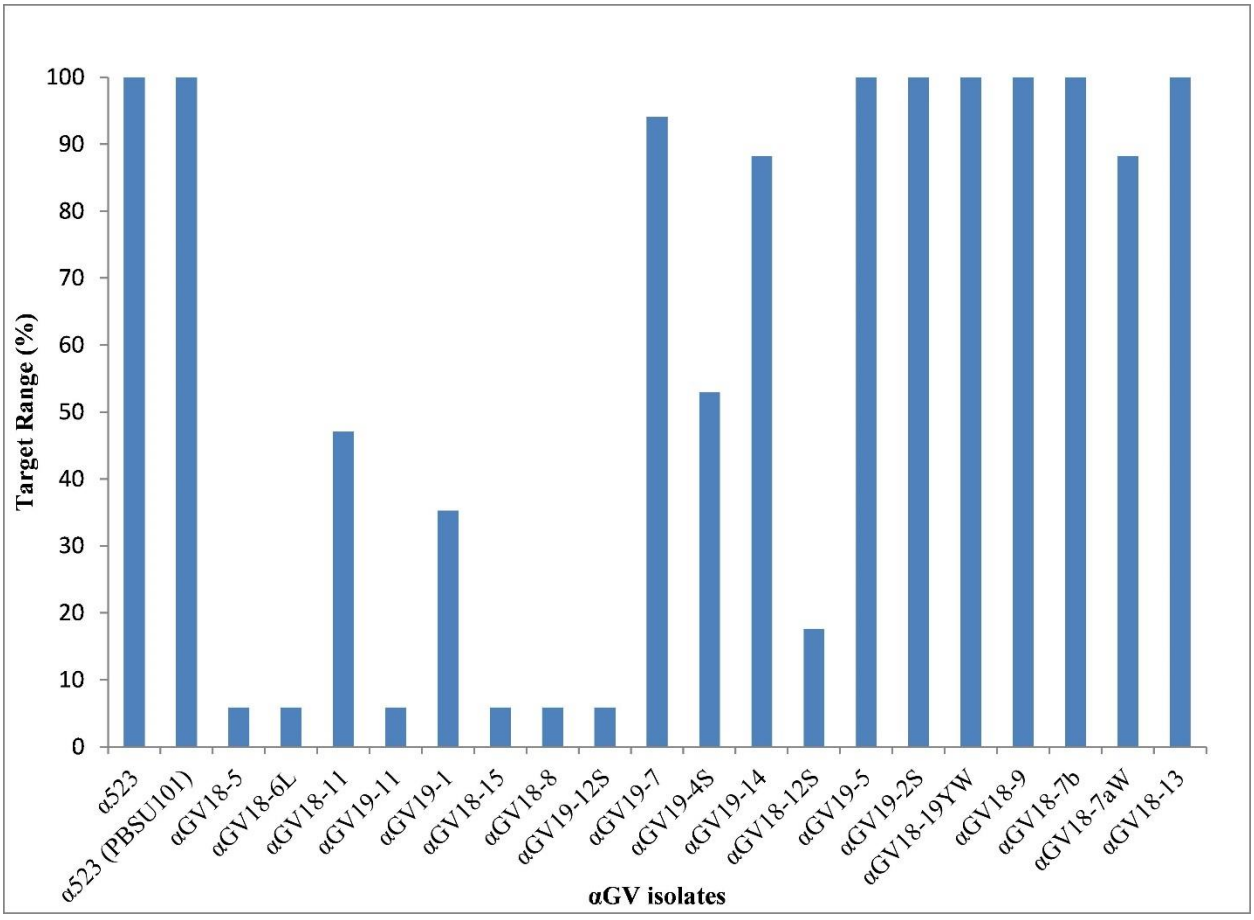

Figure 3.23 Target ranges of twenty αGV concentrated supernatants against *G. vaginalis* isolates (n=17) as determined by "drop on the lawn" experiments.

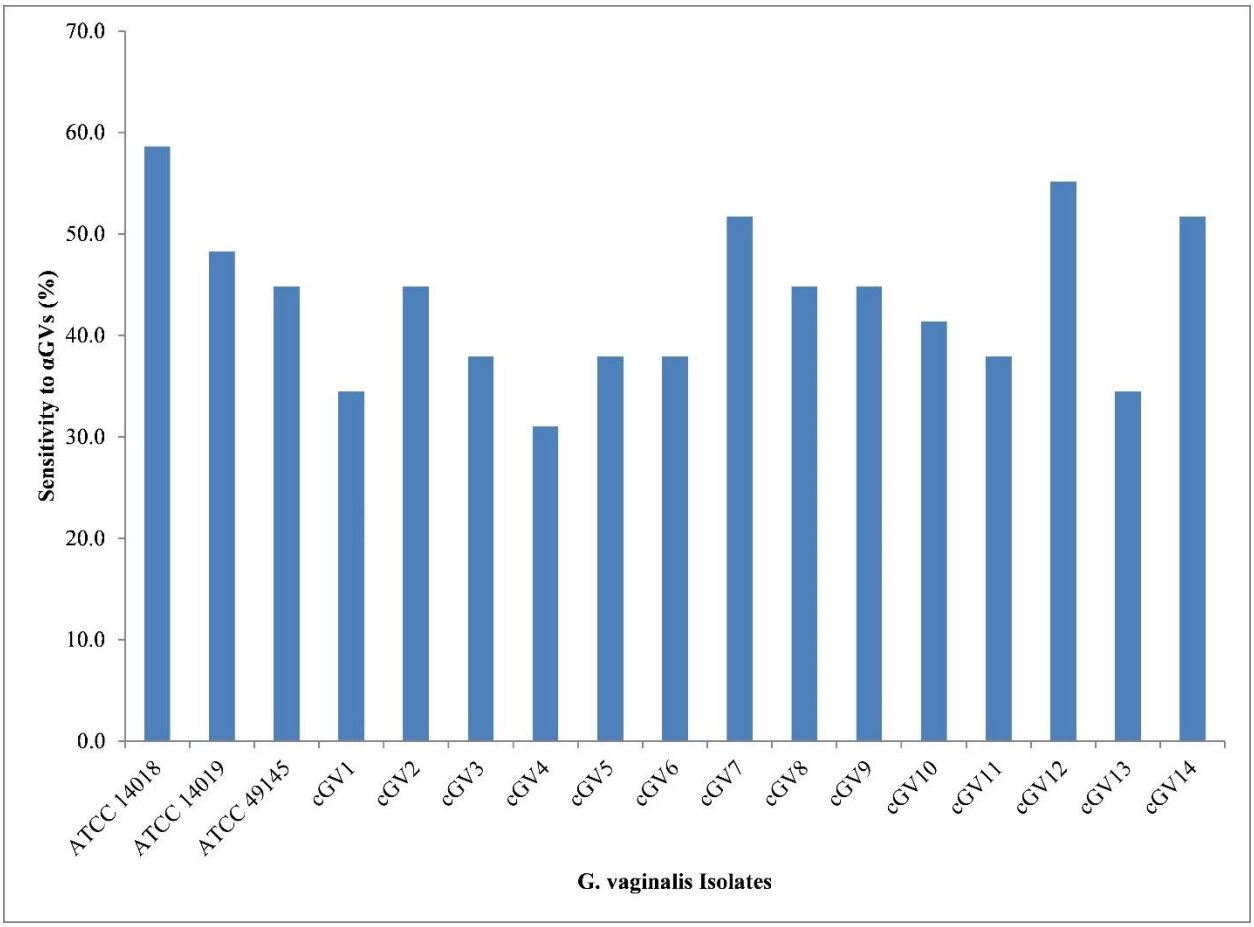

Figure 3.24 Sensitivity of the *G. vaginalis* isolates (n=17) to twenty αGV concentrated supernatants as determined by "drop on the lawn" experiments.

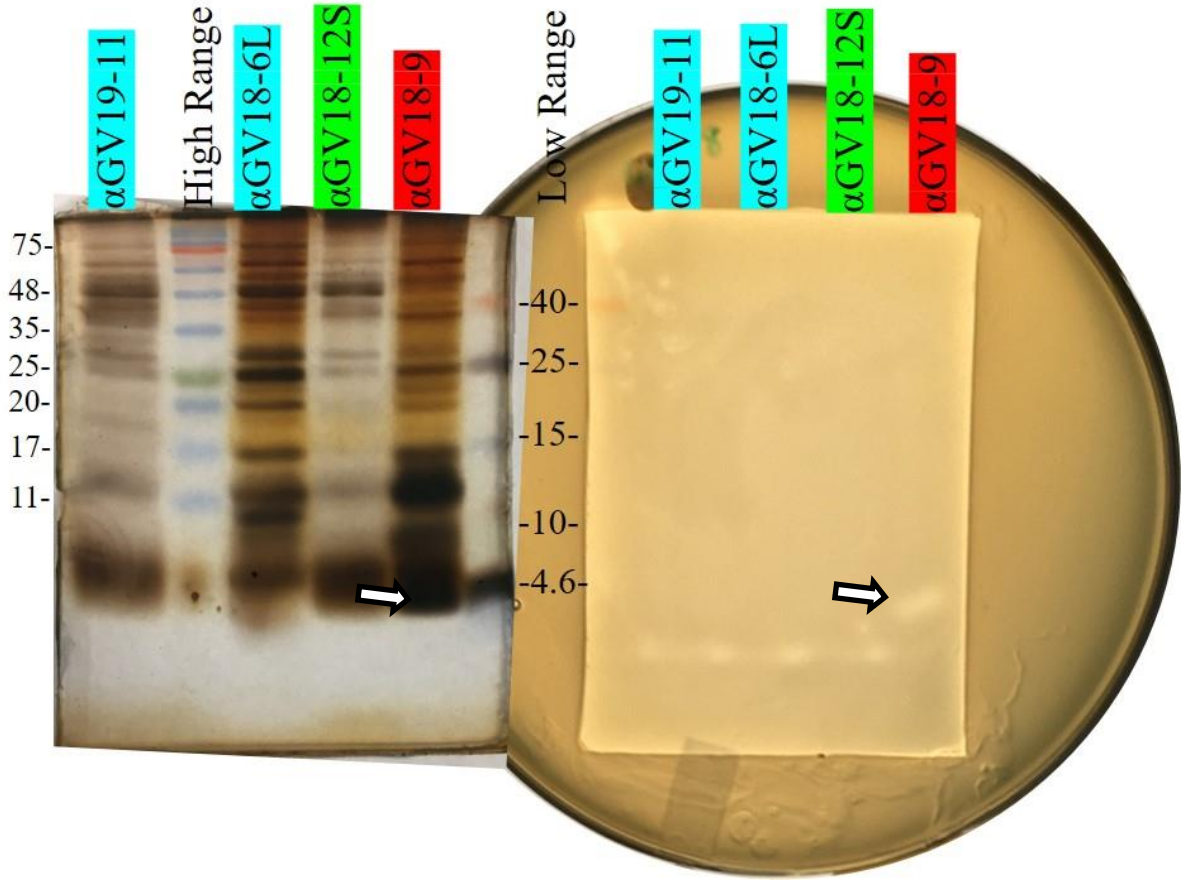

Figure 3.25 Alignment of silver stained and gel diffusion half gels. Concentrated supernatants from αGV 19-11, 18-6L, 18-12S and 18-9 were fractionated in a 15% SDS-PAGE and diffused into *G. vaginalis* ATCC 14018 lawn. Multicolor low range protein ladder was used to align the gel images (lane 6). Arrows indicate position of silver stained protein band that correspond to clearance zone in gel diffusion.

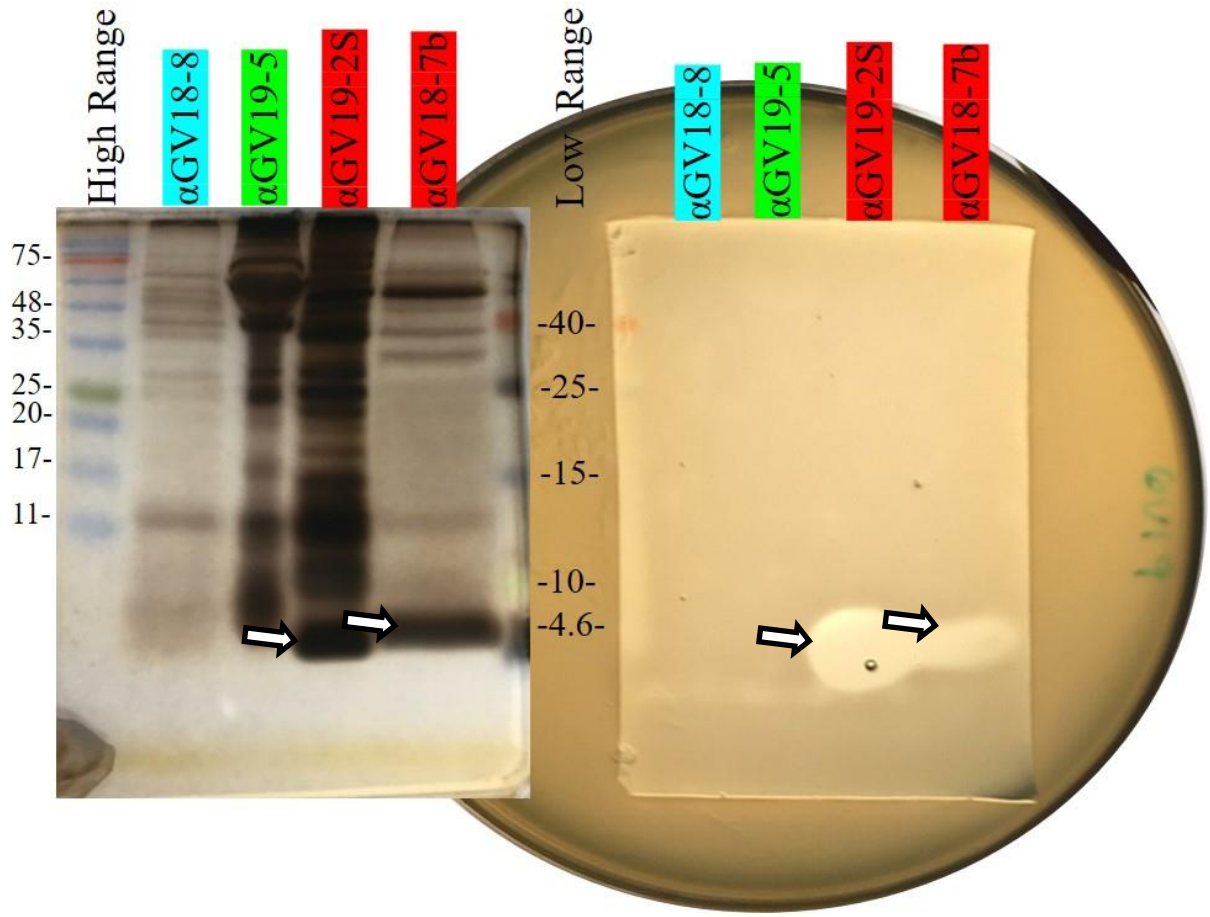

Figure 3.26 Alignment of silver stained and gel diffusion half gels. Concentrated supernatants from αGV 18-8, 19-5, 19-2S and 18-7b were fractionated in a 15% SDS-PAGE and diffused into *G. vaginalis* ATCC 14019 lawn. Multicolor low range protein ladder was used to align the gel images (lane 6). Arrows indicate position of silver stained protein bands that correspond to clearance zones in gel diffusion.

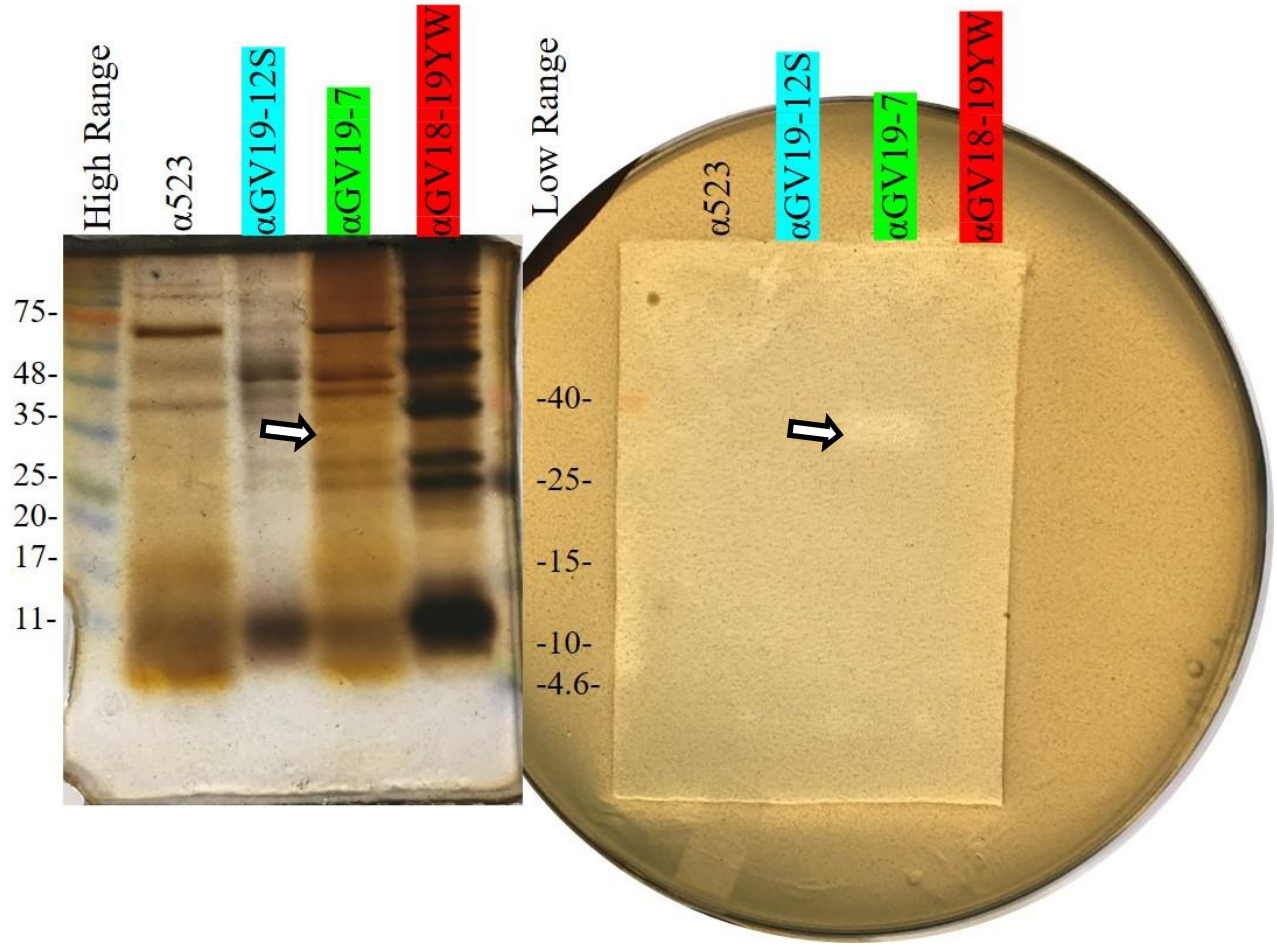

Figure 3.27 Alignment of silver stained and gel diffusion half gels. Concentrated supernatants from α523, αGV 19-12S, 19-7, and 18-19YW were fractionated in a 15% SDS-PAGE and diffused into *G. vaginalis* cGV14 lawn. Multicolor low range protein ladder was used to align the gel images (lane 6). Arrows indicate position of silver stained protein band that correspond to clearance zone in gel diffusion.

4 Discussion

4.1 Genotypic diversity among *G. vaginalis* isolates

Consistent with the earlier reports as summarized in section 1.2, our collection of *G. vaginalis* (n=17) were determined to be genotypically diverse by ARDRA analysis via *Taq*I or *Hpa*II digestion of a PCR product amplified from the 16S rRNA gene (Figure 3.5Figure 3.6). ARDRA analysis results using *Taq*I showed the existence of genotypes A and B but not C in our collection, suggestive of limited genotypic diversity among these *G. vaginalis* isolates (Ingianni *et al.*, 1997). The majority of our isolates were genotype B (82.4%), while the remaining were found to be genotype A (17.6%). In Pleckaityte et al. (2012), the distribution of *G. vaginalis* genotypes was seen with 35% genotype A (n=6) and 64.7% genotype B (n=11) and no genotype C, indicating that genotype C may be a rare type. However, among clinical isolates from Kenya and Belgium the genotypic distribution differed with most isolates having genotype A (49%), followed by genotype B (26%) and C (25%) (Lopes dos Santos Santiago *et al.*, 2011). Therefore, geographical location may play a role in diversity of the *G. vaginalis* genotypes. This observed genotypic diversity among our *G. vaginalis* collection was further supported by *Hpa*II ARDRA analysis that revealed three genotypes 1, 2 and 4 but not genotype 3 (Ingianni *et al.*, 1997). Genotype 1 made up 82.4% of the isolates. Interestingly, all previously identified genotype B isolates were determined to be genotype 1 by *Hpa*II digestion. This pattern of correspondence between *Taq*I and *Hpa*II genotypes, however, was not consistent, as the *Taq*I genotype A isolates appeared to be either genotype 3 (5.8%) or 4 (11.8%) by *Hpa*II ARDRA analysis.

In conclusion, consistent with other reports, the members of our small collection of *G. vaginalis* isolates showed genotypic diversities that might also independently apply to their other traits such as virulence factors and antimicrobial sensitivities.

4.2 Diversity in antibiotic susceptibility profiles

Antibiotic susceptibilities of our *G. vaginalis* collection was determined for eight antibiotics: metronidazole (MZ), clindamycin (CM), vancomycin (VA), linezolid (LZ), rifampicin (RI), ampicillin (AM), ciprofloxacin (CI), and tetracycline (TC) (Table 3.1). The two clinically relevant antibiotics are metronidazole and clindamycin. Metronidazole MIC values for *G. vaginalis* isolates have been shown to be in the range of 0.125µg/mL to >1024µg/mL (Kharsany *et al.*, 1993; Austin, 2006; De Backer *et al.*, 2006; Nagaraja, 2008; Togni *et al.*, 2011; Knupp de Souza *et al.*, 2016). However, there are some contradicting reports. For example, *G. vaginalis* ATCC 14018 when assessed in VDMP medium (vaginally defined medium plus peptone) containing metronidazole showed growth stimulation at lower concentrations of antibiotic while at higher concentrations (1000-4000µg/mL) there was a gradual growth inhibition (Anukam and Reid, 2008). On the contrary, the *G. vaginalis* ATCC 14018 MIC value was said to be 16µg/mL (Knupp de Souza *et al.*, 2016). There seems that the accuracy of MIC values is dependent on the method of evaluation, as well as the growth conditions. For example, incubation at strict aerobic versus microaerobic environments and the enriching supplements in the growth medium have dramatic effect on the growth kinetics of *G. vaginalis*. Suboptimal conditions can alter the growth kinetics of the *G. vaginalis* and therefore lead to reduced concentration of antibiotics

required to cause growth inhibition (MIC) and/or cell death. Gottschick *et al.* (2016) study demonstrated that the strict anaerobic condition was not optimal for *G. vaginalis* biofilm growth and that incubation should occur under 5% partial pressure for CO_2. We conducted our E-testing for our *G. vaginalis* collection according to CLSI and EUCAST guidelines with optimal growth conditions for *G. vaginalis* and agar plates consistent with De Backer *et al.* (2006) and Schuler *et al.* (2016) (section 2.2.4). The quality control strain *E. faecalis* was used to validate test performance. Based on the E-test results (Table 3.1) the metronidazole MIC range for the *G. vaginalis* collection was 8µg/mL to >256µg/mL. This accounted for 1 to 8-fold differences in MIC values amongst the isolates, indicating diversity with respect to their susceptibilities to metronidazole. Differences in the levels of susceptibilities of different isolates might be related to the physiological differences among isolates in their antibiotic uptake rates, biological drug activation of antibiotic, and DNA repair functions that mitigate the genotoxicity of metronidazole. Nevertheless, our observations are congruent with previous literature results (Kharsany *et al.*, 1993; Austin, 2006; De Backer *et al.*, 2006; Nagaraja, 2008; Togni *et al.*, 2011; Schuyler *et al.*, 2016). The distribution of metronidazole MIC values for *G. vaginalis* collection is illustrated in Figure 3.8. Since there are no standard breakpoints defined by CLSI and EUCAST for *G. vaginalis*, the suggested breakpoints in the literature, <8µg/mL for susceptible, 8-16µg/mL intermediate, and resistant is defined as either >16µg/mL or ≥32µg/mL, were used to interpret our results (Austin, 2006; De Backer *et al.*, 2006; Nagaraja, 2008; Knupp de Souza *et al.*, 2016; Schuyler *et al.*, 2016). Based on the literature-stated breakpoints, none of the *G.*

vaginalis isolates were susceptible to metronidazole, whereas 35.3% were deemed to be intermediately resistant and 64.7% of the *G. vaginalis* isolates were resistant (Table 3.1). The isolates (n=4) that were not inhibited by metronidazole at the highest concentration available on the E-strips are most likely part of an emerging population of extremely resistant strains similar to what observed in Schulyer *et al.* (2016).

4.3 Metronidazole resistance development

Additionally, within the inhibition zones of metronidazole, rifampicin and ciprofloxacin E-strips several *G. vaginalis* singular colonies were observed (Figure 3.9; Table 3.2). The ARDRA analysis of 20 purified colonies picked from the inhibition zone of metronidazole E-strips on *G. vaginalis* cGV2 isolate showed similar banding patterns to their parental isolate consistent with genotype B. This suggested that resistant colonies were derived from the metronidazole sensitive parental cGV2 isolate. Further analysis of these 20 colonies for their susceptibilities to metronidazole by E-test showed elevated MIC values of >256µg/mL (Figure 3.11) indicating that an overnight exposure to metronidazole was sufficient to select for either existing spontaneous mutants in the population or mutants were induced via exposure to metronidazole. Although the latter seems an interesting postulation given the genotoxic and mutagenic capacity of metronidazole, however, determining the molecular basis of the mechanism involved in generation of mutants in *G. vaginalis* requires further experiments. It is noteworthy that involvement of nitroreductases in stepwise genotoxic bioactivation of the nitroaromatic compounds and further generation of induced resistant mutants has been mapped to mutations in

these enzymes in *E. coli, S. thyphimurium* and *H. pylori* (De Meo *et al.*, 1992; Whiteway *et al.*, 1998; Carroll *et al.*, 2002; Nokhbeh *et al.*, 2002; Tanih *et al.*, 2011). The observation that an overnight exposure/selection of the *G. vaginalis* metronidazole mutants did not result in incremental MIC values might be an indication of involvement of a single step mechanism leading to the highest MIC values. This rapid selection/induction of highly resistant isolates in *G. vaginalis* has obvious important clinical implications, which might be relevant to the reported 20% relapse rates among BV patients within first month post-treatment with metronidazole and 70% recurrences by the third month (Swidsinski *et al.*, 2008; Mayer *et al.*, 2015). This is not specific to *G. vaginalis* since a similar increasing trend of metronidazole treatment failure has become a common place for other infections such as *C. difficile* and *H. pylori* infections, which are also treated with metronidazole (Pandya *et al.*, 2014; Barkin *et al.*, 2017; Hu *et al.*, 2017).

Regardless of the mechanism through which the metronidazole resistance developed, we asked whether the metronidazole resistant cGV2 derivatives were still sensitive to the inhibitory/killing effects of antiGV isolates. We tested the sensitivities of 20 metronidazole resistant isolates to the antiGV collection by using a high throughput target range plate assay, as detailed in section 2.2.7.1. We found no difference in their susceptibilities to killing by specific antiGV isolates (data not shown).

The diversity in the MIC values, rapid resistance acquisition rates and treatment failure further demonstrates the need for developing alternative therapeutics. Given the example of retaining the

target spectra of our αGV collection by the metronidazole-resistant mutants mentioned above highlights our approach of using bacteria against *G. vaginalis* isolates as one of the viable alternative trends that might lead to promising applications for combatting metronidazole-resistant GV.

Learning from the metronidazole experience, we were prompted to examine the susceptibilities of the *G. vaginalis* collection to other antibiotics. The MIC ranges determined by E-test for clindamycin were <0.016µg/mL to 0.064µg/mL, which is consistent with literature with most reported MIC values falling between <0.016µg/mL and 0.047µg/mL (Kharsany *et al.*, 1993; De Backer *et al.*, 2006; Nagaraja, 2008; Togni *et al.*, 2011; Knupp de Souza *et al.*, 2016). The suggested breakpoints in literature were <2µg/mL susceptible, 2-4µg/mL intermediate, and >4µg/mL resistant (Nagaraja, 2008). Based on these breakpoints all our *G. vaginalis* isolates tested (n=17) were determined to be susceptible to clindamycin.

The MIC values for the six clinically not relevant antibiotics for our *G. vaginalis* collection are shown in Table 3.1. The MIC ranges for VA (0.25µg/mL to 0.5µg/mL), and CI (1µg/mL to 4µg/mL) were consistent with MIC ranges previously reported for VA (0.12µg/mL to 0.5µg/mL), and CI(0.75µg/mL to 4µg/mL) (Kharsany *et al.*, 1993; De Backer *et al.*, 2006). Whereas MIC ranges reported here for LZ (0.125µg/mL to 0.50µg/mL), RI (0.5µg/mL to 2µg/mL), AM (<0.016µg/mL to 2µg/mL), were found to be slightly higher than the maxima of the literature-reported MIC ranges. The literature reported MIC ranges are as follows: LZ

(0.125µg/mL to 0.19µg/mL), RI (0.5µg/mL to 0.75µg/mL), and AM (<0.016µg/mL to 0.5µg/mL) (Kharsany *et al.*, 1993; De Backer *et al.*, 2006; Knupp de Souza *et al.*, 2016). There was a 2.6 fold, 2.7 fold, and 4 fold increase in MIC maxima values relative to literature values for LZ, RI, AM, respectively (Kharsany *et al.*, 1993; De Backer *et al.*, 2006; Knupp de Souza *et al.*, 2016). On the contrary, the MIC range for tetracycline (0.5µg/mL to 128µg/mL) in our collection was lower by 4 fold for minimum MIC value compared to the reported MIC range (2µg/mL to 128µg/mL) (Kharsany *et al.*, 1993). Regardless of the antibiotic MIC value differences observed between our collection and the reported values, the susceptibilities to each antibiotic among the *G. vaginalis* isolates varied with an overall 2 to 256-fold difference in MIC values, which further demonstrated the diversity among *G. vaginalis* isolates (Table 3.1).

In conclusion, it appears that our small collection of *G. vaginalis* isolates comprised of non-homogenous members with respect to their genotypic characteristics and their antibiotic susceptibility properties. These observations are congruent with the reports from other labs around the world. In addition to limited efficacy of the available antibiotics, the genetic heterogeneity and diversity in biological properties of the members of *G. vaginalis* bacteria play a determining role in contributing to the treatment failure, acquisition of resistance and consequently to the ever-increasing prevalence of this infection globally. Therefore, developing effective antimicrobials for treatment of BV cannot simply rely on synthetic derivation technology by modifying the existing antibiotics for which there is a historical failure records. Moreover, vaginal colonization and formation of sessile biofilm of *G. vaginalis*, which plays a

primer role in establishing multi-bacterial biofilm, is greatly potentiated by the concomitant antibiotic induced severe damage to the otherwise protective vaginal microflora. Therefore, it would be beneficial to avoid developing and the use of broad spectrum antibiotics in the future. It makes a logical sense that for controlling a highly diverse and plastic pathogen such as *G. vaginalis* one must either rely on multiple narrow spectrum and highly specific therapies with additive or synergistic properties, or develop smart antimicrobials with dynamic adaptation properties, eg phage therapy.

We considered the following principles in formulating our strategic approach to developing novel antibacterials against *G. vaginalis*. These principles are as follows, i) narrow spectrum and specificity against *G. vaginalis*, ii) indiscriminate inhibitory effect against metronidazole-resistant variants, iii) multiplicity and diversity among candidate antibacterials, iv) overlapping modes of actions to provide additive or synergistic effects, v) advanced refinement through evolutionary selection to reduce the chance of resistance.

One of the most attractive approaches in developing novel antibacterials is the use of bacteria against bacteria, as this has occurred throughout evolution in nature. Therefore, we screened for bacteria with potential direct antagonism against *G. vaginalis*.

4.4 Isolation and characterization of a diverse collection of AntiGV isolates

Screening raw sewage for bacteria against *G. vaginalis* ATCC strains 14018 and 14019 resulted in the isolation of a diverse group of 33 bacteria and one previously isolated bacterium (α523)

with direct inhibitory/killing effects against *G. vaginalis* (section 3.2.1). Except for two isolates that appeared rod-shaped, the majority of the αGV isolates belonged to streptococcal morphotype and differed from each other with respect to their individual cell sizes and the length of streptococcal chains, and all appeared to be catalase-negative (Figure 3.14 and Table 3.3). We used the catalase test not only as an additional phenotypic marker for diversity, but to be cautious about catalase-positive isolates, since catalase is considered to be a virulence factor for most pathogens, assisting them in avoding the immune system.

We sequenced the genomes of these 34 αGV isolates and assembled into multiple contigs and annotated (see section 2.2.6.2 and 0), however, we could not close them. Closing any genome to its final circular representation of chromosome requires further long-range sequencing using PacBio or MinIon sequencing platforms and using the long scaffolds to fill-in the gaps between assembled contigs. Nonetheless, the available sequencing data (~90% recovery) provided useful information to proceed with taxonomic identification, and further analyses to detect genomic cassettes. In addition to morphological and varying levels of effect on *G. vaginalis*, a glance at the preliminary sequencing report indicates that the antiGV isolates were diverse with respect to their GC contents, number of rRNA operons and tRNA (Table 3.3). Using the nucleotide sequences and amino acid sequences of the predicted open reading frames (ORFs) the phylogenetic trees were constructed. Alignment of the 16S rRNA genes lead to construction of a phylogenetic tree (Figure 3.15). The majority of the isolates were clustered within lactococci (28 out of 34 isolates) of which 22 of 28 lacococci were distantly related to *L. raffinolactis* that

appeared as an outgroup (Figure 3.15). The closest representative for 16 of 22 isolates in *L. raffinolactis* subgroup was identified as *Lactococcus sp.* YM05004 for which no complete genomic sequence is available, nor its accurate taxonomic binomial ID is defined. There seems that this group of 22 isolates are also divergent between each other that makes assigning accurate taxonomic identification challenging. Therefore, we speculate that these isolates might be either novel species or they might be defined as subspecies or strains of a common species. For the ease of communication, therefore, we suggest designating them as *Lactococcus sp*. followed by the numbers that we have already used in this report. The remaining 6 isolates out of 28 lactococcal group clearly clustered with *Lactococcus lactis*, a dairy fermenter (Figure 3.15). The third group of αGV isolates included two members that clustered with Enterococci and most closely with *E. faecium* and distantly with *E. durans* and our previously reported α523 isolate (Ybazeta *et al.*, 2017). For these two members we suggest assigning the representative *E. faecium* names and await full genome sequence when we can identify them at subspecies taxonomic levels, depending on the level of divergence they show compared to reference genome. The fourth group of isolates consisted of two that were clustered with *Streptococcus suis* and *Streptococcus lutetiensis* and the fifth group of two isolates were clustered with *Paenibacillus lautus*, a rod-shaped spore bearing Gram-variable bacterium.

The αGV isolates appear to be genetically diverse as shown by their 16S rRNA restriction maps; however, to gain more detailed information about diversities, we constructed a pangenomic phylogenetic tree using over 2000 protein amino acid sequences as explained in section 3.2.2.2

(Figure 3.16). Since the two rod-shaped bacteria had weak antiGV activities and the two members of Enterococci resembled by *E. faecium* (a pathogenic bacterium), and the other two isolates within *Streptococcus suis* cluster are considered emerging pathogens, we excluded these isolates from further analyses. We focused our pangenomic analysis on 28 lacotcoccal isolates. Pangenomic analysis identified three divergent clusters indicated by blue, green and red colors in Figure 3.16 and Table 3.4. As noted in the 16S rRNA phylogenetic tree, the closely related *Lactococcus raffinolactic* isolates represented the blue and green clusters, while the *Lactococcus lactic* isolates closely represented the red colored cluster. *L. piscium* and *L. garvieae*, that were included as the outgroup controls, showed clear differences with the members of each cluster (Figure 3.16). While these clusters were different from each other, the αGV members within each cluster appeared not to be the same with respect to their banding patterns suggesting diversity among these isolates and consistent with previous genetic and morphological observations.

It is noteworthy that 27 out of 28 of these isolates were also shown to carry genetic cassettes potentially coding for novel bacteriocins (Table 3.4). The predicted bacteriocin cassettes identified for each of the *Lactococcus* clusters also seemed to be linked to the pangenomic and 16S rRNA clusters as illustrated in Table 3.4. The diversity of cassette types as well as cassette molecular weights indicated that there is a probability that the αGV isolates' active agents are different from each other. Although, having an identified cassette does not indicate that the cassette is active, however, since 96% of isolates contain a cassette this might suggest that the

cassettes might have been conserved functionally. Therefore, the active agents' natures needed to be studied. In addition, the αGV bacteria producing the active agent required further characterization for the specificity and sensitivity against *G. vaginalis*.

4.5 Functional diversity of antiGV isolates against GV collection

The functional diversity of the αGV collection was demonstrated via the high throughput target range assay against the *G. vaginalis* isolates (Figure 3.17). It was noted that not only the diameter of the zones of inhibition and clearance differed among αGV isolates against an individual *G. vaginalis* isolate, but also different *G. vaginalis* isolates showed different sensitivities to a given antiGV isolate. The overall sensitivity of *G. vaginalis* isolates varied from 32% to 75% of αGV isolates (Figure 3.19). Since more than half of the αGV isolates were capable of killing at least 50% of the *G. vaginalis* isolates and only 17.6% affected more than 40% of non-*G. vaginalis* bacteria, we concluded that the αGV isolates had a narrow spectrum of effect with a relative specificity biased for the *G. vaginalis* isolates (Figure 3.18). These observations stress the correct design and effectivness of our screeenig method. Due to the limited number of the *G. vaginalis* isolates the high sensitivity ranges are promising since there are multiple effective αGV isolates with overlapping target ranges that can be included in a minimal cocktail and tested against extended collection of GV isolated in the future. Thus expanding both collections is necessary to further refine the future cocktails to target a globally representative GV isolates. Additionally, sensitivity of the non-GV bacteria to the αGV isolates was biased toward bacteria that are not part of the vaginal microflora indicating that the

lactobacillus species that were used as surrogate representatives of vaginal lactobacilli were the least affected by antiGV bacteria (0 to <30% of entire antiGV collection). This observation is promising from the futuristic applied point of view indicating that it is less likely that the antiGV isolates or their active agents might damage the vaginal microflora. To fully define the target range and sensitivity of vaginal bacteria to the αGV collection, continued research would need to be completed utilizing commensal bacteria specific to the vaginal natural microflora in the high throughput target range assay.

Overall, we concluded that the genetic diversity observed among antiGV isolates was also translated to their functional antimicrobial activities against GV isolates.

4.6 Antimicrobial agents in the supernatants of antiGV isolates

As mentioned earlier, members of a limited number of *Bacillus sp*. isolates, and many members of *Lactococcus sp.* and *Enterococcus sp.* isolates were found to carry cassettes coding for antimicrobial peptides collectively named bacteriocins, which are considered promising antimicrobials (van Belkum *et al.*, 2011; Dischinger *et al.*, 2014; Perez *et al.*, 2014; Etayash *et al.*, 2015). Bacteriocins are ribosomally synthesized and some are post translationally modified and exported via cell membrane to the extracellular environment where they deliver their antimicrobial functions. They are part of the arms-race phenomenon among bacteria to gain advantage against competitors in accessing limited resources and colonization surfaces. By virtue of their modes of action, which most often destabilize the integrity of the cell membranes of their

targeted microorganisms, they can be considered a new generation of antimicrobials. Therefore, searching for and studying the nature of such antimicrobial agents (found in the extracellular environment) was a logical next step to the projects goal of discovering alternative antimicrobials against *G. vaginalis* with the potential to be utilized in the furture as a bacterial vaginosis therapeutic treatment.

Our analysis of the genomes of antiGV isolates pointed to the existence of multiple bacteriocin cassettes of different types among 27 out of 28 lactococcal isolates together with the previously identified α523 isolate (Table 3.4). Concentrating the supernatants from these 29 isolates produced 20 preparations with activity against GV isolates tested by drop on the lawn experiments.

The lack of activity among 9 supernatants might be due to the inefficiency of the methods applied for concentrating the agents or inactivation of the active agents during extraction. It is necessary to repeat this procedure using an alternative method of extraction such as the salting in method using ammonium sulfate. It might also be that the activity of the bacteriocin is not compatible with extraction methods used here. If the active agent is not a peptide, then none of these methods would concentrate the agent in question. For example, overproduction of acids and hydrogen peroxide can exert antimicrobial activity and can be diluted out during the extraction process. Therefore, our attention will be focused on these isolates in the future.

As previously described in section 3.3.2, the target ranges of the 20 concentrated supernatants were 5% to 100% with 12 of the αGV isolates with target ranges of at least 45% against the *G.*

vaginalis isolates (Figure 3.23). Interestingly, the target ranges of the αGV isolates in the high throughput target range assay, differed for some isolates compared to the extracted and concentrated active agents. In some cases, there was a decline in target range by using supernatants compared to inoculating the cells on the lawns. This is possibly due to a limited amount of active agent excreted to the supernatant environment as opposed to continuous export of the agent by the growing antiGV bacteria growing on the indicator lawn. Opposing results also occurred with increased target ranges in the extracted supernatant compared to the active bacterial growth on the indicator lawn. The αGV isolate with the greatest increase in *G. vaginalis* specific target range was αGV19-5. This αGV isolates' target range increased by 76.5%, being able to inhibit growth of all *G. vaginalis* isolates within the collection. The reasoning for such a dramatic alteration in target range could be due to the growth rate of αGV19-5. As a slow grower, the bacteria may not have had enough time to produce a significant concentration of its active agent to effectively produce clear inhibition zone on a lawn of *G. vaginalis*. In contrast, since the active agents from the supernatants were concentrated by 57 times the active agent concentration would have greatly increased and therefore bacterial cell growth would not be the limiting factor anymore.

The methods applied to concentrate the active agents from antiGV supernatants are standard methods of choice applied to bacteriocins and other antimicrobials that are short peptides or polypeptides. To further verify the peptide nature of the active agent in the supernatant concentrates, diffusion of the fractionated proteins and peptides from polyacrylamide gels into

the indicator bacterial lawn was used. We used this approach to test the supernatant extracts previously used in drop on lawn experiments. This approach is efficient in identifying the bands with antibacterial activities that can resist heat denaturation and resist the reducing agent used in these gels. Of note, class II circular bacteriocins and some class I lantibiotics typically fit this category and produce clearing zones upon diffusion to indicator bacterial lawn. Such molecules usually show slightly enhanced molecular rates on the gel due to retaining their folded structures and additionally due to the loss of stretch of amino acids at their N-terminus during circularization (van Belkum *et al.*, 2011). As detailed in section 3.3.3, several bands matching the predicted bacteriocin molecules were detected on gels. All active bands but αGV19-7, had active agents within 1.7 to 10kD marker bands. However, αGV19-7 showed clearance on the indicator lawn at around 35kD region, highlighting the ability of this method in identifying unpredicted antimicrobial proteins. While bacteriocidins can be peptides, they can also be higher molecular weight proteins. For example, a family of heat-resistant high molecular weight bacteriocins have been described (Šmarda and Benada, 2005; Yang *et al.*, 2014). There are also antibacterial enzymes, ie. enzybiotics, that target cell membranes or cell walls of bacteria that are large in size (Nelson *et al.*, 2001; Hojckova *et al.*, 2013; Maestro and Sanz, 2016). Thus, finding a large antibacterial protein, rather than being surprising, shows the power and efficiency behind the method applied for screening such antimicrobials.

Although, application of SDS-PAGE for direct screening of active agents seems efficient, it can fail to detect some polypeptides or short peptides if they lose their function under the reducing

and denaturing conditions that these gels operate. Therefore, for further testing it is recommended to use non-denaturing gels. By no means are the gel diffusion assays complete, and we intend to expand this approach to identify further antimicrobials.

4.7 Formulation of cocktails

Based on the αGV collection, its genomic diversity, and the overlapping complimentary properties specific to *G. vaginalis* for its isolates, we are suggesting two minimal cocktail formulations for further study against a more globally representative *G. vaginalis* collection. The first cocktail formulation would consist of exponentially growing bacterial cells: αGV19-1, αGV19-6, α-GV18-12S and αGV18-7aW. Isolates αGV19-1, αGV19-6 are part of the 'blue' pangenome matrix cluster, while α-GV18-12S and αGV18-7aW are part of the 'green' and 'red' clusters, respectively. The second proposed cocktail would consist of αGV purified and concentrated active agents. Suggested active agents to further characterize as potential minimal cocktail components include αGV18-11, αGV19-7, αGV19-5, αGV19-2S and α523. Each of the clusters identified in the pangenome matrix would be represented for this formulation. With this formulation a diverse combination of bacteriocin cassettes would be represented excluding only Lanthipeptide class I orf013. The active agent of α523 would also be a part of the cocktail as it is effective against all isolates in our *G. vaginalis* collection. Thus, the cocktail would be diverse in mechanistic nature and function and consequently be affective in eliminating even antibiotic resistant *G. vaginalis* growth in BV positive women (Turovskiy *et al.*, 2009).

4.8 Future work

As a pilot study, further experiments must be completed before highly defined and effective cocktail formulations can be established. Suggested future work for this project would be to first expand the *G. vaginalis* collection to be globally representative, and then to utilize an alternative supernatant extraction method for unidentified active agents such as ammonium sulfate precipitation. Additionally, not all the extractable αGV active agents were visualized via the SDS-PAGE gel diffusion assay. Possibly non-denaturing gels could be utilized to identify the protein bands responsible for the active agent. The active agent bands would also need to be cut from the gel and be sequenced to confirm their identities. Generation of knockout mutants of the identified bacteriocin cassettes for each αGV isolate followed by replications of the gel diffusion assay could be beneficial to making a direct link between the bacteriocin in question and its activity. Further toxicological studies and proper *in vivo* studies using animal models are among some of the important steps to be taken before a novel alternative treatment option could be introduced to clinic. Positive therapeutic outcomes are also subjects to successful human trials to examine the acceptable variations in responses of individual patients to the therapy.

5 Conclusion

Although our *G. vaginalis* collection is limited to 17 isolates, the isolates were shown to have diversity among their antibiotic susceptibilities and genotypes. Acknowledging the differing properties of these isolates allows us to better comprehend the difficulties in treatment of BV. Moreover, it is highly possible that the GV isolates would not be equally susceptible to a given

antimicrobial agent. Therefore, we searched for bacteria capable of producing diverse therapeutic agents. The αGV isolates were found to be diverse in their antimicrobial activities, their genetic construction, and the type of antibacterial agent they produce. The elucidation of these active agents is necessary to demonstrate that the active agents can be isolated and concentrated to potentially be used in a cocktail without the incorporation of non-commensal bacterium to the vaginal environment. However, we cannot say much about their differences in function and mechanism of action. The αGV collection was shown to be superior to metronidazole, as regardless of *G. vaginalis* antibiotic susceptibility profiles and rapid resistance acquisition, the αGV isolates worked successfully to inhibit *G. vaginalis* growth. The αGV isolates could potentially be used with low dose antibiotics, reducing antibiotic usage and exposure and thus potentially reducing the inclining resistance rates. We speculate that the use of multiple diverse αGV isolates in combination with antibiotics will have a potential to more effectively eliminate diverse *G. vaginalis* growth. As well, synergism among αGV isolates has yet to be determined, which could further eliminate BV at an advanced rate. The potential of these natural antimicrobial agents for the therapeutic treatment of *G. vaginalis* and therefore bacterial vaginosis is promising.

6 References

Abma, J.C., Chandra, A., Mosher, W.D., Peterson, L.S., and Piccinino, L.J. (1997) Fertility, family planning, and women's health: new data from the 1995 National Survey of Family Growth. National Center for Health Statistics. *Vital Health Statistics* **23**: 1–114.

Afolabi, B.B., Moses, O.E., and Oduyebo, O.O. (2016) Bacterial Vaginosis and Pregnancy Outcome in Lagos, Nigeria. *Open Forum Infect. Dis.* **3**: ofw030.

Akinbiyi, A.A., Watson, R., and Feyi-Waboso, P. (2008) Prevalence of Candida albicans and bacterial vaginosis in asymptomatic pregnant women in South Yorkshire, United Kingdom. *Arch. Gynecol. Obstet.* **278**: 463–466.

Algburi, A., Volski, A., and Chikindas, M.L. (2015) Natural antimicrobials subtilosin and lauramide arginine ethyl ester synergize with conventional antibiotics clindamycin and metronidazole against biofilms of Gardnerella vaginalis but not against biofilms of healthy vaginal lactobacilli. *Pathog. Dis.* **73**: ftv018.

Algburi, A., Zehm, S., Netrebov, V., Bren, A.B., Chistyakov, V., and Chikindas, M.L. (2017) Subtilosin Prevents Biofilm Formation by Inhibiting Bacterial Quorum Sensing. *Probiotics Antimicrob. Proteins* **9**: 81–90.

Algburi, A., Zhang, Y., Weeks, R., Comito, N., Zehm, S., Pinto, J., et al. (2017) Gemini Cationic Amphiphiles Control Biofilm Formation by Bacterial Vaginosis Pathogens. *Antimicrob. Agents Chemother.* **61**: e00650-17.

Alice, T.N.D., Kives, S., Merovitz, L., Nitsch, R., Tessler, K., and Yudin, M.H. (2012) Screening for bacterial vaginosis at the time of intrauterine contraceptive device insertion: is there a role? *J. Obstet. Gynaecol. Can.* **34**: 179–185.

Allsworth, J.E. and Peipert, J.F. (2011) Severity of Bacterial Vaginosis and the Risk of Sexually Transmitted Infection. *Am. J. Obstet. Gynecol.* **205**: 113.e1-113.e6.

Alnaif, B. and Drutz, H.P. (2000) Bacterial vaginosis increases in pessary users. *Int. Urogynecol. J. Pelvic Floor Dysfunct.* **11**: 219–222; discussion 222-223.

Alves, P., Castro, J., Sousa, C., Cereija, T.B., and Cerca, N. (2014) Gardnerella vaginalis Outcompetes 29 Other Bacterial Species Isolated From Patients With Bacterial Vaginosis, Using in an In Vitro Biofilm Formation Model. *J. Infect. Dis.* **210**: 593–596.

Amaya, R.A., Al-Dossary, F., and Demmler, G.J. (2002) Gardnerella Vaginalis Bacteremia in a Premature Neonate. *Publ. Online 07 Oct. 2002 Doi101038sjjp7210757* **22**: 585–587.

Andrade-Rocha, F.T. (2009) Colonization of Gardnerella vaginalis in semen of infertile men: prevalence, influence on sperm characteristics, relationship with leukocyte concentration and clinical significance. *Gynecol. Obstet. Invest.* **68**: 134–136.

Andrews, S. (2010) Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data.

Anorlu, R., Imosemi, D., Odunukwe, N., Abudu, O., and Otuonye, M. (2004) Prevalence of HIV among women with vaginal discharge in a gynecological clinic. *J. Natl. Med. Assoc.* **96**: 367.

Anukam, K.C., Osazuwa, E.O., Ahonkhai, I., and Reid, G. (2006) Lactobacillus vaginal microbiota of women attending a reproductive health care service in Benin city, Nigeria. *Sex. Transm. Dis.* **33**: 59–62.

Anukam, K.C. and Reid, G. (2008) Effects of metronidazole on growth of Gardnerella vaginalis ATCC 14018, probiotic Lactobacillus rhamnosus GR-1 and vaginal isolate Lactobacillus plantarum KCA. *Microb. Ecol. Health Dis.* **20**: 48–52.

Aroutcheva, A.A., Simoes, J.A., Behbakht, K., and Faro, S. (2001) Gardnerella vaginalis Isolated from Patients with Bacterial Vaginosis and from Patients with Healthy Vaginal Ecosystems. *Clin. Infect. Dis.* **33**: 1022–1027.

Austin, M.N. (2006) Susceptibility of vaginal bacteria to metronidazole and tinidazole. *Anaerobe* **12**: 227–230.

Bahram, A., Hamid, B., and Zohre, T. (2009) Prevalence of Bacterial Vaginosis and Impact of Genital Hygiene Practices in Non-Pregnant Women in Zanjan, Iran. *Oman Med. J.* **24**: 288–293.

Balashov, S.V., Mordechai, E., Adelson, M.E., and Gygax, S.E. (2014) Identification, quantification and subtyping of Gardnerella vaginalis in noncultured clinical vaginal samples by quantitative PCR. *J. Med. Microbiol.* **63**: 162–175.

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., et al. (2012) SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. *J. Comput. Biol.* **19**: 455–477.

Barcelos, M.R.B., Vargas, P.R.M. de, Baroni, C., and Miranda, A.E. (2008) [Genital infections in women attending a Primary Unit of Health: prevalence and risk behaviors]. *Rev. Bras. Ginecol. E Obstet. Rev. Fed. Bras. Soc. Ginecol. E Obstet.* **30**: 349–354.

Barkin, J.A., Sussman, D.A., Fifadara, N., and Barkin, J.S. (2017) Clostridium difficile Infection and Patient-Specific Antimicrobial Resistance Testing Reveals a High Metronidazole Resistance Rate. *Dig. Dis. Sci.* **62**: 1035–1042.

Bautista, C.T. (2017) Association of Bacterial Vaginosis With Chlamydia and Gonorrhea Among Women in the U.S. Army. *Am J Prev Med* **52**: 632–639.

Bautista, C.T., Wurapa, E., Sateren, W.B., Morris, S., Hollingsworth, B., and Sanchez, J.L. (2016) Bacterial vaginosis: a synthesis of the literature on etiology, prevalence, risk factors, and relationship with chlamydia and gonorrhea infections. *Mil. Med. Res.* **3**:.

van Belkum, M.J., Martin-Visscher, L.A., and Vederas, J.C. (2011) Structure and genetics of circular bacteriocins. *Trends Microbiol.* **19**: 411–418.

Benito, R., Vazquez, J.A., Berron, S., Fenoll, A., and Saez-Neito, J.A. (1986) A modified scheme for biotyping Gardnerella vaginalis. *J. Med. Microbiol.* **21**: 357–359.

Berardi-Grassias, L., Roy, O., Berardi, J.C., and Furioli, J. (1988) Neonatal meningitis due to Gardnerella vaginalis. *Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol.* **7**: 406–407.

Bilardi, J., Walker, S., Mooney-Somers, J., Temple-Smith, M., McNair, R., Bellhouse, C., et al. (2016) Women's Views and Experiences of the Triggers for Onset of Bacterial Vaginosis and Exacerbating Factors Associated with Recurrence. *PLOS ONE* **11**: e0150272.

Bilardi, J.E., Walker, S., Temple-Smith, M., McNair, R., Mooney-Somers, J., Bellhouse, C., et al. (2013) The Burden of Bacterial Vaginosis: Women's Experience of the Physical, Emotional, Sexual and Social Impact of Living with Recurrent Bacterial Vaginosis. *PLoS ONE* **8**: e74378.

Bilardi, J.E., Walker, S.M., Temple-Smith, M.J., McNair, R.P., Mooney-Somers, J., Vodstrcil, L.A., et al. (2017) Women view key sexual behaviours as the trigger for the onset and recurrence of bacterial vaginosis. *PLoS ONE* **12**: e0173637.

bioMérieux (2012a) Antimicrobial agents with bactericidal and bacteriostatic modes of action. E test Customer Information Sheet (CIS006- 16263 B-en- 2012/07) bioMérieux, Marcy-l'Étoile, France.

bioMérieux (2012b) Antimicrobial Susceptibility Testing. Etest Package Insert (9302553C-en-2012/01) bioMérieux, Marcy-l'Étoile, France.

bioMérieux (2011) Etest MIC end points for Glycopeptides. E test Customer Information Sheet (CIS002-16259 A- en-2010/11). bioMérieux, Marcy-l'Étoile, France.

bioMérieux (2012c) Reading sharp and hazy endpoints. Etest Customer Information Sheet (CIS 007; 16264 B-en-2012/07) bioMérieux, Marcy-l'Étoile, France.

bioMérieux (2014) Summary of EUCAST Interpretive Criteria, Relevant Epidemiological Cut-OFFs and Etest® Quality Control Strains and Ranges. (19625B - 2014/01). bioMérieux, Marcy-l'Étoile, France.

Bjørnerem, A., Aghajani, E., Maltau, J.M., and Moi, H. (1997) [Occurrence of bacterial vaginosis among abortion seekers]. *Tidsskr. Den Nor. Laegeforening Tidsskr. Prakt. Med. Ny Raekke* **117**: 1282–1284.

Blankhart, D., Müller, O., Gresenguet, G., and Weis, P. (1999) Sexually transmitted infections in young pregnant women in Bangui, Central African Republic. *Int. J. STD AIDS* **10**: 609–614.

Boustouller, Y.L., Johnson, A.P., and Taylor-Robinson, D.T. (1986) Detection of a species-specific antigen of Gardnerella vaginalis by Western blot analysis. *J. Gen. Microbiol.* **132**: 1969–1973.

Bradshaw, C.S., Walker, J., Fairley, C.K., Chen, M.Y., Tabrizi, S.N., Donovan, B., et al. (2013) Prevalent and Incident Bacterial Vaginosis Are Associated with Sexual and Contraceptive Behaviours in Young Australian Women. *PLoS ONE* **8**: e57688.

Bradshaw, C.S., Walker, S.M., Vodstrcil, L.A., Bilardi, J.E., Law, M., Hocking, J.S., et al. (2014) The influence of behaviors and relationships on the vaginal microbiota of women and their female partners: the WOW Health Study. *J. Infect. Dis.* **209**: 1562–1572.

Braga, P.C., Dal Sasso, M., Culici, M., and Spallino, A. (2010) Inhibitory activity of thymol on native and mature Gardnerella vaginalis biofilms: in vitro study. *Arzneimittelforschung* **60**: 675–681.

Breshears, L.M., Edwards, V.L., Ravel, J., and Peterson, M.L. (2015) Lactobacillus crispatus inhibits growth of Gardnerella vaginalis and Neisseria gonorrhoeae on a porcine vaginal mucosa model. *BMC Microbiol.* **15**: 276–287.

Bretelle, F. (2015) High Atopobium vaginae and Gardnerella vaginalis Vaginal Loads Are Associated With Preterm Birth. *Clin. Infect. Dis.* **60**: 860–867.

Briselden, A.M. and Hillier, S.L. (1990) Longitudinal study of the biotypes of Gardnerella vaginalis. *J. Clin. Microbiol.* **28**: 2761–2764.

Brooks-Smith-Lowe, K. and Rodrigo, S. (2013) Prevalencia de la vaginosis bacteriana en mujeres granadinas en edad reproductiva. *West Indian Med. J.* **62**: 599–603.

Brotman, R.M., Klebanoff, M.A., Nansel, T.R., Yu, K.F., Andrews, W.W., Zhang, J., and Schwebke, J.R. (2010) Bacterial Vaginosis Assessed by Gram Stain and Diminished Colonization Resistance to Incident Gonococcal, Chlamydial, and Trichomonal Genital Infection. *J. Infect. Dis.* **202**: 1907–1915.

Budvytyte, R., Pleckaityte, M., Zvirbliene, A., Vanderah, D.J., and Valincius, G. (2013) Reconstitution of Cholesterol-Dependent Vaginolysin into Tethered Phospholipid Bilayers: Implications for Bioanalysis. *PLoS ONE* **8**: e82536.

Bushnell, B. BBMap. *sourceforge.net/projects/bbmap/*.

Caiyan, X., Weiyuan, Z., Minghui, W., and Songwen, Z. (2012) Prevalence and risk factors of lower genital tract infections among women in Beijing, China. *J. Obstet. Gynaecol. Res.* **38**: 310–315.

Calvert, L.D., Collins, M., and Bateman, J.R.M. (2005) Multiple abscesses caused by Gardnerella vaginalis in an immunocompetent man. *J. Infect.* **51**: E27-29.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden, T.L. (2009) BLAST+: architecture and applications. *BMC Bioinformatics* **10**: 421.

Carroll, C.C., Warnakulasuriyarachchi, D., Nokhbeh, M.R., and Lambert, I.B. (2002) Salmonella typhimurium mutagenicity tester strains that overexpress oxygen-insensitive nitroreductases nfsA and nfsB. *Mutat. Res.* **501**: 79–98.

Castro, E., Dominguez, M., Navarrete, P., Boggiano, G., and Zemelman, R. (1999) Prevalence of Bacterial Vaginosis in Women Attending Family Planning Clinics. *Anaerobe* **5**: 399–401.

Castro, J., Alves, P., Sousa, C., Cereija, T., França, Â., Jefferson, K.K., and Cerca, N. (2015) Using an in-vitro biofilm model to assess the virulence potential of Bacterial Vaginosis or non-Bacterial Vaginosis Gardnerella vaginalis isolates. *Sci. Rep.* **5**: 11640.

Castro, J., França, A., Bradwell, K.R., Serrano, M.G., Jefferson, K.K., and Cerca, N. (2017) Comparative transcriptomic analysis of Gardnerella vaginalis biofilms vs. planktonic cultures using RNA-seq. *Npj Biofilms Microbiomes* **3**: 3.

Castro, J., Henriques, A., Machado, A., Henriques, M., Jefferson, K.K., and Cerca, N. (2013) Reciprocal Interference between Lactobacillus spp. and Gardnerella vaginalis on Initial Adherence to Epithelial Cells. *Int. J. Med. Sci.* **10**: 1193.

Cauci, S. and Culhane, J.F. (2011) High sialidase levels increase preterm birth risk among women who are bacterial vaginosis–positive in early gestation. *Am. J. Obstet. Gynecol.* **204**: 142.e1-142.e9.

Cauci, S., Culhane, J.F., Di Santolo, M., and McCollum, K. (2008) Among pregnant women with bacterial vaginosis, the hydrolytic enzymes sialidase and prolidase are positively associated with interleukin-1β. *Am. J. Obstet. Gynecol.* **198**: 132.e1-132.e7.

Cauci, S., Guaschino, S., Driussi, S., Santo, D.D., Lanzafame, P., and Quadrifoglio, F. (2002) Correlation of Local Interleukin-8 with Immunoglobulin a against Gardnerella vaginalis Hemolysin and with Prolidase and Sialidase Levels in Women with Bacterial Vaginosis. *J. Infect. Dis.* **185**: 1614–1620.

Cauci, S., Hitti, J., Noonan, C., Agnew, K., Quadrifoglio, F., Hillier, S.L., and Eschenbach, D.A. (2002) Vaginal hydrolytic enzymes, immunoglobulin A against Gardnerella vaginalis toxin, and risk of early preterm birth among women in preterm labor with bacterial vaginosis or intermediate flora. *Am. J. Obstet. Gynecol.* **187**: 877–881.

Cauci, S., Monte, R., Driussi, S., Lanzafame, P., and Quadrifoglio, F. (1998) Impairment of the Mucosal Immune System: IgA and IgM Cleavage Detected in Vaginal Washings of a Subgroup of Patients with Bacterial Vaginosis. *J. Infect. Dis.* **178**: 1698–1706.

Cavera, V.L., Volski, A., and Chikindas, M.L. (2015) The Natural Antimicrobial Subtilosin A Synergizes with Lauramide Arginine Ethyl Ester (LAE), ε-Poly-l-lysine (Polylysine), Clindamycin Phosphate and Metronidazole, Against the Vaginal Pathogen Gardnerella vaginalis. *Probiotics Antimicrob. Proteins* **7**: 164–171.

Chaban, B., Links, M.G., Jayaprakash, T.P., Wagner, E.C., Bourque, D.K., Lohn, Z., et al. (2014) Characterization of the vaginal microbiota of healthy Canadian women through the menstrual cycle. *Microbiome* **2**: 23–23.

Chaudhary (2014) EMERGING METRONIDAZOLE RESISTANCE IN ANAEROBES AND MAPPING THEIR SUSCEPTIBILITY BEHAVIOUR. *Am. J. Infect. Dis.* **10**: 56–63.

Cherpes, T.L., Meyn, L.A., Krohn, M.A., Lurie, J.G., and Hillier, S.L. (2003) Association between Acquisition of Herpes Simplex Virus Type 2 in Women and Bacterial Vaginosis. *Clin. Infect. Dis.* **37**: 319–325.

Clinical and Laboratory Standards Institute (2004) Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria. Approved-Standard-Sixth Edition. CLSI document M100-A6. Clinical and Laboratory Standards Institute, Mayne, PA.

Clinical and Laboratory Standards Institute (2015) Performance Standards for Antimicrobial Testing. Twenty-Fifth Informational Supplement. CLSI document M100-s25 (ISBEN 1-56238-989-0). Clinical and Laboratory Standards Institute, 950 West Valey Road, Suite 2500, Wayne, Pennsylvania 19087 USA.

Cohen, C.R., Lingappa, J.R., Baeten, J.M., Ngayo, M.O., Spiegel, C.A., Hong, T., et al. (2012) Bacterial Vaginosis Associated with Increased Risk of Female-to-Male HIV-1 Transmission: A Prospective Cohort Analysis among African Couples. *PLoS Med.* **9**: e1001251.

Cottrell, B.H. (2006) Vaginal Douching Practices of Women in Eight Florida Panhandle Counties. *J. Obstet. Gynecol. Neonatal Nurs.* **35**: 24–33.

Cox, C., Watt, A., McKenna, J., and Coyle, P. (2016) Mycoplasma hominis and Gardnerella vaginalis display a significant synergistic relationship in bacterial vaginosis. *Eur. J. Clin. Microbiol. Infect. Dis.* **35**: 481–487.

Cristiano, L., Rampello, S., Noris, C., and Valota, V. (1996) Bacterial Vaginosis: Prevalence in an Italian Population of Asymptomatic Pregnant Women and Diagnostic Aspects. *Eur. J. Epidemiol.* **12**: 383–390.

Criswell, B.S., Marston, J.H., Stenback, W.A., Black, S.H., and Gardner, H.L. (1971) Haemophilus vaginalis 594, a gram-negative organism? *Can. J. Microbiol.* **17**: 865–869.

Criswell, B.S., Stenback, W.A., Black, S.H., and Gardner, H.L. (1972) Fine structure of Haemophilus vaginalis. *J. Bacteriol.* **109**: 930–932.

Crowley, T., Low, N., Turner, A., Harvey, I., Bidgood, K., and Horner, P. (2001) Antibiotic prophylaxis to prevent post-abortal upper genital tract infection in women with bacterial vaginosis: randomised controlled trial. *Br. J. Obstet. Gynaecol.* **108**: 396–402.

Culhane, J.F., Rauh, V., McCollum, K.F., Elo, I.T., and Hogan, V. (2002) Exposure to chronic stress and ethnic differences in rates of bacterial vaginosis among pregnant women. *Am. J. Obstet. Gynecol.* **187**: 1272–1276.

Dadhwal, V., Hariprasad, R., Mittal, S., and Kapil, A. (2010) Prevalence of bacterial vaginosis in pregnant women and predictive value of clinical diagnosis. *Arch. Gynecol. Obstet.* **281**: 101–104.

Dai, Q., Hu, L., Jiang, Y., Shi, H., Liu, J., Zhou, W., et al. (2010) An epidemiological survey of bacterial vaginosis, vulvovaginal candidiasis and trichomoniasis in the Tibetan area of Sichuan Province, China. *Eur. J. Obstet. Gynecol.* **150**: 207–209.

Daniele, M., Pascual, L., and Barberis, L. (2014) Curative effect of the probiotic strain Lactobacillus fermentum L23 in a murine model of vaginal infection by Gardnerella vaginalis. *Lett. Appl. Microbiol.* **59**: 93–98.

Darwish, A.M., Makarem, M.H., Alnashar, E.M., and Hamadeh, S.M. (2005) Screening for bacterial vaginosis in high-risk pregnancy: the experience of a developing country. *Acta Obstet. Gynecol. Scand.* **84**: 483–485.

De Backer, E., Verhelst, R., Verstraelen, H., Claeys, G., Verschraegen, G., Temmerman, M., and Vaneechoutte, M. (2006) Antibiotic susceptibility of Atopobium vaginae. *BMC Infect. Dis.* **6**: 51.

De Lima Soares, V., De Mesquita, A.M.T.S., Cavalcante, F.G.T., Silva, Z.P., Hora, V., Diedrich, T., et al. (2003) Sexually transmitted infections in a female population in rural north-east Brazil: prevalence, morbidity and risk factors. *Trop. Med. Int. Health* **8**: 595–603.

De Meo, M., Vanelle, P., Bernadini, E., Laget, M., Maldonado, J., Jentzer, O., et al. (1992) Evaluation of the mutagenic and genotoxic activities of 48 nitroimidazoles and related imidazole derivatives by the Ames test and the SOS chromotest. *Environ. Mol. Mutagen.* **19**: 167–181.

Decena, D.C.D., Co, J.T., Manalastas, R.M., Palaypayon, E.P., Padolina, C.S., Sison, J.M., et al. (2006) Metronidazole with Lactacyd vaginal gel in bacterial vaginosis. *J. Obstet. Gynaecol. Res.* **32**: 243–251.

Denslow, S.A., Westreich, D.J., Firnhaber, C., Michelow, P., Williams, S., and Smith, J.S. (2011) Bacterial vaginosis as a risk factor for high-grade cervical lesions and cancer in HIV-seropositive women. *Int. J. Gynecol. Obstet.* **114**: 273–277.

Desseauve, D., Chantrel, J., Fruchart, A., Khoshnood, B., Brabant, G., Ancel, P.Y., and Subtil, D. (2012) Prevalence and risk factors of bacterial vaginosis during the first trimester of pregnancy in a large French population-based study. *Eur. J. Obstet. Gynecol.* **163**: 30–34.

Dingsdag, S.A. and Hunter, N. (2018) Metronidazole: an update on metabolism, structure-cytotoxicity and resistance mechanisms. *J. Antimicrob. Chemother.* **73**: 265–279.

Dischinger, J., Basi Chipalu, S., and Bierbaum, G. (2014) Lantibiotics: promising candidates for future applications in health care. *Int. J. Med. Microbiol. IJMM* **304**: 51–62.

Dols, J.A.M., Smit, P.W., Kort, R., Reid, G., Schuren, F.H.J., Tempelman, H., et al. (2011) Microarray-based identification of clinically relevant vaginal bacteria in relation to bacterial vaginosis. *Am. J. Obstet. Gynecol.* **204**: 305.e1–7.

Donders, G.G., Van Bulck, B., Caudron, J., Londers, L., Vereecken, A., and Spitz, B. (2000) Relationship of bacterial vaginosis and mycoplasmas to the risk of spontaneous abortion. *Am. J. Obstet. Gynecol.* **183**: 431–437.

Doyle, C. (2015) Sexually Transmitted Pathogens, Depression, and Other Manifestations Associated with Premenstrual Syndrome. *Hum. Nat.* **26**: 277–291.

Dukes, C.D. and Gardner, H.L. (1961) IDENTIFICATION OF HAEMOPHILUS VAGINALIS. *J. Bacteriol.* **81**: 277.

Dunkelberg, W.E. and McVeigh, I. (1969) Growth requirements of Haemophilus vaginalis. *Antonie Van Leeuwenhoek.* **35**: 129–145.

Dunstone, M.A. and Tweten, R.K. (2012) Packing a punch: the mechanism of pore formation by cholesterol dependent cytolysins and membrane attack complex/perforin-like proteins. *Curr. Opin. Struct. Biol.* **22**: 342–349.

Durugbo, I.I., Nyengidiki, T.K., Bassey, G., and Wariso, K.T. (2015) Bacterial vaginosis among women with tubal factor infertility in Nigeria. *Int. J. Gynecol. Amp Obstet.* **131**: 133–136.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**: 1792–1797.

Edmunds, P.N. (1962) The biochemical, serological and hæmag-glutinating reactions of "Hæmophilus vaginalis." *J. Pathol. Bacteriol.* **83**: 411–422.

Edmunds, P.N. (1960) The growth requirements of Haemophilus vaginalis. *J Pathol Bacteriol* **80**: 325–335.

El Aila, N.A., Tency, I., Claeys, G., Verstraelen, H., Saerens, B., Lopes dos Santos Santiago, G., et al. (2009) Identification and genotyping of bacteria from paired vaginal and rectal samples from pregnant women indicates similarity between vaginal and rectal microflora. *BMC Infect. Dis.* **9**: 167.

Eren, A.M., Borisy, G.G., Huse, S.M., and Welch, J.L.M. (2014) Oligotyping analysis of the human oral microbiome. *Proc. Natl. Acad. Sci.* **111**: E2875–E2884.

Eren, A.M., Maignien, L., Sul, W.J., Murphy, L.G., Grim, S.L., Morrison, H.G., and Sogin, M.L. (2013) Oligotyping: differentiating between closely related microbial taxa using 16S rRNA gene data. *Methods Ecol. Evol.* **4**: 1111–1119.

Eren, A.M., Zozaya, M., Taylor, C.M., Dowd, S.E., Martin, D.H., and Ferris, M.J. (2011) Exploring the diversity of Gardnerella vaginalis in the genitourinary tract microbiota of monogamous couples through subtle nucleotide variation. *PLoS ONE* **6**: e26732.

Eriksson, K., Adolfsson, A., Forsum, U., and Larsson, P.-G. (2010) The prevalence of BV in the population on the Åland Islands during a 15- year period. *APMIS* **118**: 903–908.

Etayash, H., Azmi, S., Dangeti, R., and Kaur, K. (2015) Peptide Bacteriocins--Structure Activity Relationships. *Curr. Top. Med. Chem.* **16**: 220–241.

EUCAST (2016) The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0. http://www.eucast.org. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 6.0. http://www.eucast.org.

Fang, X., Zhou, Y., Yang, Y., Diao, Y., and Li, H. (2007) Prevalence and risk factors of trichomoniasis, bacterial vaginosis, and candidiasis for married women of child-bearing age in rural Shandong. *Jpn. J. Infect. Dis.* **60**: 257–261.

Fastring, D.R., Amedee, A., Gatski, M., Clark, R.A., Mena, L.A., Levison, J., et al. (2014) Co-occurrence of Trichomonas vaginalis and Bacterial Vaginosis and Vaginal Shedding of HIV-1 RNA: *Sex. Transm. Dis.* **41**: 173–179.

Fethers, K., Marks, C., Mindel, A., and Estcourt, C. (2000) Sexually transmitted infections and risk behaviours in women who have sex with women. *Sex. Transm. Infect.* **76**: 345–349.

Fethers, K.A., Fairley, C.K., Hocking, J.S., Gurrin, L.C., and Bradshaw, C.S. (2008) Sexual risk factors and bacterial vaginosis: a systematic review and meta-analysis. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **47**: 1426–1435.

Fethers, K.A., Fairley, C.K., Morton, A., Hocking, J.S., Hopkins, C., Kennedy, L.J., et al. (2009) Early Sexual Experiences and Risk Factors for Bacterial Vaginosis. *J. Infect. Dis.* **200**: 1662–1670.

Forcey, D.S., Walker, S.M., Vodstrcil, L.A., Fairley, C.K., Bilardi, J.E., Law, M., et al. (2014) Factors Associated with Participation and Attrition in a Longitudinal Study of Bacterial Vaginosis in Australian Women Who Have Sex with Women. *PLOS ONE* **9**: e113452.

Fosch, S., Fogolín, N., Azzaroni, E., Pairetti, N., Dana, L., Minacori, H., et al. (2006) [Vulvovaginitis: correlation with predisposing factors, clinical manifestations and microbiological studies]. *Rev. Argent. Microbiol.* **38**: 202–205.

Frohlich, J.A., Abdool Karim, Q., Mashego, M.M., Sturm, A.W., and Abdool Karim, S.S. (2007) Opportunities for treating sexually transmitted infections and reducing HIV risk in rural South Africa. *J. Adv. Nurs.* **60**: 377–383.

Gallo, M.F., Macaluso, M., Warner, L., Fleenor, M.E., Hook, E.W., Brill, I., and Weaver, M.A. (2012) Bacterial Vaginosis, Gonorrhea, and Chlamydial Infection Among Women Attending a Sexually Transmitted Disease Clinic: A Longitudinal Analysis of Possible Causal Links. *Ann. Epidemiol.* **22**: 213–220.

García, P.J., Chavez, S., Feringa, B., Chiappe, M., Li, W., Jansen, K.U., et al. (2004) Reproductive tract infections in rural women from the highlands, jungle, and coastal regions of Peru. *Bull. World Health Organ.* **82**: 483–492.

Gardner, H.L. and Dukes, C.D. (1954) New etiologic agent in nonspecific bacterial vaginitis. *Science.* **120**: 853.

Gelber, S.E., Aguilar, J.L., Lewis, K.L.T., and Ratner, A.J. (2008) Functional and Phylogenetic Characterization of Vaginolysin, the Human-Specific Cytolysin from Gardnerella vaginalis. *J. Bacteriol.* **190**: 3896–3903.

Ghiasi, M., Fazaeli, H., Kalhor, N., Sheykh-Hasan, M., and Tabatabaei-Qomi, R. (2014) Assessing the prevalence of bacterial vaginosis among infertile women of Qom city. *Iran. J. Microbiol.* **6**: 404–408.

Gilbert, N.M., Lewis, W.G., and Lewis, A.L. (2013) Clinical Features of Bacterial Vaginosis in a Murine Model of Vaginal Infection with Gardnerella vaginalis. *PLoS ONE* **8**: e59539.

Gillet, E., Meys, J.F., Verstraelen, H., Bosire, C., De Sutter, P., Temmerman, M., and Broeck, D.V. (2011) Bacterial vaginosis is associated with uterine cervical human papillomavirus infection: a meta-analysis. *BMC Infect. Dis.* **11**: 10.

Gillet, E., Meys, J.F.A., Verstraelen, H., Verhelst, R., Sutter, P.D., Temmerman, M., and Broeck, D.V. (2012) Association between Bacterial Vaginosis and Cervical Intraepithelial Neoplasia: Systematic Review and Meta-Analysis. *PLOS ONE* **7**: e45201.

Go, V.F., Quan, V.M., Celentano, D.D., Moulton, L.H., and Zenilman, J.M. (2006) Prevalence and risk factors for reproductive tract infections among women in rural Vietnam. *Southeast Asian J. Trop. Med. Public Health* **37**: 185–189.

Goldenberg, R.L., Klebanoff, M.A., Nugent, R., Krohn, M.A., Hillier, S., and Andrews, W.W. (1996) Bacterial colonization of the vagina during pregnancy in four ethnic groups. Vaginal Infections and Prematurity Study Group. *Am. J. Obstet. Gynecol.* **174**: 1618–1621.

Goldstein, E.J.C., Citron, D.M., Merriam, C.V., Warren, Y.A., Tyrrell, K.L., and Fernandez, H.T. (2002) In Vitro Activities of Garenoxacin (BMS 284756) against 108 Clinical Isolates of Gardnerella vaginalis. *Antimicrob. Agents Chemother.* **46**: 3995–3996.

Gondo, F., da Silva, M.G., Polettini, J., Tristao, A.R., Peracoli, J.C., Witkin, S.S., and Rudge, M.V.C. (2010) Vaginal Flora Alterations and Clinical Symptoms in Low-Risk Pregnant Women. *Gynecol. Obstet. Invest.* **71**: 158–162.

Goto, A., Nguyen, Q.V., Pham, N.M., Kato, K., Cao, T.P.N., Le, T.H.C., et al. (2005) Prevalence of and Factors Associated with Reproductive Tract Infections among Pregnant Women in Ten Communes in Nghe An Province, Vietnam. *J. Epidemiol.* **15**: 163–172.

Gottschick, C., Deng, Z.-L., Vital, M., Masur, C., Abels, C., Pieper, D.H., et al. (2017) Treatment of biofilms in bacterial vaginosis by an amphoteric tenside pessary-clinical study and microbiota analysis. *Microbiome* **5**: 119.

Gottschick, C., Szafranski, S.P., Kunze, B., Sztajer, H., Masur, C., Abels, C., and Wagner-Döbler, I. (2016) Screening of Compounds against Gardnerella vaginalis Biofilms. *PLoS One* **11**: e0154086.

Gouy, M., Guindon, S., and Gascuel, O. (2010) SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* **27**: 221–224.

Govender, L., Hoosen, A.A., Moodley, J., Moodley, P., and Sturm, A.W. (1996) Bacterial vaginosis and associated infections in pregnancy. *Int. J. Gynaecol. Obstet. Off. Organ Int. Fed. Gynaecol. Obstet.* **55**: 23–28.

Graham, S., Howes, C., Dunsmuir, R., and Sandoe, J. (2009) Vertebral osteomyelitis and discitis due to Gardnerella vaginalis. *J. Med. Microbiol.* **58**: 1382–1384.

Gratacós, E., Figueras, F., Barranco, M., Ros, R., Andreu, A., Alonso, P.L., and Cararach, V. (1999) Prevalence of bacterial vaginosis and correlation of clinical to Gram stain diagnostic criteria in low risk pregnant women. *Eur. J. Epidemiol.* **15**: 913–916.

Gray, R.H., Kigozi, G., Serwadda, D., Makumbi, F., Nalugoda, F., Watya, S., et al. (2009) The effects of male circumcision on female partner's genital tract symptoms and vaginal infections in a randomized trial in Rakai, Uganda. *Am. J. Obstet. Gynecol.* **200**: 42.e1.

Greenwood, J.R. and Pickett, M.J. (1980) Transfer of Haemophilus vaginalis Gardner and Dukes to a New Genus, Gardnerella: G. vaginalis (Gardner and Dukes) comb. nov. *Int. J. Syst. Evol. Microbiol.* **30**: 170–178.

Guerreiro, D., Gigante, M.A., and Teles, L.C. (1998) Sexually transmitted diseases and reproductive tract infections among contraceptive users. *Int. J. Gynaecol. Obstet. Off. Organ Int. Fed. Gynaecol. Obstet.* **63 Suppl 1**: S167-173.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* **59**: 307–321.

Guo, Y., You, K., Qiao, J., Zhao, Y., and Geng, L. (2012) Bacterial vaginosis is conducive to the persistence of HPV infection. *Int. J. STD AIDS* **23**: 581–584.

Gupta, A., Garg, P., and Nigam, S. (2013) Bacterial Vaginosis in Pregnancy (<28 Weeks) and its Effect on Pregnancy Outcome: A Study from a Western UP City. *Obstet Gynecol* **23**: 740–744.

Haggerty, C.L., Hillier, S.L., Bass, D.C., and Ness, R.B. (2004) Bacterial Vaginosis and Anaerobic Bacteria Are Associated with Endometritis. *Clin. Infect. Dis.* **39**: 990–995.

Hardy, L., Jespers, V., Abdellati, S., De Baetselier, I., Mwambarangwe, L., Musengamana, V., et al. (2016) A fruitful alliance: the synergy between Atopobium vaginae and Gardnerella vaginalis in bacterial vaginosis-associated biofilm. *Sex. Transm. Infect.* **92**: 487–491.

Hardy, L., Jespers, V., Bulck, M.V. den, Buyze, J., Mwambarangwe, L., Musengamana, V., et al. (2017) The presence of the putative Gardnerella vaginalis sialidase A gene in vaginal specimens is associated with bacterial vaginosis biofilm. *PLOS ONE* **12**: e0172522.

Hardy, L., Jespers, V., Dahchour, N., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M., and Crucitti, T. (2015) Unravelling the Bacterial Vaginosis-Associated Biofilm: A Multiplex Gardnerella vaginalis and Atopobium vaginae Fluorescence In Situ Hybridization Assay Using Peptide Nucleic Acid Probes. *PLoS ONE* **10**: e0136658.

Harikarnpukdee, D., Sirimai, K., Kiriwat, O., and Boriboonhirunsarn, D. (2004) Prevalence of bacterial vaginosis in Thai women at.pdf. *J Med Assoc Thai* **87**: S8-11.

Harper, J.J. and Davis, G.H.G. (1982) Cell wall analysis of Gardnerella vaginalis (Haemophilus vaginalis). *Int. J. Syst. Evol. Microbiol.* **32**: 48–50.

Harwich, M.D., Alves, J.M., Buck, G.A., Strauss, J.F., Patterson, J.L., Oki, A.T., et al. (2010) Drawing the line between commensal and pathogenic Gardnerella vaginalis through genome analysis and virulence studies. *BMC Genomics* **11**: 375.

Hay, P.E., Lamont, R.F., Taylor-Robinson, D., Morgan, D.J., Ison, C., and Pearson, J. (1994) Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. *BMJ* **308**: 295–298.

van Heel, A.J., de Jong, A., Montalbán-López, M., Kok, J., and Kuipers, O.P. (2013) BAGEL3: Automated identification of genes encoding bacteriocins and (non-)bactericidal posttranslationally modified peptides. *Nucleic Acids Res.* **41**: W448-453.

Hickey, R.J., Zhou, X., Settles, M.L., Erb, J., Malone, K., Hansmann, M.A., et al. (2015) Vaginal Microbiota of Adolescent Girls Prior to the Onset of Menarche Resemble Those of Reproductive-Age Women. *mBio* **6**: e00097-15.

Hoffmann, J.N., You, H.M., Hedberg, E.C., Jordan, J.A., and McClintock, M.K. (2014) Prevalence of Bacterial Vaginosis and Candida among Postmenopausal Women in the United States. *J. Gerontol. B. Psychol. Sci. Soc. Sci.* **69**: S205–S214.

Hojckova, K., Stano, M., and Klucar, L. (2013) phiBIOTICS: catalogue of therapeutic enzybiotics, relevant research studies and practical applications. *BMC Microbiol.* **13**: 53–53.

Hooven, T.A. (2012) Retrocyclin inhibits Gardnerella vaginalis biofilm formation and toxin activity. *J. Antimicrob. Chemother.* **67**: 2870–2872.

Hotze, E.M. and Tweten, R.K. (2012) Membrane assembly of the cholesterol-dependent cytolysin pore complex. *Biochim. Biophys. Acta BBA - Biomembr.* **1818**: 1028–1038.

Hu, Y., Zhu, Y., and Lu, N.-H. (2017) Primary Antibiotic Resistance of Helicobacter pylori in China. *Dig. Dis. Sci.* **62**: 1146–1154.

Hukić, M., Seljmo, D., Ramovic, A., Ibrišimović, M.A., Dogan, S., Hukic, J., and Feric Bojic, E. (2017) The Effect of Lysozyme on Reducing Biofilms by Staphylococcus aureus, Pseudomonas aeruginosa, and Gardnerella vaginalis: An In Vitro Examination. *Microb. Drug Resist. Larchmt. N.*

Hymes, S.R., Randis, T.M., Sun, T.Y., and Ratner, A.J. (2013) DNase inhibits Gardnerella vaginalis biofilms in vitro and in vivo. *J. Infect. Dis.* **207**: 1491–1497.

Ingianni, A., Petruzzelli, S., Morandotti, G., and Pompei, R. (1997) Genotypic differentiation of Gardnerella vaginalis by amplified ribosomal DNA restriction analysis (ARDRA). *FEMS Immunol Med Microbiol.* **18**: 61–66.

Islam, A., Safidar, A., and Malik, A. (2009) Bacterial Vaginosis. *J Pak Med Assoc* **59**: 601–604.

Ison, C.A., Harvey, D.G., Tanna, A., and Easmon, C.S. (1987) Development and evaluation of scheme for serotyping Gardnerella vaginalis. *Genitourin. Med.* **63**: 196–201.

Jang, S.-E., Jeong, J.-J., Choi, S.-Y., Kim, H., Han, M., and Kim, D.-H. (2017) Lactobacillus rhamnosus HN001 and Lactobacillus acidophilus La-14 Attenuate Gardnerella vaginalis-Infected Bacterial Vaginosis in Mice. *Nutrients* **9**: 531.

Janulaitiene, M., Paliulyte, V., Grinceviciene, S., Zakareviciene, J., Vladisauskiene, A., Marcinkute, A., and Pleckaityte, M. (2017) Prevalence and distribution of Gardnerella vaginalis subgroups in women with and without bacterial vaginosis. *BMC Infect. Dis.* **17**: 394.

Joesoef, M.R., Karundeng, A., Runtupalit, C., Moran, J.S., Lewis, J.S., and Ryan, C.A. (2001) High rate of bacterial vaginosis among women with intrauterine devices in Manado, Indonesia. *Contraception* **64**: 169–172.

Jones, F.R., Miller, G., Gadea, N., Meza, R., Leon, S., Perez, J., et al. (2007) Prevalence of bacterial vaginosis among young women in low-income populations of coastal Peru. *Int. J. STD AIDS* **18**: 188–192.

Joshi, N. and Fass, J. (2011) Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files.

Kaewsrichan, J., Peeyananjarassri, K., and Kongprasertkit, J. (2006) Selection and identification of anaerobic lactobacilli producing inhibitory compounds against vaginal pathogens. *FEMS Immunol. Med. Microbiol.* **48**: 75–83.

Kalinka, J., Hanke, W., Wasiela, M., and Laudański, T. (2002) Socioeconomic and environmental risk factors of bacterial vaginosis in early pregnancy. *J. Perinat. Med.* **30**: 467–475.

Kamara, P., Hylton-Kong, T., Brathwaite, A., Del Rosario, G.R., Kristensen, S., Patrick, N., et al. (2000) Vaginal infections in pregnant women in Jamaica: prevalence and risk factors. *Int. J. STD AIDS* **11**: 516–520.

Kaźmierczak, W., Wnek, M., and Kamiński, K. (2004) [Frequency of vaginal infections in pregnant women in the Department of Perinatology and Gynaecology in Zabrze]. *Ginekol. Pol.* **75**: 932–936.

Keane, F.E., Thomas, B.J., Whitaker, L., Renton, A., and Taylor-Robinson, D. (1997) An association between non-gonococcal urethritis and bacterial vaginosis and the implications for patients and their sexual partners. *Genitourin. Med.* **73**: 373–377.

Keshavarz, H., Duffy, S.W., Sadeghi-Hassanabadi, A., Zolghadr, Z., and Oboodi, B. (2001) Risk factors for and relationship between bacterial vaginosis and cervicitis in a high risk population for cervicitis in Southern Iran. *Eur. J. Epidemiol.* **17**: 89–95.

Kharsany, A.B., Hoosen, A.A., and Van den Ende, J. (1993) Antimicrobial susceptibilities of Gardnerella vaginalis. *Antimicrob. Agents Chemother.* **37**: 2733–2735.

Kinghorn, G.R., Jones, B.M., Chowdhury, F.H., and Geary, I. (1982) Balanoposthitis associated with Gardnerella vaginalis infection in men. *Br. J. Vener. Dis.* **58**: 127–129.

Kirakoya-Samadoulougou, F., Nagot, N., Defer, M.-C., Yaro, S., Fao, P., Ilboudo, F., et al. (2011) Epidemiology of herpes simplex virus type 2 infection in rural and urban Burkina Faso. *Sex. Transm. Dis.* **38**: 117–123.

Kirakoya-Samadoulougou, F., Nagot, N., Defer, M.-C., Yaro, S., Meda, N., and Robert, A. (2008) Bacterial vaginosis among pregnant women in Burkina Faso. *Sex. Transm. Dis.* **35**: 985–989.

Klatt, N.R., Cheu, R., Birse, K., Zevin, A.S., Perner, M., Noël-Romas, L., et al. (2017) Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* **356**: 938–945.

Kleindienst, S., Grim, S., Sogin, M., Bracco, A., Crespo-Medina, M., and Joye, S.B. (2016) Diverse, rare microbial taxa responded to the Deepwater Horizon deep-sea hydrocarbon plume. *ISME J.* **10**: 400–415.

Knupp de Souza, D.M., Diniz, C.G., Filho, D.S.C., Andrade de Oliveira, L.M., Coelho, D.M., Talha, L.D.S., et al. (2016) Antimicrobial susceptibility and vaginolysin in Gardnerella vaginalis from healthy and bacterial vaginosis diagnosed women. *J. Infect. Dev. Ctries.* **10**: 913–919.

Koumans, E.H., Sternberg, M., Bruce, C., McQuillan, G., Kendrick, J., Sutton, M., and Markowitz, L.E. (2007) The Prevalence of Bacterial Vaginosis in the United States, 2001-2004; Associations With Symptoms, Sexual Behaviors, and Reproductive Health: *Sex. Transm. Dis.* **34**: 864–869.

Kurewa, N.E., Mapingure, M.P., Munjoma, M.W., Chirenje, M.Z., Rusakaniko, S., and Stray-Pedersen, B. (2010) The burden and risk factors of Sexually Transmitted Infections and Reproductive Tract Infections among pregnant women in Zimbabwe. *BMC Infect. Dis.* **10**: 127.

Lan, P.T., Lundborg, C.S., Phuc, H.D., Sihavong, A., Unemo, M., Chuc, N.T.K., et al. (2008) Reproductive tract infections including sexually transmitted infections: a population-based study of women of reproductive age in a rural district of Vietnam. *Sex. Transm. Infect.* **84**: 126–132.

Larsson, P.-G., Brandsborg, E., Forsum, U., Pendharkar, S., Andersen, K.K., Nasic, S., et al. (2011) Extended antimicrobial treatment of bacterial vaginosis combined with human lactobacilli to find the best treatment and minimize the risk of relapses. *BMC Infect. Dis.* **11**: 223–223.

Larsson, P.-G., Stray-Pedersen, B., Ryttig, K.R., and Larsen, S. (2008) Human lactobacilli as supplementation of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study. *BMC Womens Health* **8**: 3–3.

Lassey, A.T., Newman, M.J., and Opintan, J.A. (2005) Vaginal flora of first time urban family planning attendants in Accra, Ghana. *West Afr. J. Med.* **24**: 219–222.

Lata, I., Pradeep, Y., Sujata, and Jain, A. (2010) Estimation of the incidence of bacterial vaginosis and other vaginal infections and its consequences on maternal/fetal outcome in pregnant women attending an antenatal clinic in a tertiary care hospital in North India. *Indian J. Community Med.* **35**: 285.

Laue, C., Papazova, E., Liesegang, A., Pannenbeckers, A., Arendarski, P., Linnerth, B., et al. (2017) Effect of a yoghurt drink containing Lactobacillus strains on bacterial vaginosis in women - a double-blind, randomised, controlled clinical pilot trial. *Benef. Microbes* 1–16.

Leite, S.R.R.F., Amorim, M.M.R., Sereno, P.F.B., Leite, T.N.F., Ferreira, J. a. C., and Ximenes, R. a. A. (2011) Randomized clinical trial comparing the efficacy of the vaginal use of metronidazole with a Brazilian pepper tree (Schinus) extract for the treatment of bacterial vaginosis. *Braz. J. Med. Biol. Res.* **44**: 245–252.

Leitich, H., Bodner-Adler, B., Brunbauer, M., Kaider, A., Egarter, C., and Husslein, P. (2003) Bacterial vaginosis as a risk factor for preterm delivery: A meta-analysis. *Am. J. Obstet. Gynecol.* **189**: 139–147.

Leopold, S. (1953) Heretofore undescribed organism isolated from the genitourinary system. *U. S. Armed Forces Med J.* **4**: 263–266.

Lewis, W.G., Robinson, L.S., Gilbert, N.M., Perry, J.C., and Lewis, A.L. (2013) Degradation, Foraging, and Depletion of Mucus Sialoglycans by the Vagina-adapted Actinobacterium Gardnerella vaginalis. *J. Biol. Chem.* **288**: 12067–12079.

Lewis, W.G., Robinson, L.S., Perry, J., Bick, J.L., Peipert, J.F., Allsworth, J.E., and Lewis, A.L. (2012) Hydrolysis of Secreted Sialoglycoprotein Immunoglobulin A (IgA) in ex Vivo and Biochemical Models of Bacterial Vaginosis. *J. Biol. Chem.* **287**: 2079–2089.

Li, X.-D., Wang, C.-C., Zhang, X.-J., Gao, G.-P., Tong, F., Li, X., et al. (2014) Risk factors for bacterial vaginosis: results from a cross-sectional study having a sample of 53,652 women. *Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol.* **33**: 1525–1532.

Li, Xiu- De, Tong, F., Zhang, X., Pan, W., Chen, M., Wang, C., et al. (2015) Incidence and risk factors of bacterial vaginosis among pregnant women: A prospective study in Maanshan city, Anhui Province, China. *J. Obstet. Gynaecol. Res.* **41**: 1214–1222.

Lillo G, E., Lizama I, S., Medel C, J., T, M., and Angélica, M. (2010) Diagnosis of bacterial vaginosis in women attending a family planning clinic in the Metropolitan Region of Chile. *Rev. Chil. Infectol.* **27**: 199–203.

Lim, K.H., Brooks, H., Mcdougal, R., Burton, J., Devenish, C., and De Silva, T. (2010) Is there a correlation between bacterial vaginosis and preterm labour in women in the Otago region of New Zealand? *Aust. N. Z. J. Obstet. Gynaecol.* **50**: 226–229.

Löfmark, S., Edlund, C., and Nord, C.E. (2010) Metronidazole Is Still the Drug of Choice for Treatment of Anaerobic Infections. *Clin. Infect. Dis.* **50**: S16–S23.

Lopes dos Santos Santiago, G., Deschaght, P., El Aila, N., Kiama, T.N., Verstraelen, H., Jefferson, K.K., et al. (2011) Gardnerella vaginalis comprises three distinct genotypes of which only two produce sialidase. *Am. J. Obstet. Gynecol.* **204**: 450.e1-450.e7.

Lu, H., Jiang, P.-C., Zhang, X.-D., Hou, W.-J., Wei, Z.-H., Lu, J.-Q., et al. (2015) Characteristics of bacterial vaginosis infection in cervical lesions with high risk human papillomavirus infection. *Int. J. Clin. Exp. Med.* **8**: 21080.

Luong, M.-L., Libman, M., Dahhou, M., Chen, M.F., Kahn, S.R., Goulet, L., et al. (2010) Vaginal douching, bacterial vaginosis, and spontaneous preterm birth. *J. Obstet. Gynaecol. Can. JOGC J. Obstet. Gynecol. Can. JOGC* **32**: 313–320.

Machado, A., Jefferson, K.K., and Cerca, N. (2013) Interactions between Lactobacillus crispatus and Bacterial Vaginosis (BV)-Associated Bacterial Species in Initial Attachment and Biofilm Formation. *Int. J. Mol. Sci.* **14**: 12004–12012.

Machado, D., Castro, J., Martinez-de-Oliveira, J., Nogueira-Silva, C., and Cerca, N. (2017) Prevalence of bacterial vaginosis in Portuguese pregnant women and vaginal colonization by Gardnerella vaginalis. *PeerJ* **5**:.

Machado, D., Gaspar, C., Palmeira-de-Oliveira, A., Cavaleiro, C., Salgueiro, L., Martinez-de-Oliveira, J., and Cerca, N. (2017) Thymbra capitata essential oil as potential therapeutic agent against Gardnerella vaginalis biofilm-related infections. *Future Microbiol.* **12**: 407–416.

Madhivanan, P., Krupp, K., Hardin, J., Karat, C., Klausner, J.D., and Reingold, A.L. (2009) Simple and inexpensive point-of-care tests improve diagnosis of vaginal infections in resource constrained settings. *Trop. Med. Int. Health TM IH* **14**: 703–708.

Maestro, B. and Sanz, J.M. (2016) Choline Binding Proteins from Streptococcus pneumoniae: A Dual Role as Enzybiotics and Targets for the Design of New Antimicrobials. *Antibiotics* **5**:.

Magaña- Contreras, M., Contreras- Paredes, A., Chavez- Blanco, A., Lizano, M., Cruz- Hernandez, Y.D. la, and Cruz- Hernandez, E.D. la (2015) Prevalence of sexually transmitted pathogens associated with HPV infection in cervical samples in a Mexican population. *J. Med. Virol.* **87**: 2098–2105.

Marconi, C., Duarte, M.T.C., Silva, D.C., and Silva, M.G. (2015) Prevalence of and risk factors for bacterial vaginosis among women of reproductive age attending cervical screening in southeastern Brazil. *Int. J. Gynecol. Obstet.* **131**: 137–141.

Marcotte, H., Krogh Andersen, K., Lin, Y., Zuo, F., Zeng, Z., Larsson, P.G., et al. (2017) Characterization and complete genome sequences of L. rhamnosus DSM 14870 and L. gasseri DSM 14869 contained in the EcoVag® probiotic vaginal capsules. *Microbiol. Res.* **205**: 88–98.

Marrazzo, J.M., Fiedler, T.L., Srinivasan, S., Thomas, K.K., Liu, C., Ko, D., et al. (2012) Extravaginal Reservoirs of Vaginal Bacteria as Risk Factors for Incident Bacterial Vaginosis. *J. Infect. Dis.* **205**: 1580–1588.

Marrazzo, J.M., Thomas, K.K., Agnew, K., and Ringwood, K. (2010) Prevalence and risks for bacterial vaginosis in women who have sex with women. *Sex. Transm. Dis.* **37**: 335–339.

Marrazzo, J.M., Thomas, K.K., Fiedler, T.L., Ringwood, K., and Fredricks, D.N. (2010) Risks for Acquisition of Bacterial Vaginosis Among Women Who Report Sex with Women: A Cohort Study. *PLOS ONE* **5**: e11139.

Marrazzo, J.M., Thomas, K.K., and Ringwood, K. (2011) A behavioural intervention to reduce persistence of bacterial vaginosis among women who report sex with women: results of a randomised trial. *Sex. Transm. Infect.* **87**: 399–405.

Marrazzo, J.M., Wiesenfeld, H.C., Murray, P.J., Busse, B., Meyn, L., Krohn, M., and Hillier, S.L. (2006) Risk Factors for Cervicitis among Women with Bacterial Vaginosis. *J. Infect. Dis.* **193**: 617–624.

Martin, H.L., Richardson, B.A., Nyange, P.M., Lavreys, L., Hillier, S.L., Chohan, B., et al. (1999) Vaginal Lactobacilli, Microbial Flora, and Risk of Human Immunodeficiency Virus Type 1 and Sexually Transmitted Disease Acquisition. *J. Infect. Dis.* **180**: 1863–1868.

Martínez de Tejada, B., Coll, O., de Flores, M., Hillier, S.L., and Landers, D.V. (1998) [Prevalence of bacterial vaginosis in an obstetric population of Barcelona]. *Med. Clin. (Barc.)* **110**: 201–204.

Masoudi, M., Miraj, S., and Rafieian-Kopaei, M. (2016) Comparison of the Effects of Myrtus Communis L, Berberis Vulgaris and Metronidazole Vaginal Gel alone for the Treatment of Bacterial Vaginosis. *J. Clin. Diagn. Res. JCDR* **10**: QC04-07.

Massad, L.S., Evans, C.T., Kang, R., Hotton, A., Greenblatt, R., Minkoff, H., et al. (2017) Correlates of Bacterial Vaginosis Over Long-Term Follow-Up: Impact of HIV Infection. *AIDS Res. Hum. Retroviruses* **33**: 432–439.

Mayer, B.T., Srinivasan, S., Fiedler, T.L., Marrazzo, J.M., Fredricks, D.N., and Schiffer, J.T. (2015) Rapid and Profound Shifts in the Vaginal Microbiota Following Antibiotic Treatment for Bacterial Vaginosis. *J. Infect. Dis.* **212**: 793–802.

McGregor, J.A., French, J.I., and Seo, K. (1993) Premature rupture of membranes and bacterial vaginosis. *Am. J. Obstet. Gynecol.* **169**: 463–466.

Melkumyan, A.R., Priputnevich, T.V., Ankirskaya, A.S., Murav'eva, V.V., and Lubasovskaya, L.A. (2015) Effects of antibiotic treatment on the lactobacillus composition of vaginal microbiota. *Bull. Exp. Biol. Med.* **158**: 766–768.

Melo, J., Folgosa, E., Manjate, D., Osman, N., Francois, I., Temmerman, M., et al. (2008) Low prevalence of HIV and other sexually transmitted infections in young women attending a youth counselling service in Maputo, Mozambique. *Trop. Med. Int. Health* **13**: 17–20.

Menard, J.-P. (2008) Molecular Quantification of Gardnerella vaginalis and Atopobium vaginae Loads to Predict Bacterial Vaginosis. *Clin. Infect. Dis.* **47**: 33–43.

Meriwether, K.V., Rogers, R.G., Craig, E., Peterson, S.D., Gutman, R.E., and Iglesia, C.B. (2015) The effect of hydroxyquinoline-based gel on pessary-associated bacterial vaginosis: a multicenter randomized controlled trial. *Am. J. Obstet. Gynecol.* **213**: 729.e1-729.e9.

Milani, M. (2000) Effect on vaginal pH of a polycarbophil vaginal gel compared with an acidic douche in women with suspected bacterial vaginosis: a randomized, controlled study. *Curr. Ther. Res.* **61**: 781–788.

Miranda, A.E., Merçon-de-Vargas, P.R., Corbett, C.E.P., Corbett, J.F., and Dietze, R. (2009) Perspectives on sexual and reproductive health among women in an ancient mining area in Brazil. *Rev. Panam. Salud Pública* **25**: 157–161.

Mitchell, C.M., Hitti, J.E., Agnew, K.J., and Fredricks, D.N. (2009) Comparison of oral and vaginal metronidazole for treatment of bacterial vaginosis in pregnancy: impact on fastidious bacteria. *BMC Infect. Dis.* **9**: 89.

Mogha, K.V. and Prajapati, J.B. (2017) Evaluation of probiotic cream by in-vitro tests for treatment of bacterial vaginosis conditions: *Rev. Med. Microbiol.* **28**: 43–48.

Moncla, B.J., Chappell, C.A., Debo, B.M., and Meyn, L.A. (2016) The Effects of Hormones and Vaginal Microflora on the Glycome of the Female Genital Tract: Cervical-Vaginal Fluid. *PLoS ONE* **11**: e0158687.

Moncla, B.J., Chappell, C.A., Mahal, L.K., Debo, B.M., Meyn, L.A., and Hillier, S.L. (2015) Impact of Bacterial Vaginosis, as Assessed by Nugent Criteria and Hormonal Status on Glycosidases and Lectin Binding in Cervicovaginal Lavage Samples. *PLOS ONE* **10**: e0127091.

Moncla, B.J. and Pryke, K.M. (2009) Oleate lipase activity in Gardnerella vaginalis and reconsideration of existing biotype schemes. *BMC Microbiol.* **9**: 78.

Morison, L., Ekpo, G., West, B., Demba, E., Mayaud, P., Coleman, R., et al. (2005) Bacterial vaginosis in relation to menstrual cycle, menstrual protection method, and sexual intercourse in rural Gambian women. *Sex. Transm. Infect.* **81**: 242.

Msuya, S.E., Mbizvo, E., Stray-Pedersen, B., Sundby, J., Sam, N.E., and Hussain, A. (2002) Reproductive tract infections among women attending primary health care facilities in Moshi, Tanzania. *East Afr. Med. J.* **79**: 16–21.

Msuya, S.E., Uriyo, J., Hussain, A., Mbizvo, E.M., Jeansson, S., Sam, N.E., and Stray-Pedersen, B. (2009) Prevalence of sexually transmitted infections among pregnant women with known HIV status in northern Tanzania. *Reprod. Health* **6**: 4.

Mucci, M.J., Cuestas, M.L., Cervetto, M.M., Landaburu, M.F., and Mujica, M.T. (2016) A prospective observational study of vulvovagintis in pregnant women in Argentina, with special reference to candidiasis. *Mycoses* **59**: 429–435.

Mukherjee, S., Huntemann, M., Ivanova, N., Kyrpides, N.C., and Pati, A. (2015) Large-scale contamination of microbial isolate genomes by Illumina PhiX control. *Stand. Genomic Sci.* **10**: 18.

Muzny, C.A., Harbison, H.S., Austin, E.L., Schwebke, J.R., Van Der Pol, B., and Hook, E.W. (2017) Sexually Transmitted Infection Risk among Women Is Not Fully Explained by Partner Numbers. *South. Med. J.* **110**: 161–167.

Muzny, C.A., Sunesara, I.R., Austin, E.L., Mena, L.A., and Schwebke, J.R. (2013) Bacterial vaginosis among African American women who have sex with women. *Sex. Transm. Dis.* **40**: 751–755.

Muzny, C.A., Sunesara, I.R., Kumar, R., Mena, L.A., Griswold, M.E., Martin, D.H., et al. (2013) Characterization of the Vaginal Microbiota among Sexual Risk Behavior Groups of Women with Bacterial Vaginosis. *PLOS ONE* **8**: e80254.

Myer, L., Denny, L., Telerant, R., de Souza, M., Wright, T.C., and Kuhn, L. (2005) Bacterial Vaginosis and Susceptibility to HIV Infection in South African Women: A Nested Case-Control Study. *J. Infect. Dis.* **192**: 1372–1380.

Myhre, A.K., Bevanger, L.S., Berntzen, K., and Bratlid, D. (2002) Anogenital bacteriology in non-abused preschool children: a descriptive study of the aerobic genital flora and the isolation of anogenital Gardnerella vaginalis. *Acta Paediatr. Oslo Nor. 1992* **91**: 885–891.

Myhre, A.K., Myklestad, K., and Adams, J.A. (2010) Changes in Genital Anatomy and Microbiology in Girls between Age 6 and Age 12 Years: A Longitudinal Study. *J. Pediatr. Adolesc. Gynecol.* **23**: 77–85.

Nagaraja, P. (2008) Antibiotic resistance of Gardnerella vaginalis in recurrent bacterial vaginosis. *Indian J. Med. Microbiol.* **26**: 155–157.

Nansel, T.R., Riggs, M.A., Yu, K.F., Andrews, W.W., Schwebke, J.R., and Klebanoff, M.A. (2006) The association of psychosocial stress and bacterial vaginosis in a longitudinal cohort. *Am. J. Obstet. Gynecol.* **194**: 381–386.

Nelson, D., Loomis, L., and Fischetti, V.A. (2001) Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. *Proc. Natl. Acad. Sci. U. S. A.* **98**: 4107–4112.

Neri, P., Salvolini, S., Giovannini, A., and Mariotti, C. (2009) Retinal Vasculitis Associated with Asymptomatic Gardnerella vaginalis Infection: A New Clinical Entity. *Ocul. Immunol. Inflamm.* **17**: 36–40.

Ness, R.B. (2005) A Cluster Analysis of Bacterial Vaginosis-associated Microflora and Pelvic Inflammatory Disease. *Am. J. Epidemiol.* **162**: 585–590.

Ness, R.B., Hillier, S.L., Richter, H.E., Soper, D.E., Stamm, C., Bass, D.C., et al. (2003) Why women douche and why they may or may not stop. *Sex. Transm. Dis.* **30**: 71–74.

Ness, R.B., Hillier, S.L., Richter, H.E., Soper, D.E., Stamm, C., McGregor, J., et al. (2002) Douching in relation to bacterial vaginosis, lactobacilli, and facultative bacteria in the vagina. *Obstet. Gynecol.* **100**: 765.

von Nicolai, H., Hammann, R., Salehnia, S., and Zilliken, F. (1984) A newly discovered sialidase from Gardnerella vaginalis. *Zentralbl. Bakteriol. Mikrobiol. Hyg. [A]* **258**: 20–26.

Nilsson, U., Hellberg, D., Shoubnikova, M., Nilsson, S., and Mårdh, P.A. (1997) Sexual behavior risk factors associated with bacterial vaginosis and Chlamydia trachomatis infection. *Sex. Transm. Dis.* **24**: 241–246.

Nokhbeh, M.R., Boroumandi, S., Pokorny, N., Koziarz, P., Paterson, E.S., and Lambert, I.B. (2002) Identification and characterization of SnrA, an inducible oxygen-insensitive nitroreductase in Salmonella enterica serovar Typhimurium TA1535. *Mutat. Res.* **508**: 59–70.

Noll, K.S., Sinko, P.J., and Chikindas, M.L. (2011) Elucidation of the Molecular Mechanisms of Action of the Natural Antimicrobial Peptide Subtilosin Against the Bacterial Vaginosis-associated Pathogen Gardnerella vaginalis. *Probiotics Antimicrob. Proteins* **3**: 41–47.

Nunez, J.T. (2005) Low-dose secnidazole in the treatment of bacterial vaginosis. *Int. J. Gynecol. Obstet.* **88**: 281–285.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A.A., Korobeynikov, A., Lapidus, A., et al. (2013) Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *J. Comput. Biol. J. Comput. Mol. Cell Biol.* **20**: 714–737.

Oakeshott, P., Hay, P., Hay, S., Steinke, F., Rink, E., and Kerry, S. (2002) Association between bacterial vaginosis or chlamydial infection and miscarriage before 16 weeks' gestation: prospective community based cohort study. *BMJ* **325**: 1334.

O'Donnell, A.G., Minnikin, D.E., Goodfellow, M., and Piot, P. (1984) Fatty acid, polar lipid and wall amino acid composition of Gardnerella vaginalis. *Arch. Microbiol.* **138**: 68–71.

Ojala, T., Kankainen, M., Castro, J., Cerca, N., Edelman, S., Westerlund-Wikström, B., et al. (2014) Comparative genomics of Lactobacillus crispatus suggests novel mechanisms for the competitive exclusion of Gardnerella vaginalis. *BMC Genomics* **15**: 1070.

Oliveira, F.A., Pfleger, V., Lang, K., Heukelbach, J., Miralles, I., Fraga, F., et al. (2007) Sexually transmitted infections, bacterial vaginosis, and candidiasis in women of reproductive age in rural Northeast Brazil: a population-based study. *Mem. Inst. Oswaldo Cruz* **102**: 751–756.

Onderdonk, A.B., Delaney, M.L., and Fichorova, R.N. (2016) The Human Microbiome during Bacterial Vaginosis. *Clin. Microbiol. Rev.* **29**: 223–238.

Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T.G., et al. (2015) Roary: rapid large-scale prokaryote pan genome analysis. *Bioinforma. Oxf. Engl.* **31**: 3691–3693.

Pandya, H.B., Agravat, H.H., Patel, J.S., and Sodagar, N.R.K. (2014) Emerging antimicrobial resistance pattern of Helicobacter pylori in central Gujarat. *Indian J. Med. Microbiol.* **32**: 408–413.

Passey, M., Mgone, C.S., Lupiwa, S., Suve, N., Tiwara, S., Lupiwa, T., et al. (1998) Community based study of sexually transmitted diseases in rural women in the highlands of Papua New Guinea: prevalence and risk factors. *Sex. Transm. Infect.* **74**: 120–127.

Patel, V., Weiss, H.A., Mabey, D., West, B., D'Souza, S., Patil, V., et al. (2006) The burden and determinants of reproductive tract infections in India: a population based study of women in Goa, India. *Sex. Transm. Infect.* **82**: 243–249.

Pathak, M., Turner, M., Palmer, C., and Coombes, A.G.A. (2014) Evaluation of polycaprolactone matrices for the intravaginal delivery of metronidazole in the treatment of bacterial vaginosis. *J. Biomater. Appl.* **29**: 354–363.

Patterson, J.L., Girerd, P.H., Karjane, N.W., and Jefferson, K.K. (2007) Effect of biofilm phenotype on resistance of Gardnerella vaginalis to hydrogen peroxide and lactic acid. *Am. J. Obstet. Gynecol.* **197**: 170.e1-170.e7.

Pendharkar, S., Brandsborg, E., Hammarström, L., Marcotte, H., and Larsson, P.-G. (2015) Vaginal colonisation by probiotic lactobacilli and clinical outcome in women conventionally treated for bacterial vaginosis and yeast infection. *BMC Infect. Dis.* **15**: 255.

Penney, G.C., Thomson, M., Norman, J., McKenzie, H., Vale, L., Smith, R., and Imrie, M. (1998) A randomised comparison of strategies for reducing infective complications of induced abortion. *Br. J. Obstet. Gynaecol.* **105**: 599–604.

Perez, R.H., Zendo, T., and Sonomoto, K. (2014) Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microb. Cell Factories* **13**: S3.

Pessoa, W.F.B., Melgaço, A.C.C., de Almeida, M.E., Ramos, L.P., Rezende, R.P., and Romano, C.C. (2017) In Vitro Activity of Lactobacilli with Probiotic Potential Isolated from Cocoa Fermentation against Gardnerella vaginalis. *BioMed Res. Int.* **2017**: 1–10.

Piot, P., Dyck, E.V., Peeters, M., Hale, J., Totten, P.A., and Holmes, K.K. (1984) Biotypes of Gardnerella vaginalis. *J. Clin. Microbiol.* **20**: 677–679.

Piot, P., Dyck, E.V., Totten, P.A., and Holmes, K.K. (1982) Identification of Gardnerella (Haemophilus) vaginalis. *J. Clin. Microbiol.* **15**: 19.

Platz- Christensen, J.J., Sundström, E., and Larsson, P. (1994) Bacterial vaginosis and cervical intraepithelial neoplasia. *Acta Obstet. Gynecol. Scand.* **73**: 586–588.

Pleckaityte, M., Janulaitiene, M., Lasickiene, R., and Zvirbliene, A. (2012) Genetic and biochemical diversity of Gardnerella vaginalis strains isolated from women with bacterial vaginosis. *FEMS Immunol. Med. Microbiol.* **65**: 69–77.

Pleckaityte, M., Mistiniene, E., Lasickiene, R., Zvirblis, G., and Zvirbliene, A. (2011) Generation of recombinant single-chain antibodies neutralizing the cytolytic activity of vaginolysin, the main virulence factor of Gardnerella vaginalis. *BMC Biotechnol.* **11**: 100.

Poteat, T.C. (2015) Stigma, sexual health, and human rights among women who have sex with women in Lesotho. *Reprod Health Matters* **23**: 107–116.

Puapermpoonsiri, S., Kato, N., Watanabe, K., Ueno, K., Chongsomchai, C., and Lumbiganon, P. (1996) Vaginal microflora associated with bacterial vaginosis in Japanese and Thai pregnant women. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **23**: 748–752.

Pybus, V. and Onderdonk, A.B. (1997) Evidence for a Commensal, Symbiotic Relationship between Gardnerella vaginalis and Prevotella bivia Involving Ammonia: Potential Significance for Bacterial Vaginosis. *J. Infect. Dis.* **175**: 406–413.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* **41**: D590–D596.

Rahman, S., Garland, S., Currie, M., Tabrizi, S.N., Rahman, M., Nessa, K., and Bowden, F.J. (2008) Prevalence of Mycoplasma genitalium in health clinic attendees complaining of vaginal discharge in Bangladesh. *Int. J. STD AIDS* **19**: 772–774.

Randis, T.M., Kulkarni, R., Aguilar, J.L., and Ratner, A.J. (2009) Antibody-Based Detection and Inhibition of Vaginolysin, the Gardnerella vaginalis Cytolysin. *PLoS ONE* **4**: e5207.

Randis, T.M., Zaklama, J., LaRocca, T.J., Los, F.C.O., Lewis, E.L., Desai, P., et al. (2013) Vaginolysin Drives Epithelial Ultrastructural Responses to Gardnerella vaginalis. *Infect. Immun.* **81**: 4544–4550.

Rathod, S.D., Krupp, K., Klausner, J.D., Arun, A., Reingold, A.L., and Madhivanan, P. (2011) Bacterial Vaginosis and Risk for Trichomonas vaginalis Infection: A Longitudinal Analysis. *STD* **38**: 882–886.

Ratner, A.J., Hippe, K.R., Aguilar, J.L., Bender, M.H., Nelson, A.L., and Weiser, J.N. (2006) EPITHELIAL CELLS ARE SENSITIVE DETECTORS OF BACTERIAL PORE-FORMING TOXINS. *J. Biol. Chem.* **281**: 12994.

Reboul, C.F., Whisstock, J.C., and Dunstone, M.A. (2014) A New Model for Pore Formation by Cholesterol-Dependent Cytolysins. *PLoS Comput. Biol.* **10**: e1003791.

Riazi, S. (2012) Mode of action and safety of lactosporin, a novel antimicrobial protein produced by Bacillus coagulans ATCC 7050. **113**: 714–722.

Riduan, J.M., Hillier, S.L., Utomo, B., Wiknjosastro, G., Linnan, M., and Kandun, N. (1993) Bacterial vaginosis and prematurity in Indonesia: association in early and late pregnancy. *Am. J. Obstet. Gynecol.* **169**: 175–178.

Romoren, M., Velauthapillai, M., Rahman, M., Sundby, J., Klouman, E., and Hjortdahl, P. (2007) Trichomoniasis and bacterial vaginosis in pregnancy: inadequately managed with the syndromic approach. *Bull. World Health Organ.* **85**: 297–304.

Rossjohn, J., Feil, S.C., McKinstry, W.J., Tweten, R.K., and Parker, M.W. (1997) Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* **89**: 685–692.

Ryen, A., Birch-Andersen, A., and Lapage, S.P. (1966) An electron microscope study of thin sections of Haemophilus vaginalis (Gardner and Dukes) and some possibly related species. *Can J Microbiol.* **12**: 1125–1136.

Sadhu, K., Dominque, P.A.G., Chow, A.W., Nelligan, J., Cheng, N., and Costerton, J.W. (1989) Gardnerella vaginalis has a gram-positive cell-wall ultrastructure and lacks classical cell-wall lipopolysaccharide. *J Med Microbiol.* **29**: 223–235.

Sánchez, S.E., Koutsky, L.A., Sánchez, J., Fernández, A., Casquero, J., Kreiss, J., et al. (1998) Rapid and inexpensive approaches to managing abnormal vaginal discharge or lower abdominal pain: an evaluation in women attending gynaecology and family planning clinics in Peru. *Sex. Transm. Infect.* **74 Suppl 1**: S85-94.

Saunders, S., Bocking, A., Challis, J., and Reid, G. (2007) Effect of Lactobacillus challenge on Gardnerella vaginalis biofilms. *Colloids Surf. B Biointerfaces* **55**: 138–142.

Schellenberg, J.J., Jayaprakash, T.P., Gamage, N.W., Patterson, M.H., Vaneechoutte, M., and Hill, J.E. (2016) Gardnerella vaginalis Subgroups Defined by cpn 60 Sequencing and Sialidase Activity in Isolates from Canada, Belgium and Kenya. *PLOS ONE* **11**: e0146510.

Schleifer, K.H. and Kandler, O. (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**: 407.

Schuyler, J.A., Mordechai, E., Adelson, M.E., Sobel, J.D., Gygax, S.E., and Hilbert, D.W. (2016) Identification of intrinsically metronidazole-resistant clades of Gardnerella vaginalis. *Diagn. Microbiol. Infect. Dis.* **84**: 1–3.

Schwebke, J.R., Lee, J.Y., Lensing, S., Philip, S.S., Wiesenfeld, H.C., Seña, A.C., et al. (2016) Home Screening for Bacterial Vaginosis to Prevent Sexually Transmitted Diseases. *Clin. Infect. Dis.* **62**: 531–536.

Seemann, T. (2014) Prokka: rapid prokaryotic genome annotation. *Bioinforma. Oxf. Engl.* **30**: 2068–2069.

Shashikala, A., Nagasrilatha, B., Sasidhar, M., Manmohan, B., and Suseela, T.L. (2015) BACTERIAL VAGINOSIS IN PRETERM LABOUR AT A TERTIARY CARE CENTRE. *J. Evol. Med. Dent. Sci.* **4**: 8806–8817.

Shatursky, O., Heuck, A.P., Shepard, L.A., Rossjohn, J., Parker, M.W., Johnson, A.E., and Tweten, R.K. (1999) The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell* **99**: 293–299.

Shepard, L.A., Heuck, A.P., Hamman, B.D., Rossjohn, J., Parker, M.W., Ryan, K.R., et al. (1998) Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin Clostridium perfringens perfringolysin O: an alpha-helical to beta-sheet transition identified by fluorescence spectroscopy. *Biochemistry (Mosc.)* **37**: 14563–14574.

Shimano, S., Nishikawa, A., Sonoda, T., and Kudo, R. (2004) Analysis of the prevalence of bacterial vaginosis and Chlamydia trachomatis infection in 6083 pregnant women at a hospital in Otaru, Japan. *J. Obstet. Gynaecol. Res.* **30**: 230–236.

Shobeiri, F. and Nazari, M. (2006) A prospective study of genital infections in Hamedan, Iran. *Southeast Asian J. Trop. Med. Public Health* **37 Suppl 3**: 174–177.

Shrestha, S., Tuladhar, N.R., Basnyat, S., Acharya, G.P., Shrestha, P., and Kumar, P. (2011) Prevalence of vaginitis among pregnant women attending Paropakar Maternity and Women's Hospital, Thapathali, Kathmandu, Nepal. *Nepal Med. Coll. J. NMCJ* **13**: 293–296.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., et al. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **7**: 539.

Sihavong, A., Phouthavane, T., Lundborg, C.S., Sayabounthavong, K., Syhakhang, L., and Wahlström, R. (2007) Reproductive tract infections among women attending a gynecology outpatient department in Vientiane, Lao PDR. *Sex. Transm. Dis.* **34**: 791–795.

Silva *Httpswwwarb-Silvade*.

Simoes, J.A., Hashemi, F.B., Aroutcheva, A.A., Heimler, I., Spear, G.T., Shott, S., and Faro, S. (2001) Human immunodeficiency virus type 1 stimulatory activity by Gardnerella

vaginalis: relationship to biotypes and other pathogenic characteristics. *J. Infect. Dis.* **184**: 22–27.

Sivadon-Tardy, V., Roux, A.-L., Piriou, P., Herrmann, J.-L., Gaillard, J.-L., and Rottman, M. (2009) Gardnerella vaginalis Acute Hip Arthritis in a Renal Transplant Recipient. *J. Clin. Microbiol.* **47**: 264–265.

Skidmore-Roth, L. (2015) Mosby's Drug Guide for Nursing Students 11th ed. Elsevier, St. Louis, Missouri.

Skowrońska-Jóźwiak, E., Lebiedzińska, K., Smyczyńska, J., Lewandowski, K.C., Głowacka, E., and Lewiński, A. (2014) Effects of maternal vitamin D status on pregnancy outcomes, health of pregnant women and their offspring. *Neuro Endocrinol. Lett.* **35**: 367–372.

Šmarda, J. and Benada, O. (2005) Phage Tail-Like (High-Molecular-Weight) Bacteriocins of Budvicia aquatica and Pragia fontium (Enterobacteriaceae). *Appl. Environ. Microbiol.* **71**: 8970–8973.

Soltani, C.E., Hotze, E.M., Johnson, A.E., and Tweten, R.K. (2007) Structural elements of the cholesterol-dependent cytolysins that are responsible for their cholesterol-sensitive membrane interactions. *Proc. Natl. Acad. Sci. U. S. A.* **104**: 20226–20231.

Srinivasan, S., Morgan, M.T., Fiedler, T.L., Djukovic, D., Hoffman, N.G., Raftery, D., et al. (2015) Metabolic Signatures of Bacterial Vaginosis. *mBio* **6**: e00204-15.

Stoner, K.A. (2011) Recalcitrance of bacterial vaginosis among herpes- simplex- virus- type- 2- seropositive women. **38**: 77–83.

Storti-Filho, A., Souza, P.C., Souza, R.J.S., Pereira, M.W., Mello, I.C.J., Svidizinski, T.I.E., and Lopes-Consolaro, M.E. (2011) Prevalence of clue cells suggestive for Gardnerella vaginalis in population-based cervical screening in the public versus private health care in Maringá, Paraná, Brazil. *Arch. Gynecol. Obstet.* **283**: 781–785.

Svare, J., Schmidt, H., Hansen, B., and Lose, G. (2006) Bacterial vaginosis in a cohort of Danish pregnant women: prevalence and relationship with preterm delivery, low birthweight and perinatal infections. *BJOG Int. J. Obstet. Gynaecol.* **113**: 1419–1425.

Swidsinski, A., Loening-Baucke, V., Swidsinski, S., and Verstraelen, H. (2015) Polymicrobial Gardnerella biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis: a preliminary report. *Arch. Gynecol. Obstet.* **291**: 605–609.

Swidsinski, A., Mendling, W., Loening-Baucke, V., Ladhoff, A., Swidsinski, S., Hale, L.P., and Lochs, H. (2005) Adherent biofilms in bacterial vaginosis. *Obstet. Gynecol.* **106**: 1013–1023.

Swidsinski, A., Mendling, W., Loening-Baucke, V., Swidsinski, S., Dörffel, Y., Scholze, J., et al. (2008) An adherent Gardnerella vaginalis biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am. J. Obstet. Gynecol.* **198**: 97.e1-97.e6.

Tabata, A., Ohkura, K., Ohkubo, Y., Tomoyasu, T., Ohkuni, H., Whiley, R.A., and Nagamune, H. (2014) The diversity of receptor recognition in cholesterol-dependent cytolysins: Receptor recognition diversity in CDCs. *Microbiol. Immunol.* **58**: 155–171.

Tanih, N.F., Ndip, L.M., and Ndip, R.N. (2011) Characterisation of the genes encoding resistance to metronidazole (rdxA and frxA) and clarithromycin (the 23S-rRNA genes) in South African isolates of Helicobacter pylori. *Ann. Trop. Med. Parasitol.* **105**: 251–259.

Tankovic, J., Timinskas, A., Janulaitiene, M., Zilnyte, M., Baudel, J.-L., Maury, E., et al. (2017) Gardnerella vaginalis bacteremia associated with severe acute encephalopathy in a young female patient. *Anaerobe* **47**: 132–134.

Taylor-Robinson, D. and Boustouller, Y.L. (2011) Damage to oviduct organ cultures by Gardnerella vaginalis. *Int. J. Exp. Pathol.* **92**: 260.

Tchoudomirova, K. (1998) Gynaecological and microbiological findings in women attending for a general health check-up. *J. Obstet. Gynaecol.* **18**: 556–560.

Thammalangsy, S., Sihavong, A., Phouthavane, T., Sayabounthavong, K., Puapermpoonsiri, S., Kitayaporn, D., et al. (2006) The prevalence of lower genital tract infections among ante-natal care (ANC) clinic patients in two central hospitals, Vientiane, Lao People's Democratic Republic. *Southeast Asian J. Trop. Med. Public Health* **37**: 190–199.

Thellin, O., Zorzi, W., Zorzi, D., Delvenne, P., Heinen, E., ElMoualij, B., and Quatresooz, P. (2016) Lysozyme as a cotreatment during antibiotics use against vaginal infections: An in vitro study on Gardnerella vaginalis biofilm models. *Int. Microbiol. Off. J. Span. Soc. Microbiol.* **19**: 101–107.

Thorsen, P., Vogel, I., Molsted, K., Jacobsson, B., Arpi, M., Møller, B.R., and Jeune, B. (2006) Risk factors for bacterial vaginosis in pregnancy: a population-based study on Danish women. *Acta Obstet. Gynecol. Scand.* **85**: 906–911.

Tilley, S.J., Orlova, E.V., Gilbert, R.J.C., Andrew, P.W., and Saibil, H.R. (2005) Structural basis of pore formation by the bacterial toxin pneumolysin. *Cell* **121**: 247–256.

Tita, A.T.N. (2007) Clinical trial of interconceptional antibiotics to prevent preterm birth: subgroup analyses and possible adverse antibiotic-microbial interaction. *Am. J. Obstet. Gynecol.* **197**: 367-e6.

Togni, G., Battini, V., Bulgheroni, A., Mailland, F., Caserini, M., and Mendling, W. (2011) In Vitro Activity of Nifuratel on Vaginal Bacteria: Could It Be a Good Candidate for the Treatment of Bacterial Vaginosis?▽ . *Antimicrob. Agents Chemother.* **55**: 2490–2492.

Tolosa, J.E., Chaithongwongwatthana, S., Daly, S., Maw, W.W., Gaitán, H., Lumbiganon, P., et al. (2006) The International Infections in Pregnancy (IIP) study: Variations in the prevalence of bacterial vaginosis and distribution of morphotypes in vaginal smears among pregnant women. *Am. J. Obstet. Gynecol.* **195**: 1198–1204.

Tomusiak, A., Heczko, P.B., Janeczko, J., Adamski, P., Pilarczyk-Zurek, M., and Strus, M. (2013) Bacterial infections of the lower genital tract in fertile and infertile women from the southeastern Poland. *Ginekol. Pol.* **84**: 352–358.

Tomusiak, A., Magdalena, S., and Bogumi\l, H.P. (2011) Lekowrażliwość szczepów Gardnerella vaginalis wyizolowanych z przypadków bakteryjnej waginozy. *Ginekol Pol* **82**: 900–904.

Tosheva-Daskalova, K., Strateva, T.V., Mitov, I.G., and Gergova, R.T. (2017) Multiplex PCR detection of problematic pathogens of clinically heterogeneous bacterial vaginosis in Bulgarian women. *Turk. J. Med. Sci.* **47**: 1492–1499.

Turner, A.N., Carr Reese, P., Chen, P.L., Kwok, C., Jackson, R.D., Klebanoff, M.A., et al. (2016) Serum vitamin D status and bacterial vaginosis prevalence and incidence in Zimbabwean women. *Am. J. Obstet. Gynecol.* **215**: 332.e1-332.e10.

Turovskiy, Y., Ludescher, R.D., Aroutcheva, A.A., Faro, S., and Chikindas, M.L. (2009) Lactocin 160, a Bacteriocin Produced by Vaginal Lactobacillus rhamnosus, Targets Cytoplasmic Membranes of the Vaginal Pathogen, Gardnerella vaginalis. *Probiotics Antimicrob. Proteins* **1**: 67–74.

Unzeitig, V., Bucek, R., Cupr, Z., and Vachek, S. (1991) [Epidemiology and diagnosis in the vaginal environment. III. Diagnosis and epidemiology of bacterial vaginosis]. *Cesk. Gynekol.* **56**: 247–252.

Vaca, M., Guadalupe, I., Erazo, S., Tinizaray, K., Chico, M., Cooper, P., and Hay, P. (2010) High prevalence of bacterial vaginosis in adolescent girls in a tropical area of Ecuador. *BJOG Int. J. Obstet. Gynaecol.* **117**: 225–228.

Verstraelen, H., Verhelst, R., Vaneechoutte, M., and Temmerman, M. (2010) The epidemiology of bacterial vaginosis in relation to sexual behaviour. *BMC Infect. Dis.* **10**: 81.

Vicariotto, F., Mogna, L., and Del Piano, M. (2014) Effectiveness of the two microorganisms Lactobacillus fermentum LF15 and Lactobacillus plantarum LP01, formulated in slow-release vaginal tablets, in women affected by bacterial vaginosis: a pilot study. *J. Clin. Gastroenterol.* **48**: S106–S112.

Vice, J.L. and Smaron, M.F. (1973) Indirect Fluorescent-Antibody Method for the Identification of Corynebacterium vaginale. *Appl. Microbiol.* **25**: 908–916.

Vodstrcil, L.A., Hocking, J.S., Law, M., Walker, S., Tabrizi, S.N., Fairley, C.K., and Bradshaw, C.S. (2013) Hormonal Contraception Is Associated with a Reduced Risk of Bacterial Vaginosis: A Systematic Review and Meta-Analysis. *PLOS ONE* **8**: e73055.

Vodstrcil, L.A., Twin, J., Garland, S.M., Fairley, C.K., Hocking, J.S., Law, M.G., et al. (2017) The influence of sexual activity on the vaginal microbiota and Gardnerella vaginalis clade diversity in young women. *PLOS ONE* **12**: e0171856.

Vodstrcil, L.A., Walker, S.M., Hocking, J.S., Law, M., Forcey, D.S., Fehler, G., et al. (2015) Incident bacterial vaginosis (BV) in women who have sex with women is associated with behaviors that suggest sexual transmission of BV. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **60**: 1042–1053.

Walraven, G., Scherf, C., West, B., Ekpo, G., Paine, K., Coleman, R., et al. (2001) The burden of reproductive-organ disease in rural women in The Gambia, West Africa. *The Lancet* **357**: 1161–1167.

Wang, L., Koppolu, S., Chappell, C., Moncla, B.J., Hillier, S.L., and Mahal, L.K. (2015) Studying the Effects of Reproductive Hormones and Bacterial Vaginosis on the Glycome of Lavage Samples from the Cervicovaginal Cavity. *PLOS ONE* **10**: e0127021.

Wells, J.I. and Goei, S.H. (1981) Rapid identification of Corynebacterium vaginale in non-purulent vaginitis. *J. Clin. Pathol.* **34**: 917.

Wenman, W.M., Joffres, M.R., Tataryn, I.V., Pearson, R., and et al. (2004) A prospective cohort study of pregnancy risk factors and birth outcomes in Aboriginal women. *Can. Med. Assoc. J. CMAJ Ott.* **171**: 585–589.

Wenman, W.M., Tataryn, I.V., Joffres, M.R., Pearson, R., Grace, M.G.A., Albritton, W.L., and Prasad, E. (2002) Demographic, clinical and microbiological characteristics of maternity patients: A Canadian clinical cohort study. *Can. J. Infect. Dis.* **13**: 311–318.

Whiteway, J., Koziarz, P., Veall, J., Sandhu, N., Kumar, P., Hoecher, B., and Lambert, I.B. (1998) Oxygen-insensitive nitroreductases: Analysis of the roles ofnfsA and nfsB in development of resistance to 5-Nitrofuran derivatives in Escherichia coli. *J. Bacteriol.* **180**: 5529–5539.

Wiesenfeld, H.C., Hillier, S.L., Krohn, M.A., Landers, D.V., and Sweet, R.L. (2003) Bacterial Vaginosis Is a Strong Predictor of Neisseria gonorrhoeae and Chlamydia trachomatis Infection. *Clin. Infect. Dis.* **36**: 663–668.

Wiggins, R., Hicks, S., Soothill, P., Millar, M., and Corfield, A. (2001) Mucinases and sialidases: their role in the pathogenesis of sexually transmitted infections in the female genital tract. *Sex. Transm. Infect.* **77**: 402–408.

Wilson, J.D., Lee, R.A., Balen, A.H., and Rutherford, A.J. (2007) Bacterial vaginal flora in relation to changing oestrogen levels. *Int. J. STD AIDS* **18**: 308–311.

Xu, C., Zhang, W., Wu, M., and Zhang, S. (2010) [Prevalence and determinants of lower reproductive tract infections among women aged 25 - 54 years in Beijing]. *Zhonghua Liu Xing Bing Xue Za Zhi Zhonghua Liuxingbingxue Zazhi* **31**: 138–141.

Yang, S.-C., Lin, C.-H., Sung, C.T., and Fang, J.-Y. (2014) Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Front. Microbiol.* **5**:.

Ybazeta, G., Douglas, L., Graham, J., Fraleigh, N.L., Murad, Y., Perez, J., et al. (2017) Complete Genome Sequence of Enterococcus thailandicus Strain a523 Isolated from Urban Raw Sewage. *Genome Announc.* **5**: e01298-17.

Yen, S., Shafer, M.-A., Moncada, J., Campbell, C.J., Flinn, S.D., and Boyer, C.B. (2003) Bacterial vaginosis in sexually experienced and non-sexually experienced young women entering the military. *Obstet. Gynecol.* **102**: 927–933.

Yeoman, C.J., Yildirim, S., Thomas, S.M., Durkin, A.S., Torralba, M., Sutton, G., et al. (2010) Comparative Genomics of Gardnerella vaginalis Strains Reveals Substantial Differences in Metabolic and Virulence Potential. *PLOS ONE* **5**: e12411.

Yongjun, T., Samuelson, J., Qingsheng, D., Ali, M.M., Li, X., Yanjian, M., et al. (2009) The prevalence of sexually transmitted and other lower reproductive tract infections among rural women in Sichuan Province, China. *Southeast Asian J. Trop. Med. Public Health* **40**: 1038–1047.

Younus, N.K., Gopinath, R., Jegasothy, R., Nordin, S.A., van Belkum, A., Mary, N., and Neela, V.K. (2017) An update on Gardneralla vaginalis associated bacterial vaginosis in Malaysia. *Asian Pac. J. Trop. Biomed.* **7**: 831–835.

Ziaei, S., Sadrkhanlu, M., Moeini, A., and Faghihzadeh, S. (2006) Effect of Bacterial Vaginosis on Premature Rupture of Membranes and Related Complications in Pregnant Women with a Gestational Age of 37–42 Weeks. *Gynecol. Obstet. Invest.* **61**: 135–138.

Zilnyte, M., Venclovas, Č., Zvirbliene, A., and Pleckaityte, M. (2015) The Cytolytic Activity of Vaginolysin Strictly Depends on Cholesterol and Is Potentiated by Human CD59. *Toxins* **7**: 110–128.

Zinnemann, K. and Turner, G.C. (1963) The taxonomic position of "Haemophilus vaginalis" [Corynebacterium vaginale]. *J. Pathol.* **85**: 213–219.

Zvirbliene, A., Pleckaityte, M., Lasickiene, R., Kucinskaite-Kodze, I., and Zvirblis, G. (2010) Production and characterization of monoclonal antibodies against vaginolysin: Mapping of a region critical for its cytolytic activity. *Toxicon* **56**: 19–28.

Appendix A Epidemiology

A.1 Bacterial vaginosis prevalence

Table A.1 Reported prevalence rates of Bacterial vaginosis by location, date and population.

Continent	Country	Study Location	Date	Population	Age Range or Mean Age	BV Prevalence %	Reference
North America	Canada	Edmonton, Alberta	1994-1995	2047 pregnant women attending 1 of 4 obstetrical office or the University of Alberta Hospital	18-45	14	Wenman *et al.*, 2002
		Edmonton, Alberta	1995-1996	2047 pregnant women attending 1 of 4 obstetrical office or the University of Alberta Hospital (4.3% Aboriginal, 95.7% non-aboriginal)	NS	13.6 Overall 33 Aboriginal 13 Non-aboriginal	Wenman *et al.*, 2004
		Toronto, Ontario	2008-2009	70 women attending one of two Toronto University associated hospitals (St. Michael's hospital and Sunnybrook Health Sciences Centre) for an IUD insertion (Alice et al., 2012)	18-45	7.1 Baseline 9 Follow-up	Alice *et al.*, 2012
	Grenada	St. George's	2009-2011	2677 non-menopausal women, obtaining pap smears	15-49	16.1 - 21.4	Brooks-Smith-Lowe and Rodrigo, 2013
	Jamaica	Kingston	1999	269 pregnant women in 4 prenatal clinics. (2^{nd} or 3^{rd} trimester)	14-40	44.1	Kamara *et al.*, 2000
	Mexico	Villahermosa, Tabasco	2013-2014	201 women attending outpatient clinic for routine gynecological checkups	16-74	25.9	Magaña- Contreras *et al.*, 2015
	United States	United States	1984-1989	13,747 of predominantly low-socioeconomic pregnant women (23-26 weeks gestation) attending 7 urban medical centers based on ethnic origin	NS	6.1-22.7	Goldenberg *et al.*, 1996
		Alabama	1995-1998	584 women at baseline and at follow-up visits while attending STD clinics	18-35	16.1-16.6	Gallo *et al.*, 2012
		Chicago	1998-1999	117 woman attending Rush-Presbyterian-St. Luke's Medical Center's gynecology clinic	NS	27.4	Aroutcheva *et al.*, 2001
		Pittsburgh, Pennsylvania	1998-2001	255 women attending sexually transmitted diseases clinic	15-30	46	Wiesenfeld *et al.*, 2003
		US Marines	1999	1938 women entering Us Marines Corp	17-33	27	Yen *et al.*, 2003

		Philadelphia, Pennsylvania	1999-2001	69 pregnant women attending antenatal clinic. (18-35 weeks gestation)	15-46	5.8	Tolosa *et al.*, 2006
		Birmingham, Alabama	1999-2002	3,620 women	15-44	37.96	Brotman *et al.*, 2010
		United States	2001-2004	3739 women participating in the National Health and Nutrition Examination Survey	14-49	29.2	Koumans *et al.*, 2007
		Eight Florida Panhandle Counties	2003-2004	483 women, self-proclaimed douching practices and medical records	14-45	31.3 - 70.5	Cottrell, 2006
		Mississippi	2009-2010	African American women attending Mississippi State Department of Health STD Clinic.	18-45	47.4	(Muzny, Sunesara, Austin, *et al.*, 2013)
		United States	2005-2006 & 2010-2011	1,016 postmenopausal women at first time point and 883 at second time point	57-85	23.3 in 2005-2006 38.0 in 2010-2011	Hoffmann *et al.*, 2014
		United States	2006-2012	463,496 United States Army Women.	NS	16.6	Bautista, 2017
		United States	1994-2015	3,730 women participating in the Women's Interagency HIV Study	NS	48.7 cumulative	Massad *et al.*, 2017
		Baltimore, Maryland; Birmingham, Alabama; Durhamand Raleigh, North Carolina; Pittsburgh, Pennsylvania; and San Francisco, California	Pre-2016	1,365 women attending 1 of 10 family planning clinics, obstetrics-gynecology clinics, clinical research clinics, or STD clinics. All women are asymptomatic for BV	15-25	87 baseline 83-93 follow-up	Schwebke *et al.*, 2016
South America	Argentina	Santa Fe	2001-2003	400 women	15-55	13.5	Fosch *et al.*, 2006
		Buenos Aires	2012-2013	210 pregnant women attending 'Ramon Sarda' Maternity Hospital. (symptomatic n=80; asymptomatic n=130)	10-42	18.7 symptomatic 17.6 asymptomatic	Mucci *et al.*, 2016
	Brazil	Alagoas	1997	341 women from 4 villages	15-63	15.3	De Lima Soares *et al.*, 2003
		Vitoria, Espirito Santo	2003-2004	290 women attending Primary Health Unit	14-49	21.3	Barcelos *et al.*, 2008
		Serra Pelada, Para State	2004	209 women from mining village	NS	18.7	(Miranda *et al.*, 2009)
		Maringá, Paraná	2004-2006	133,966 cytology examinations of women from public and private health system for the presences of clue cells	13-80	4.8	(Storti-Filho *et al.*, 2011)

		Pacoti, Ceara	Pre-2007	579 women from 7 hamlets in Pacoti municipality	12-49	20	Oliveira *et al.*, 2007
		Botucatu, Sao Paulo	2006-2007	245 low-risk pregnant women (5-40 weeks gestation) attending 1 of 8 health clinics	12-44	21.6	Gondo *et al.*, 2010
		South-Eastern Brazil	2012-2013	1519 women attending cervical screening	14-54	30.1	(Marconi *et al.*, 2015)
	Chile	Concepción	1997-1998	242 women attending family clinics	NS	33.1	Castro *et al.*, 1999
	Chile	Santiago	2006	100 women attending family planning clinics	15-49	32.0	Lillo G *et al.*, 2010
	Colombia	Bogota	1999-2001	155 pregnant women attending antenatal clinic. (18-35 weeks gestation)	14-43	9.0	Tolosa *et al.*, 2006
	Ecuador	La Concordia	Pre-2010	213 adolescent females attending 1 of 2 high schools	13-17	31.5	Vaca *et al.*, 2010
	Peru	Lima	Pre-1998	630 women attending family planning clinic and/or gynecologist	15-35	30.0	(Sánchez *et al.*, 1998)
	Peru	Coastal, Highlands, Jungle Regions	1997-1998	754 women from 18 villages through community-based organization	18-67	40.8	García *et al.*, 2004
	Peru	Lima, Trujillo, Chiclayo	2001	779 low-income women	18-30	26.6	Jones *et al.*, 2007
Europe	Bulgaria	Plovdiv	Pre-1998	200 women attending dermatology and venereology clinics.	17-34	17.5	Tchoudomirova, 1998
Europe	Czech Republic	Brno	1990	600 women	NS	11.5	Unzeitig *et al.*, 1991
Europe	Denmark	Odense	1992-1994	2,927 pregnant women (17-24 weeks gestation)	NS	13.7	Thorsen *et al.*, 2006
Europe	Denmark	Denmark	1998-2002	3,526 pregnant women attending antenatal care (2nd trimester, <20 weeks gestation)	NS	16.0	Svare *et al.*, 2006
Europe	England	Bristol & Taunton	1996-1998	1111 women terminating a pregnancy at 1 of 3 hospitals; Southmead Hospital (Bristol), St. Michael's Hospital (Bristol) and Musgrove Park Hospital (Taunton)	16-44	29.3	Crowley *et al.*, 2001
Europe	England	Paddington, London	Pre-1997	38 women whose husbands attend genitourinary medicine clinics	NS	44.7	Keane *et al.*, 1997
Europe	England	London	1998-2000	1201 pregnant women attending 1 of 34 general practices or 5 family planning clinics (<10 weeks gestation)	16-48	14.5	(Oakeshott *et al.*, 2002)

		South Yorkshire	Pre-2008	1,073 asymptomatic pregnant women (15-24 weeks gestation)	11 - >40	3.5	Akinbiyi *et al.*, 2008
	Finland	Aland Islands	1993-2008	Cervical cancer screening for all women aged 20, 25, 30, 35, 40, 45, 50, 55, 60 the years of 1993 (n=819), 1998 (n=824), 2003 (n=790) and 2008 (n=771)	20-60	15.6 in 1993 11.9 in 1998 8.7 in 2003 8.6 in 2008	Eriksson *et al.*, 2010
	France	Nord-Pas-de Calais	2006-2008	14,193 pregnant women (1st trimester: before 14 weeks gestation)	NS	7.1	Desseauve *et al.*, 2012
	Ireland	Dublin	1999-2001	203 pregnant women attending antenatal clinic (18-35 weeks gestation)	16-44	5.9	Tolosa *et al.*, 2006
	Italy	Lombardo	1989-1994	1,441 asymptomatic pregnant women (3rd trimester: 8-9 months gestation)	NS	4.9	Cristiano *et al.*, 1996
	Lithuania	Vilnius & Marijampole	Pre-2017	116 women attending private gynecology clinics (119 samples)	22-53	24.4	(Janulaitiene *et al.*, 2017)
	Norway	Tromso	1996	168 pregnant women whom had applied for abortions in their 1st trimester	NS	24.0	Bjørnerem *et al.*, 1997
	Poland	Lodz region	2001	196 pregnant women from 10 district maternity units (8-16 weeks gestation)	NS	28.1	Kalinka *et al.*, 2002
		Zabrze	2001-2003	450 pregnant women attending antenatal clinics (6-36 weeks gestation)	NS	19.1	Kaźmierczak *et al.*, 2004
		South-Eastern Poland	Pre-2013	161 women; Attending PARENS infertility centre in Cracow, Poland (n=101), fertile (n=60)	20-40	7 infertile women	Tomusiak *et al.*, 2013
	Portugal	Lisbon	1993-1994	840 women attending clinics	22-39	7.0	Guerreiro *et al.*, 1998
		Braga & Senhora da Hora	2014-2016	Pregnant women attending either Hospital of Braga and Unidada Local de Saúde de Matosinhos.	M=30 +/- 5.16	3.8	Machado *et al.*, 2017
	Scotland	Aberdeen, Dundee & Glasgow	1995-1996	1599 women obtaining abortions at 1 of 4 Scotland hospitals (Aberdeen Royal Infirmary, Ninewells Hospital, Glasgow Royal Infirmary and Western Infirmary)	NS	17.5	Penney *et al.*, 1998
	Spain	Barcelona	1995-1996	293 pregnant women attending hospital clinic	M=29.9	7.5	Martínez de Tejada *et al.*, 1998
		Barcelona	1998	492 pregnant women (<28 weeks gestation)	M=27 +/- 5.5	4.5	Gratacós *et al.*, 1999

	Sweden	Stockholm & Eskilstuna	1989-1991	956 women attending youth and family planning clinics	M=25.7 +/- 6.9	13.7	(Nilsson *et al.*, 1997)
Asia	Bangladesh	Dhaka	2001-2002	399 married women attending 1 of 5 family planning clinics	NS	23.2	(Rahman *et al.*, 2008)
	China	Sichuan	2003-2004	2000 married women	20-49	15.4	Yongjun *et al.*, 2009
		Shandong Province	2004	4,039 married women from rural location	20-49	5.9	Fang *et al.*, 2007
		Tibetan region of Sichuan	2007	397 women	18-72	51.6	Dai *et al.*, 2010
		Beijing	2009	6,339 married women from 12 districts	15-54	8.7	Caiyan *et al.*, 2012
		Beijing	Pre-2010	6,337 women from 137 communes	25-54	8.7	(Xu *et al.*, 2010)
		Anhui Province	2010	53,286 married women from rural Anhui Province, China	18-49	11.9	(Li *et al.*, 2014)
		Maanshan city, Anhui Province	2012	793 pregnant women attending prenatal care	20-≥30	15.6	(Li *et al.*, 2015)
		Shanghai	2009-2013	3502 women attending Fengxian Hospital, Southern Medical University	NS	9.3	(Lu *et al.*, 2015)
	India	Goa	2001-2003	2494 women who lived near the Aldona primary health centre	18-45	17.8	Patel *et al.*, 2006
		Chennai	2002	487 women from urban slum area	18-40	24.6	Tolosa *et al.*, 2006
		New Delhi	2003-2004	502 pregnant symptomatic and asymptomatic women (14-28 weeks gestation)	18-35	11.2 symptomatic 7 asymptomatic	Dadhwal *et al.*, 2010
		Mysore	2005-2006	898 women attending 1 of 2 reproductive health clinics	15-30	19.1	Madhivanan *et al.*, 2009
		Lucknow	Pre-2010	200 pregnant women attending antenatal clinic in tertiary hospital	18-45	13	Lata *et al.*, 2010
		Meerut, Uttar Pradesh	2009-2010	500 pregnant women attending either Lala Lajpat Rai Memorial Medical College or associated Sardar Vallabhbhai Patel Hospital (<28 weeks gestation)	21-31	19.6	Gupta *et al.*, 2013
	Indonesia	Jakarta	1989-1990	490 pregnant women attending 1 of 3 hospitals (16-20 and 28-32 weeks gestation)	NS	17	Riduan *et al.*, 1993
		Manado	1999	357 women with intrauterine devices, attending a family planning clinic	NS	32.5	Joesoef *et al.*, 2001
	Iran	Fars Province	1996-1997	Married Qashqa'i women (n=839), married Mamasani Lor women (n=274) and married women from Shiraz	NS	40-50	Keshavarz *et al.*, 2001

		Hamedan	2005	540 women attending university hospital (Symptomatic n=270, control; asymptomatic n=270)	NS	28.5 symptomatic 0.4 asymptomatic	Shobeiri and Nazari, 2006
		NS	Pre-2006	425 pregnant women attending a prenatal clinic (37-42 weeks gestation)	NS	30.5	Ziaei *et al.*, 2006
		Zanjan	Pre-2009	500 married women attending 1 of 5 primary health care clinics	15-45	16.2	Bahram *et al.*, 2009
		Qom	Pre-2014	73 women attending infertility clinics in Qom City	21.1-38.5	70.3	Ghiasi *et al.*, 2014
	Japan	Otaru, Hokkaido	1993-2000	6,038 pregnant women attending Otaru Kyokai Hospital	14-46	13.6 in 1993 21.4 in 2000	Shimano *et al.*, 2004
	Japan	Gifu	1995	118 pregnant women attending the prenatal clinic at Iwasa Hospital	21-37	13.6	Puapermpoonsiri *et al.*, 1996
	Kenya	Mombasa	Pre-1999	HIV-1 seronegative female sex workers attending an STD clinic. Enrollment (n=647); Follow-up visits (n=3685)	18-48	36 enrollment 33 follow-up visit	Martin *et al.*, 1999
	Laos	Vientiane	2000-2001	1125 women attending gynecology outpatient department at Sethathirath Hospital	15-49	25	Sihavong *et al.*, 2007
	Laos	Vientiane	2001-2002	500 pregnant women attending 1 of 2 hospitals for antenatal care (≤ 20 weeks gestation)	17-40	22	Thammalangsy *et al.*, 2006
	Malaysia	Kuala Lumpur	2012	207 women admitted to Hospital Kuala Lumpur.	NS	30.9	Younus *et al.*, 2017
	Myanmar	Yangon	1999-2001	227 pregnant women attending antenatal clinic (18-35 weeks gestation)	11-42	15.6	Tolosa *et al.*, 2006
	Nepal	Kathmandu	2006	200 pregnant women attending Paropakar Maternity and Women's Hospital	NS	28	(Shrestha *et al.*, 2011)
	Pakistan	Rawalpindi	2007-2008	100 women who had preterm labour	NS	21.0	Islam *et al.*, 2009
	Philippines	Manila	1999-2001	202 pregnant women attending antenatal clinic (18-35 weeks gestation)	15-43	7.5	Tolosa *et al.*, 2006
	Thailand	Khon Kaen	1995	208 pregnant women attending the clinic at Khon Kaen University's hospital, Srinagarind Hospital	15-40	15.9	Puapermpoonsiri *et al.*, 1996
	Thailand	Khon Kaen	1999-2001	200 pregnant women attending antenatal clinic (18-35 weeks gestation	16-42	11.5	Tolosa *et al.*, 2006

		Bangkok	1999-2001	227 pregnant women attending antenatal clinic (18-35 weeks gestation	16-42	12.5	Tolosa *et al.*, 2006
		Bangkok	2003	300 women with IUDs, attending family planning clinic at Siriraj Hospital	NS	20.3	Harikarnpukdee *et al.*, 2004
	Vietnam	Nghe An Province	2004	505 pregnant women attending antenatal clinics	15-49	7.0	(Goto *et al.*, 2005)
		Haiphong	Pre-2006	197 women from rural village	18-49	27.4	Go *et al.*, 2006
		Bavi District	2006	1012 married women from rural district	18-49	11.0	Lan *et al.*, 2008
Africa	Botswana	Gaborone	2000	703 pregnant women attending 1 of 13 antenatal clinics	15-43	38.1	Romoren *et al.*, 2007
	Burkina Faso	Boulgou, Poni, Seno and Yatenga Provinces	2003	2133 pregnant women from 96 antenatal clinics	15-49	6.4	Kirakoya-Samadoulougou *et al.*, 2008
		Ouagodougou	2003	2018 pregnant women and 883 non-pregnant women	15-49	7.9	Kirakoya-Samadoulougou *et al.*, 2011
	Central African Republic	Bangui	1996	481 pregnant women attending 1 of 3 antenatal clinics	< 22	29.1	Blankhart *et al.*, 1999
	Egypt	Assiut	2001-2002	468 3rd trimester pregnant women who are at high-risk for Premature rupture of the membranes (PROM) and/or preterm labour	NS	33.3	Darwish *et al.*, 2005
	Ghana	Accra	2001	100 women attending family planning clinic	19-48	25	Lassey *et al.*, 2005
	Mozambique	Maputo	2002-2003	435 women attending SAAJ clinic at Maputo Central Hospital for the 1st time	14-24	12.9	Melo *et al.*, 2008
	Nigeria	Lagos & Yaba-Lagos	2000	140 women with vaginal discharge Cervical Cytology Clinic of the Lagos University Teaching Hospital.	18-50	18.6	Anorlu *et al.*, 2004
		Araba, Lagos	2012-2013	270 pregnant women attending antenatal clinic at Lagos University University Teaching Hospital (14-36 weeks gestation)	22-40	26.0	Afolabi *et al.*, 2016
		Port Harcourt	2014	356 women (178 fertile, 178 tubal factor infertility)	NS	17.8	Durugbo *et al.*, 2015
	South Africa	Durban	1994-1995	168 pregnant women (30 weeks or less gestation)	16-44	52.0	(Govender *et al.*, 1996)
		Khayelitsha	2000-	5,110 women participating in cervical	35-65	58.3	Myer *et al.*, 2005

			2002	cancer screening trial, follow-up with 3,199 women and 86 HIV seropositive women			
		Rural KwaZulu	2002	226 women attending family planning clinics and 48 women attending antenatal clinic	14-52	58.4	(Frohlich *et al.*, 2007)
		Johannesburg	2005-2009	1,954 HIV-seropositive women attending Themba Lethu Clinic	18-65	54.0	Denslow *et al.*, 2011
		Elandsdoorn	Pre-2011	101 women attending Ndlovu Medical Centre, an HIV testing centre	NS	33.7	Dols *et al.*, 2011
	Tanzania	Moshi	1999	382 women attending maternal child health and family planning clinics	16-46	33.9	Msuya *et al.*, 2002
		Moshi	2002-2004	2654 pregnant women attending 1 of 2 primary healthcare clinics. (3^{rd} trimester)	14-43	20.9	Msuya *et al.*, 2009
	The Gambia	Farafenni	1999	1348 women from 20 villages	15-54	37	Walraven *et al.*, 2001
		NS	2000	30 married women from 5 villages	20-53	37	(Morison *et al.*, 2005)
	Uganda	Rakai	Pre-2006	1,264 married women with circumcised and uncircumcised husbands	NS	30.5 circumcised husbands 38.3 uncircumcised husbands	Gray *et al.*, 2009
	Zimbabwe	Harare	1999-2001	210 pregnant women attending antenatal care. (18-35 weeks gestation)	12-41	24.4	Tolosa *et al.*, 2006
		Harare	2002-2003	678 pregnant women partaking in Prevention of Mother to Child Transmission program (36 weeks gestation)	M=24.2 +/- 5.1	32.6	Kurewa *et al.*, 2010
		NS	Pre-2016	571 women attending family planning and general health care clinics utilized in in Hormonal Contraception and Risk of HIV Acquisition Study	18-35	30.8	Turner *et al.*, 2016
Australia	Australia	Melbourne	2008	528 women attending Melbourne University	17-21	4.7	Fethers *et al.*, 2009
		NS	Pre-2013	1,093 women attending 29 primary care facilities	16-25	11.8	(Bradshaw *et al.*, 2013)
Oceania	Papua New Guinea	Asaro Valley	1995	201 women from 16 rural villages	15-45	9.0	(Passey *et al.*, 1998)
	New Zealand	Otago Region	2005-2007	69 pregnant women attending Queen Mary Maternity Services	M=32	8.7	Lim *et al.*, 2010

Appendix B Supplements, Reagents and Culture Media

B.1 Supplements and reagents

5 mg/mL hemin working solution:

A volume of 0.5g of hemin powder was added to 10mL of M NaOH and mixed until dissolved. A volume of 900mL of sterile ddH_2O was added to the solution and sterilized by autoclave, using liquid cycle at 121°C for 15 minutes. Solution was allowed to cool to room temperature then stored in the dark at 4°C for a maximum of 30 days.

95 % (v/v) ethanol working solution:

A volume of 5mL sterile ddH_2O was added to 95mL anhydrous ethanol and mixed well by inversion.

10 mg/mL vitamin K_1 stock solution:

A volume of 0.2mLVitamin K_1 solution was added to 20mL 95% EtOH and stored in the dark at 4°C.

1 mg/mL vitamin K_1 working solution:

A volume of 1mL Vitamin K_1 stock solution (10mg/mL) was added to 9mL sterile ddH_2O and mix by inversion. Solution stored in the dark at 4°C for a maximum of 30 days.

20% (v/v) glycerol:

A volume of 100mL glycerol was added to 400mL of sterile ddH_2O. The solution was sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

Laked, defiberated sheep blood:

Defibrinated sheep blood was frozen at -20°C for a minimum of 8 hours and then warmed to 37°C.

B.2 Culture media

BHI broth:

Approximate formula per litre:

Casein Peptone	15.0 g
Meat peptone/brain heart infusion	12.0 g
Dipotassium phosphate	2.5 g
Dextrose	2.0 g
Yeast extract	5.0 g
Sodium chloride	5.5 g

A total of 42g dehydrated BHI broth media and 1g soluble starch was dissolved in 1L ddH_2O. The media was sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

BHI top agar (0.7%) *supplemented with 1% starch:*

7g of agar was added with 42g of dehydrated BHI broth media and 1g soluble starch to 1L of ddH_2O. Media was mixed and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

BHI agar (1.5%) supplemented with 1% starch:

15g of agar was added with 42g of dehydrated BHI broth media and 1g soluble starch to 1L of ddH_2O. Media was mixed with magnetic stir bar and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

BHI agar (1.5%) supplemented with 1% starch and 10% FBS:

15g of agar was added with 42g of dehydrated BHI broth media and 1g soluble starch to 900mL of ddH_2O. Media was mixed with magnetic stir bar and sterilized by autoclave, using liquid

cycle at 121°C and 15 Psi for 30 minutes. Once media cooled to approximately 50°C 100mL of FBS was added.

BHI agar (1.5%) supplemented with hemin and vitamin K_1:

15g of agar was added with 42g of dehydrated BHI broth media 100mL of ddH_2O. 1mL hemin working solution (5mg/mL) and 1 mL vitamin K_1 working solution (1mg/mL) were added to the media. Media was mixed with magnetic stir bar and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes. Once media cooled to approximately 50°C 100mL of FBS was added.

BHI Bilayer plates (1.5%):

For the bottom layer 7g of agar was added with 42g of dehydrated BHI broth media and 1g souble starch to 1L of ddH_2O. Media was mixed with magnetic stir bar and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes. Once media cooled to approximately 50°C antibiotic working solutions for, 1mL of nalidixic aid sodium salt (20mg/mL) and 150µL of amphotericin B (20mg/mL) were added to the media. In a 30mm petri dishes, 15mL of media was dispensed.

For the top layer 7g of agar was added with 42g of dehydrated BHI broth media and 1g soluble starch to 900mL of ddH_2O. Media was mixed and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes. Once media cooled to approximately 50°C antibiotic working solutions for, 1mL of nalidixic aid sodium salt (20mg/mL) and 150µL of amphotericin B (20mg/mL) were added to the media. Additionally, 100mL of pre-warmed (37°C) human whole blood were added to the media and mixed with magnetic stir bar. Once bottom layer was solidified 10mL of media was dispensed into petri dishes.

TSA supplemented with 5% sheep blood:

Approximate formula per litre:

Pancreatic digest of casein	17.0 g

Papaic digest of soybean	3.0 g
Dextrose	2.5 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5g

15g of agar was added with 30g of dehydrated TSA media was dissolved in 950mL ddH_2O. The Solution was sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes. Once media cooled 500mL of pre- warmed (37°C) laked, defiberated sheep blood was mixed into the media with magnetic stir bar. In a 92mm petri dishes, 42mL of media was dispensed to create a nutrient agar depth of 4mm +/- 0.5mm.

CB broth:

Formula per litre:

Special peptone mix	23.0 g
Sodium chloride	5.0 g
L-cysteine	0.1 g
Sodium carbonate	0.6 g
Dextrose	2.5 g
Magnesium sulfate	0.1 g
Ferrous sulfate	0.02 g
Tris	0.83 g
Tris, hydrochloride	2.86 g

A total of 35g dehydrated CB broth media was dissolved in 1L ddH_2O. The media was sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

CB agar (1.5%):

15g of agar was added with 35g of dehydrated CB broth media to 1L of ddH_2O. Media was mixed and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

MH broth:

Approximate formula per litre:

Dehydrated infusion from beef	300.0 g
Casein hydrolysate	17.5 g
Starch	1.5 g

A total of 21g dehydrated MH broth media was dissolved in 1L ddH_2O. Media was mixed with magnetic stir bar and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

MH agar (1.5%):

15g of agar was added with 21g dehydrated MH broth media was dissolved in 1L ddH_2O. Media was mixed with magnetic stir bar and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes. In a 92mm petri dishes, 42mL of media was dispensed to create a nutrient agar depth of 4mm +/- 0.5mm.

MRS broth:

Approximate Formula per litre:

Proteose peptone no. 3	10.0g
Beef extract	10.0g
Yeast extract	5.0g
Dextrose	20.0g
Polysorbate 80	1.0g
Ammonium citrate	2.0g
Sodium Acetate	5.0g
Magnesium sulfate	0.1g
Manganese sulfate	0.05g

Dipotassium phosphate	2.0g

A total of 55g dehydrated MRS broth media was dissolved in 1L ddH_2O. The media was sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

MRS agar (1.5%):

15g of agar was added with 55g dehydrated MRS broth media was dissolved in 1L ddH_2O. Media was mixed with magnetic stir bar and sterilized by autoclave, using liquid cycle at 121°C and 15 Psi for 30 minutes.

Appendix C Molecular Techniques: Buffer and Reagent Preparations

C.1 Common buffers

1M Tris-HCl, pH 8.0:

121.1g Tris base was dissolved in 800ml sterile ddH_2O. Concentrated HCl was added dropwise until a pH 8.0 was achieved. Additional sterile ddH_2O was added to bring the final volume to 1L and mixed well.

0.5M EDTA, pH 8.0:

186.12 g of $EDTA.Na_2.2H_2O$ was mixed with 800 ml ddH_2O.While mixing vigorously, NaOH was added into the solution until a pH 8.0 was achieved. Additional sterile ddH_2O was added to bring the final volume to 1L and mixed well.

C.2 Bacterial cell lysis

Cell lysis buffer, pH 8.0:

250 ml 0.1M Tris-HCl pH 8.0 was added to 735mL of sterile ddH_2O. Additionally, 5mL of 0.5 mM EDTA pH, 8.0 and 10 ml 100% Triton X-100 were added. Solution was mixed well.

10% Sodium dodecyl sulfate (SDS):

100g of SDS was added to 800 ml sterile ddH_2O and stirred on a magnetic stirrer until dissolved. Additional sterile ddH_2O was added to bring the final volume to 1L and mixed well.

C.3 Agarose gel electrophoresis

50X Tris acetate (TAE), pH 8.3:

242g of Tris base was dissolved in 500mL of sterile ddH_2O. 57.1mL of 1M Glacial acetic acid and 100mL 0.5M EDTA pH, 8.0 were also added. Additional sterile ddH_2O was added to bring the final volume to 1L and mixed well.

1X TAE running buffer:
80 mL 50X TAE concentrated stock solution was mixed with 3.92L of sterile ddH_2O.

0.7% (w/v) agarose gel:
0.7g of electrophoresis grade agarose was added to 100mL of 1X TAE running buffer. Solution was mixted well and heated until boiling point reached, and agarose was dissolved.

2.0% (w/v) agarose gel:
2g of electrophoresis grade agarose was added to 100mL of 1X TAE running buffer. Solution was mixed well and heated until boiling point reached, and agarose was dissolved.

6X DNA loading buffer pH 8.0:
2mL of sterile ddH_2O was combined with 6mL of glycerol, 1mL of 0.5M EDTA pH 8.0 and 1mL of 1% bromophenol blue in ddH_2O. A volume of 1mL was transferred to 2mL cryotubes and stored at -20°C.

C.4 SDS-PAGE electrophoresis

Acrylamide/Bis acrylamide (30%, 29:1):
145g of acrylamide was added with 5g of bis-acrylamide in 500mL of sterile ddH_2O. Solution was mixed until dissolved, filtered and stored at 4°C.

4X buffer A (1.5M Tris-HCl, pH 8.8):
54.51g of tris base was added to 150mL of sterile ddH_2O. Concentrated HCl was added dropwise until a pH 8.0 was achieved. Additional sterile ddH_2O was added to bring the final volume to 300mL and mixed well.

4X buffer B (0.5M Tris, pH 6.8):

6g of tris base was added to 60mL of sterile ddH_2O. Concentrated HCl was added dropwise until a pH 8.0 was achieved. Additional sterile ddH_2O was added to bring the final volume to 100mL and mixed well.

10% Sodium dodecyl sulfate (SDS):

Refer to B.2. Bacterial cell lysis

10% Ammonium persulfate (APS):

0.1g of ammonium persulfate was dissolved in 1mL of sterile ddH_2O.

Sample buffer 2X:

A volume of 2.5mL of 4X buffer B, 2.0mL of glycerol, 2.5mL of 10% SDS, β-mercaptoethanol, 0.2mL of 0.2% bromophenol blue and 2.3mL of ddH_2O.

5X Running Buffer:

15.14g of tris base, 7g of glycine, 5g of SDS was dissolved into 1L of ddH_2O.

1X Running Buffer:

A volume of 100mL of 5X running buffer was diluted to 400mL of ddH_2O.

12% separating gel:

The following reagents are added in order of appearance:

Acrylamide/Bis acrylamide (30%, 29:1)	4mL
ddH_2O	3.3mL

4X Buffer A	2.5mL
10% SDS	100µL
10% APS	50µL
TEMED	5µL

Mixed by inversion and pipette into gel apparatus.

15% separating gel:

The following reagents are added in order of appearance:

Acrylamide/Bis acrylamide (30%, 29:1)	5mL
ddH_2O	2.345mL
4X Buffer A	2.5mL
10% SDS	100µL
10% APS	50µL
TEMED	5µL

Mixed by inversion and pipette into gel apparatus.

4% stacking gel:

The following reagents are added in order of appearance:

Acrylamide/Bis acrylamide (30%, 29:1)	533µL
ddH_2O	2.4mL
4X Buffer B	1mL
10% SDS	40µL
10% APS	20µL
TEMED	4µL

Mixed by inversion and pipette into gel apparatus.

Appendix D Antibiotic Susceptibility

D.1 Quality control standards

Table A.2 Expected and observed MIC values and susceptibility ratings for quality control strain, *E. faecalis* ATCC 29212. S: Susceptible; I: Intermediate; R: Resistant; "-": No Defined Standards.

	Expected				**Observed**	
Antibiotic	MIC Ranges (μg/mL)	S	I	R	MIC (μg/mL)	Susceptibility
Clindamycin (CM)	4-16	-	-	-	12-16	-
Linezoid (LZ)	1-4	≤4	-	>4	3	S
Vancomycin (VA)	1-4	≤4	-	>4	3	S
Rifampicin (RI)	0.5-4	-	-	-	0.5	-
Metronidazole (MZ)	-	-	-	-	>256	-
Ciprofloxacin (CI)	-	-	-	-	0.38	-
Ampillicin (AM)	0.5-4	≤4	-	8	0.75	S
Tetracycline (TC)	8-32	-	-	-	8-16	-

Appendix E Plate Layouts: Bacterial and Supernatant

E.1 High throughput target range assay

—	—	—	—	—	—	—	—	—	—	—	—
BHI	—	α523	—	αGV18-1S	—	αGV18-2S	—	αGV18-3	—	αGV18-5	—
—	αGV18-6L	—	αGV18-7aW	—	αGV18-7b	—	αGV18-8	—	αGV18-9	—	αGV18-11
αGV18-12S	—	αGV18-13	—	αGV18-14S	—	αGV18-15	—	αGV18-16	—	αGV18-17	—
—	αGV18-18	—	αGV18-19W	—	αGV18-19YW	—	αGV18-20	—	αGV19-1	—	αGV19-2S
αGV19-3	—	αGV19-4S	—	αGV19-4L	—	αGV19-5	—	αGV19-6	—	αGV19-7	—
—	αGV19-8	—	αGV19-9	—	αGV19-10L	—	αGV19-11	—	αGV19-12S	—	αGV19-13
αGV19-14	—	—	—	—	—	—	—	—	—	—	—

Figure E.1 Layout of bacterial cultures in 96 well plate utilized in high throughput target range assay.

E.2 Drop on the lawn

BHI	α523	α523 (PBSU101)	αGV18-20	αGV18-1S	αGV18-5
αGV18-6L	αGV18-11	αGV19-10L	αGV19-11	αGV19-1	–
αGV18-15	αGV19-9	αGV19-13	αGV18-8	αGV18-18	αGV18-17

Plate 1

BHI	α523	α523 (PBSU101)	αGV19-6	αGV19-12S	αGV19-3
αGV19-7	αGV19-4S	αGV19-14	αGV18-12S	αGV19-2S	–
αGV18-19YW	αGV18-9	αGV18-7b	αGV18-7aW	αGV18-13	–

Plate 2

Figure E.2 Layout of chloroform extracted supernatants for drop on the lawn experiment.

BHI	α523 (PBSU101)	αGV18-20	αGV18-1S
αGV18-5	αGV18-6L	αGV18-11	αGV19-10L
αGV19-11	αGV19-1	αGV19-15	αGV19-9

Plate 1

BHI	αGV19-13	αGV18-8	αGV18-18	αGV18-17
αGV19-6	αGV19-12S	αGV19-3	αGV19-7	–
αGV19-4S	αGV19-14	αGV18-12S	αGV19-5	–

Plate 2

Figure E.3 Layout of methanol extracted supernatants for drop on the lawn experiment.