EVALUATION OF PROBIOTIC AND VAGINAL *LACTOBACILLUS* SPECIES FOR THE TREATMENT OF BACTERIAL VAGINOSIS AND PROMOTION OF VAGINAL HEALTH IN SOUTH AFRICAN WOMEN

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PLAGIARISM DECLARATION

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

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Science is not about certainty. Science is about finding the most reliable way of thinking at the present level of knowledge.

Carlo Rovel

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ABSTRACT

Background: Bacterial vaginosis (BV) increases women's risk for adverse reproductive outcomes and acquisition of sexually transmitted infections (STIs), including HIV. The etiology of BV is still unclear, and it has been hypothesized that lytic or temperate *Lactobacillus* bacteriophages may contribute. The current standard of care for BV are antibiotics like metronidazole or clindamycin, although these are not effective long-term, as rates of BV recurrence are high. There is thus an urgent need for durable treatment of BV to be developed. Probiotics administered adjunctively to antibiotics may improve efficacy and durability of BV treatment, but no randomized trial comparing antibiotic treatment to probiotics as an adjunct to antibiotics has been performed in South Africa, despite BV rates >50%. The South African Health Products Regulatory Authority (SAHPRA) regulates drugs and health supplements, into which probiotics are categorized locally. However, no probiotic product has yet been registered with SAHPRA. Further, there have been no recent surveys of the availability of probiotics for vaginal health in South Africa; neither has their suitability to be used in the treatment for BV been evaluated.

Aims: The specific aims of this dissertation were (1) to survey the South African probiotic market and evaluate locally available products marketed explicitly for vaginal health *in vitro*, (2) to determine the efficacy of the most promising local over-the-counter (OTC) probiotic for vaginal health for BV treatment in South African women in a pilot randomized clinical trial that is approved by the regulatory authorities in South Africa, in order to explore the local regulatory landscape for future trials; (3) to screen and thoroughly characterize vaginal *Lactobacillus* strains isolated from healthy South African women *in vitro* for the development of a geographically-specific probiotic for vaginal health; and (4) to evaluate the role of bacteriophages in the etiology of BV and *Lactobacillus* spp. survival *in vitro*.

Approach and results: To review probiotics available on the South African retail market, a cross-sectional survey using two-stage cluster sampling was conducted in Durban and Cape Town. Of the 104 unique probiotic products identified, only four were explicitly for vaginal health, although they contained bacterial species commonly found in the gastro-intestinal tract (GIT) and not lower female genital tract

(FGT). The probiotics marketed for vaginal health were analysed for the bacterial contents, concentration per dose, growth kinetics, influence of bacterial growth on culture pH, adhesion to cervical cells, production of L-and D-lactic acid as well as H_2O_2, inhibitory activity against *G. vaginalis* and *P. bivia* and Group B *Streptococcus* (GBS), their susceptibility to antibiotics, and mucosal safety (using cytokine biomarkers). Batch testing revealed that they mainly contained the bacterial species and dose as claimed by the manufacturers. The contained bacterial isolates had promising probiotic characteristics, but there was some variability in the biological characteristics of isolates from different lot numbers of some of the products.

A single-blind, randomised SAHPRA-approved trial enrolling BV positive, STI negative (including discharge-causing STIs; *C. trachomatis, T. vaginalis, M. genitalium* and *A. vaginae*) South African women was initiated to compare standard of care (SOC, MetroGel™ V, n=20) to a combination of metronidazole and a commercially-available probiotic (Vagiforte® PLUS Combo Pack, containing *L. rhamnosus, L. acidophilus, B. longum and B. bifidum* in oral capsules and vaginal spay, treatment duration 15 days) marketed for vaginal health, including treatment of BV and vaginal thrush, in South Africa (n=30, intervention group). The primary endpoint of the pilot study was BV cure one month after treatment completion. BV was assessed by Nugent scoring, vaginal pH was measured, IL-1α concentrations in cervicovaginal fluid were measured by ELISA as a biomarker of genital inflammation, and quantitative PCR (qPCR) was performed to measure the abundance of several vaginal *Lactobacillus* spp. (including *L. crispatus, L. gasseri, L. jensenii, L. vaginalis, L. mucosae* and *L. iners*), in addition to key BV-associated bacteria (including *G. vaginalis, P. bivia, A. vaginae*, BVAB2 and *Megasphaera* 1), the bacterial species contained in Vagiforte® (including *L. acidophilus, L. rhamnosus, B. bifidum* and *B. longum*) and *Candida* spp. (including *C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. lusitaniae, C. parapsilosis,* and *C. tropicalis*), as a common side effect of metronidazole treatment is vaginal thrush. An interim analysis of the first 24 participants who have completed the trial is included in this dissertation, as the trial is still ongoing. The probiotic was found to be well accepted and no product related adverse events were reported, although women commonly experienced vaginal *Candida* infections after topical metronidazole use. In the interim analysis, BV cure rates were similar between the SOC and intervention group, as was vaginal

pH and the abundances of *Lactobacillus* spp. and most BV-associated bacteria. Women randomized to the intervention group had higher levels of *B. bifidum* and *B. longum* after treatment, which tended to go along with increased levels of *Candida* spp. and some BV-associated bacteria, while *L. crispatus* levels were lower in these women. This shows the urgent need to develop a vaginal probiotic containing *Lactobacillus* strains that are commensal to the FGT, to ensure achieving the desired effect of adjunctive probiotics in BV treatment.

Thus, the characteristics of the commercially in South Africa available probiotic strains were compared to clinical vaginal *Lactobacillus* strains isolated from healthy South African women. A weighted scoring system was developed to select candidate strains for the development of a vaginal probiotic. Towards this aim, 57 *Lactobacillus* strains were isolated from healthy South African women (including 10 *L. crispatus,* 9 *L. gasseri,* 18 *L. jensenii,* 8 *L. vaginalis,* and 12 *L. mucosae* strains) which were distinct to the commercially available probiotic strains, and several isolates exhibited better probiotic characteristics *in vitro* than the commercially available probiotic bacterial strains (such as ability to lower pH and adherence to cervical cells), although this appeared to be highly strain- and not species specific. Based on weighted scores, two *L. crispatus*, two *L. jensenii*, and one *L. vaginalis* and one *L. gasseri* strain isolated from BV and STI negative South African women were selected for the development of a local probiotic for vaginal health.

The presence of bacteriophages that target vaginal *Lactobacillus* spp. in cervicovaginal secretions of women with and without BV was evaluated using serial bacteriophage transfer and plaque assays. No lytic bacteriophages that targeted vaginal *Lactobacillus* spp. (including *L. crispatus, L. gasseri, L. jensenii, L. vaginalis* and *L. mucosae*) were isolated from FGT secretions, although CRISPR loci were common in publically available full *Lactobacillus* genome sequences. However, temperate bacteriophages were induced from the majority (71.8%) of the clinical *Lactobacillus* strains and 61.1% of the probiotic *Lactobacillus* strains screened using Mitomycin C, which was confirmed by transmission electron microscopy. Based on their morphology, these *Lactobacillus* bacteriophages belonged to the families of *Sipho*-, *Myo*- and *Podoviridae*.

Conclusions: There are very few probiotics for vaginal health on the South African market, and the development of a probiotic containing commensals of the lower FGT should urgently be considered. Lytic bacteriophages targeting *Lactobacillus* spp. were not found in this study, although temperate bacteriophages were common and could influence *Lactobacillus* survival *in vivo*. Screening women with vaginal discharge for the SAHPRA-acknowledged pilot probiotic trial of Vagiforte PLUS® confirmed a high burden of STIs in Cape Town, South Africa, and that the symptom vaginal discharge is a very poor predictor for BV. The pilot trial showed that large double-blind, randomized, placebo-controlled trials with adequate screening and enrolment algorithms and sample sizes, using a product containing vaginal *Lactobacillus* spp., are needed to determine the efficacy of adjunctive probiotics on BV cure and recurrence in South African women. Finally, while the products currently being marketed for vaginal health in South Africa and worldwide mostly do not contain *Lactobacillus* spp. commonly found in the lower FGT, several promising candidates from the FGTs of healthy, young, HIV- and BV- South African women were isolated and characterized that may prove more efficacious in treating BV. These have the potential to make a big impact on reproductive outcomes and HIV risk in young South African women.

LIST OF ABBREVIATIONS

α	Alpha
ADP	Adenosine 5'-diphosphate
AE	Adverse event
AGYM	Adolescent girls and young women
ATP	Adenosine 5'-triphosphate
AUC	Area under the curve
β	Beta
BHI	Brain heart infusion
BPDE	benzo(a) Pyrene diol epoxide
BV	Bacterial vaginosis
CAGR	Compound annual growth rate
$CaCl_2$	Calcium chloride
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming units
CI	Confidence interval
COX	Cyclooxygenase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CV	Coefficient of variation
DAEC	Diffusely adherent *E. coli*
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DOH	Department of Health
dsDNA	double stranded DNA
DTHF	Desmond Tutu HIV Foundation
EaggEC	Enteroaggregative *E. coli*
EB	Elementary body
EFSA	European Food Safety Authority
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *E. coli*
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ex/em	Excitation/emission
FCS	Fetal calf serum
FDA	Food and Drug Administration
FGT	Female genital tract
Foxp3	Forkhead box P3
γ	Gamma
g	Gramm
GBS	Group B Streptococcus
GCP	Good Clinical Practice
GDP	Gross Domestic Product
GI	Gastrointestinal
GIT	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practice
GRO	Growth-regulated oncogene

H_2O_2	Hydrogen peroxide
H_2O	Water
HBD	Human beta defensin
HIV	Human immunodeficiency virus
HPV	Human Papillomavirus
HREC	Human Research Ethics Committee
HRP	Horseradish peroxidase
HSV	Herpes Simplex Virus
IFU	Inclusion-forming unit
IL	Interleukin
IL1-RA	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
IP	Interferon gamma-induced protein
IS	Immune system
ITT	Intention-to-treat
kB	kilobase
LB	Luria broth
LPS	Lipopolysaccharide
MDC	Macrophage-derived chemokine
mg	Milligram
mL	Millilitre
mM	Millimolar
$MgSO_4$	Magnesium sulfate
µg	Microgram
µL	Microliter
µM	Micromolar
MIC	Minimum inhibitory concentration
MIF	Macrophage migration inhibitory factor
min	Minutes
MIP	Macrophage inflammatory protein
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NHLS	National Health Laboratory Service
NHREC	National Health Research Ethics Council
nm	Nanometre
NPV	Negative predictive value
L	Litre
OD	Optical density
OR	Odds ratio
OTC	Over-the-counter
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PID	Pelvic inflammatory disease
pK_a	Negative base-10 logarithm of the acid dissociation constant
PP	Per-protocol
PPP	Preferred product profile
PPV	Positive predictive value
PSA	Prostate-specific antigen
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted

RCT	Randomized controlled trial
RORγt	Retinoic-acid-receptor-related orphan nuclear receptor gamma
RR	Relative risk
rRNA	Ribosomal ribonucleic acid
SA	South Africa
SAHPRA	South African Health Products Regulatory Authority
SANAS	South African National Accreditation System
SB	Schaedler medium supplemented with 5% blood
SOC	Standard of care
Sp.	Singular species
Spp.	Plural species
SPG	Sucrose phosphate glutamate
STEC	Shiga-toxin producing *E. coli*
STI	Sexually transmitted infection
TB	Tuberculosis
TEM	Transmission electron microscopy
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T Cells
TS	Tryptic soy
UCT	University of Cape Town
US	United States
USD	United States dollar
UV	Ultraviolet
v/v%	Volume/volume percent
WGS	Whole genome sequencing
WISH	Women's Initiative in Sexual Health
WHO	World Health Organization
ZAR	South African Rand
ZOI	Zone-of-inhibition
%	per cent
°C	Degrees Celsius

Chapter 1

Literature review

1.1 The female genital tract

1.1.1 Microbiota associated with vaginal health and bacterial vaginosis

Maintenance of vaginal health is thought to play an important role in protecting women from reproductive complications and acquisition of sexually transmitted infections (STIs; Nelson *et al*. 2014; Haggerty *et al*. 2016; Cohen *et al.* 2012; Van de Wijgert *et al*. 2008; Anahtar *et al*. 2016; Brotman *et al*. 2010). The lower female genital tract (FGT) of healthy women is generally considered to have a pH < 4.5 (Boskey *et al*. 1999; A. Aroutcheva *et al*. 2001) and a microbiota dominated by *Lactobacillus* species (spp.), with *L. crispatus*, *L. gasseri*, *L. jensenii*, *L. iners* and *L. vaginalis* being the most prevalent (Ravel *et al*. 2011; Gajer *et al*. 2012).

Commonly, however, the lower FGT microbiome shifts from one that is dominated by commensal *Lactobacillus* spp. (Eschenbach *et al*. 1989; Macklaim *et al*. 2013) to a polymicrobial dysbiosis that is called bacterial vaginosis (BV), defined by intense overgrowth of a diverse range of pathogenic anaerobic bacteria (100 to 1000-fold above normal; Srinivasan *et al.*, 2012; Macklaim *et al.*, 2013), and corresponding to an elevated vaginal pH ≥ 4.5 (Taha *et al*. 1998; Macklaim *et al*. 2013; Srinivasan *et al*. 2012). Women with BV are typically colonized by a spectrum of anaerobes, including Gram-positive anaerobic bacteria like *Gardnerella vaginalis*, *Atopobium vaginae*, *Eggerthella* spp., *Peptoniphilus* spp. and BV-associated bacteria (BVAB, BVAB1-3, Clostridia-like bacteria), anaerobic Gram-negative bacteria like *Mobiluncus* spp., *Prevotella* spp., *Fusobacterium nucleatum*, and *Megasphaera* type 1, and bacteria without cell walls, like *Mycoplasma hominis* and *Ureaplasma urealyticum* (Africa *et al*. 2014; Lennard *et al*. 2017; Srinivasan *et al*. 2012).

Microbial changes associated with BV are sometimes accompanied by clinical symptoms, including a thin, greyish-white, malodorous discharge, and the presence of clue cells (vaginal epithelial cells covered with bacteria, Taha *et al*. 1998). However, approximately 85% of women with BV are asymptomatic (Koumans *et al*. 2007; Anukam *et al*. 2006), therefore do not seek health care, and crucially, are not treated. The fact that a large majority of BV cases are asymptomatic does not, however, mean that the risk of severe clinical outcomes is lower, as asymptomatic BV produces levels of genital inflammation equivalent to symptomatic BV (Mlisana *et al*. 2012).

Studies have suggested that the bacterial species colonizing the lower FGT in healthy women may differ by geographic location and ethnicity (Ravel et al. 2011; Zhou et al. 2007; Anahtar et al. 2015a). *Lactobacillus* dominance differed by ethnicity in women living in the United States (US), with white and Asian women being more likely to have vaginal microbiota dominated by *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii* and lower vaginal pHs than black and Hispanic women (Ravel *et al*. 2011). Similarly, Fettweis *et al*. (2014) reported that black women in the US were more likely to be colonised with more diverse organisms, including *L. iners*, *G. vaginalis*, *Sneathia, Prevotella, Atopobium, Mycoplasma hominis, Aerococcus,* 1 BVAB1, BVAB2 and other anaerobes, than white women. In three different geographic locations in South Africa (KwaZulu Natal, Johannesburg and Cape Town), the predominant *Lactobacillus* sp. found in young women was *L. iners* (Anahtar et al. 2015b; Lennard et al. 2017; Klatt et al. 2017).

1.1.2 Temporal changes in vaginal microbial communities

The majority of studies describing the composition of bacterial species in the lower FGT sampled at only one time point and a single location (mostly lateral vaginal wall), and do not take microbial changes over time or by location into account. Dunstan and Johnson (2006) have previously concluded that unstable bacterial communities are more susceptible to the invasion of pathogens, a concept broadly noted in microbial ecology. Gajer *et al*. (2012) described temporal dynamics in the composition of vaginal bacterial communities of US women over 16 weeks (~4 menstrual cycles), and found that some bacterial communities where less stable over short periods (primarily in women with *L. iners* dominated biota), whereas others were relatively stable (primarily in women with *L. crispatus* or *L. gasseri* dominated biota). Microbiome changes coincided best with phase of the menstrual cycle and recent sexual activity (Gajer *et al*. 2012; Noyes *et al*. 2018; Vodstrcil *et al*. 2017). Similarly, Srinivasan *et al.* (2010) found that the *Lactobacillus*-dominated vaginal microbiota of healthy women changed over a month of follow-up, with quantities of *L. jensenii* and *L. crispatus* decreasing and levels of *G. vaginalis* inversely increasing with onset of menstruation (Srinivasan *et al*. 2010). This emphasises the importance of understanding the drivers of microbial community change, and factors favouring stability in this ecological niche.

1.1.3 Bacterial vaginosis (BV)

1.1.3.1 Diagnosis of BV

There are currently two different approaches that are used for the diagnosis of BV: (1) Amsel criteria are more commonly used in clinical practice, and require the presence of ≥ 3 clinical criteria to diagnose BV (Amsel *et al*. 1983), including evidence of a thin, homogeneous vaginal discharge; a vaginal pH ≥ 4.5; a "fishy" odour of vaginal fluid before or after addition of 10% potassium hydroxide (whiff test); and the presence of clue cells on microscopic slides; or (2) Nugent scoring (Nugent *et al*. 1991), which is based on Gram staining of vaginal smears and microscopic quantitation of bacterial morphotypes (*Lactobacillus*, *Gardnerella*/*Bacteroides* and curved Gram variable rods), with scores ranging from zero to ten. A Nugent score of ≤3 is considered normal (predominantly *Lactobacillus* rods, absence of *Gardnerella*/*Bacteroides* and curved Gram variable rods), a Nugent score of 4–6 is considered to indicate presence of "intermediate" microbiota (mixture of all bacterial morphotypes), and a Nugent score of ≥7 is considered BV positive (predominantly *Gardnerella* or *Bacteroides* and curved Gram variable rods, in addition to absence of *Lactobacillus* rods).

Studies have shown that these methods correlate well in some cases, although Amsel criteria generally diagnose fewer women with BV than Nugent scoring (Bala *et al*. 2017; Rao *et al*. 2016), with a sensitivity of <40% but a specificity of 99% for diagnosing BV (Sha Chen, *et al*. 2005) compared to Nugent scoring, probably because of the large proportion of asymptomatic BV infections that would be missed by a purely symptom-based scoring algorithm. However, it needs to be emphasised that Amsel criteria can be used to diagnose BV in asymptomatic women, mainly in clinical studies where Amsel criteria are used outside of a clinical visit prompted by symptoms reported by the patient. Similarly, Nugent criteria can be used to diagnose both asymptomatic and symptomatic BV if reported symptoms are available.

1.1.3.2 BV prevalence in South Africa and worldwide

The prevalence of BV varies between and within countries (Kenyon *et al*. 2013), likely to be influenced by a number of social and biomedical factors (Holzman *et al*. 2001). BV prevalence appears to be lowest in Asia and Europe (up to 24%) and

highest in Sub-Saharan Africa (up to 58%) (Kenyon *et al*. 2013). Earlier studies from South Africa found that ~70% of female sex workers had BV (Kenyon *et al*. 2013; Johnson *et al*. 2005).

1.1.3.3 Causes of BV

Machado and Cerca (2015) dynamically modelled factors associated with emergence of BV, which suggested that sexual transmission of *G. vaginalis* displace resident *Lactobacillus* spp. from their ecological niche in the FGT by laying down a biofilm that allows other anaerobic pathogenic bacteria to colonize the genital tract. *G. vaginalis* also secretes sialidase, a mucolytic protein that degrades sialic acids in vaginal mucus, which allows *G. vaginalis* and other pathogenic bacteria to attach to the vaginal epithelium that would normally be protected by vaginal mucous, thereby enabling the formation of stable biofilms (Hardy *et al*. 2017). This model has significant merit but it has not yet been scientifically proven, and it does not explain recurrence, which might happen without sexual transmission, as *G. vaginalis* is already present in the FGT, albeit at low concentration/proportion. Thus, a disrupting event could lead to another episode of BV without sexual transmission, and it is unclear if a second transmission event does lead to BV.

Although the etiology of BV is still largely unclear, several risk factors have been identified. These include: (1) ethnicity (Cherpes *et al*. 2008; Simhan *et al*. 2008); (2) age and hormonal changes associated with adolescence, menstruation, and menopause (Cauci *et al.* 2002; Gajer *et al.* 2012); (3) hormonal contraception (Riggs *et al*. 2007); (4) concomitant STIs (Cherpes *et al*. 2008); (5) psycho-social stress (Nansel *et al*. 2006); (6) smoking (Hellberg *et al*. 2000; Brotman *et al*. 2014; Cherpes *et al*. 2008); (7) having multiple sexual partners, a recent new partner, and frequent sexual intercourse (Fethers *et al*. 2008; Yen *et al*. 2003; Verstraelen *et al*. 2010); (8) vaginal cleansing habits (Merchant *et al*. 1999; Brotman *et al*. 2008); and (9) the presence of bacteriophages in the lower FGT that target *Lactobacillus* spp. (section 1.1.3.4). This dissertation will focus in some detail on evidence that bacteriophages play a role in alterations in the vaginal microbiome.

1.1.3.4 Evidence that bacteriophages may modulate the host microbiome during BV

Pavlova *et al.* (1997) suggested that bacteriophage-mediated lysis of vaginal *Lactobacillus* spp. causes depletion of *Lactobacillus* spp. during the development of BV, based on their finding that temperate bacteriophages were inducible from purified vaginal *Lactobacillus* isolates using mitomycin C. Furthermore, temperate bacteriophages were detected less commonly in *Lactobacillus* isolates from healthy women than in isolates from women with BV (Pavlova *et al*. 1997). Temperate bacteriophages undergo a lysogenic lifecycle, whereby prophages integrated into a bacterial genome can be induced under stress conditions, although they are not expressed nor do they cause host cell lysis under normal environmental conditions (reviewed by Hobbs & Abedon 2016). Mitomycin C is a chemotherapeutic antibiotic that binds DNA covalent, which is commonly used to induce prophages (reviewed by Hobbs & Abedon 2016). Blackwell *et al*. (1999) suggested that BV could be considered a "vaginal bacterial phaginosis", whereby bacteriophages of *Lactobacillus*, possibly transmitted during sex, destroy the endogenous healthy *Lactobacillus*-dominated vaginal microbiome and thereby allow overgrowth of endogenous anaerobic bacteria, like *G. vaginalis*. Blackwell *et al*. (1999) further argued that BV manifests epidemiologically like a STI, although BV recurrence rates were not affected by treating male partners with antibiotics.

There is also evidence that virulent bacteriophages against *Lactobacillus* spp. exist in nature. Virulent bacteriophages undergo a lytic lifecycle, which is defined as a bacteriophage replication that necessarily ends with the lysis of the bacterial host cells (Hobbs & Abedon 2016). The first virulent bacteriophage targeting *Lactobacillus* spp. was isolated in 1934 from sewage water collected in New York City (Kopeloff 1934). Since then, virulent bacteriophages targeting *Lactobacillus* spp. have been isolated from dairy products, bread, fermented vegetables and meat products, as well as human gut samples, indicating that these bacteriophages are common in the environment (reviewed by Villion & Moineau 2009).

While no virulent bacteriophages have yet been isolated from vaginal secretions (Martín *et al*. 2009), several studies have described that temperate bacteriophages can be induced from vaginal *Lactobacillus* isolates. From 37 vaginal *Lactobacillus*

isolated from women with and without BV, Pavlova *et al.* (1997) induced seven temperate bacteriophages using mitomycin C. Of these, two had a broad host range infecting almost all of the vaginal *Lactobacillus* isolates that were screened. Interestingly, an intestinal *Lactobacillus* bacteriophage used as control also lysed several vaginal *Lactobacillus* strains, hinting that intestinal *Lactobacillus* bacteriophages might reach the FGT via ano-genital transfer, where they may target vaginal *Lactobacillus* spp. Temperate bacteriophages appeared to be less common in *Lactobacillus* isolates from healthy women than women with BV, although there was no difference in the sensitivity of the *Lactobacillus* isolates to bacteriophages depending on the BV status of the isolate donor (Pavlova *et al*. 1997). Similarly, Raya *et al*. (1989) induced a temperate bacteriophage (using both mitomycin C and UV light) from a strain of *L. gasseri*, commonly present in the lower FGT of healthy women. This *Siphoviridae* bacteriophage had a linear dsDNA genome of 41.7 kb, with a hexagonal head, and a long, non-contractile, flexible tail (**Figure 1.1**A). Environmental pH was further shown to influence the viability of this bacteriophage, as their ability to infect *L. gasseri* (and form plaques) was optimal between pH 5.2-5.5, declining at pHs greater than 6.1 or less than 5.2 (Raya *et al*., 1989). Kilic *et al.* (2001) reported that 28% (30/107) of the *Lactobacillus* isolates they tested from American women and 36% (37/102) of *Lactobacillus* strains they had isolated from Turkish women released bacteriophages, following mitomycin C treatment. All of these bacteriophages were infectious against a comprehensive panel of *Lactobacillus* strains isolated from both Turkish and American women, suggesting that these bacteriophages were virulent regardless of the geographic origin of the vaginal *Lactobacillus* isolates. Based on their morphology, these bacteriophages belonged to *Myo*- and *Siphoviridae*, as they has hexagon-shaped heads and long tails with or without a contractile tail sheath, with genome sizes ranging from 34 to 55 kb (**Figure**

1.1B). A study done in South Africa similarly showed that as many as 77% of vaginal *L. crispatus* isolates and 29% of *L. jensenii* isolates released temperate bacteriophages, following mitomycin C treatment (Damelin *et al*. 2011; **Figure 1.1**C).

1.1.3.4.1 Temperate bacteriophages can be induced using "natural" agents

While mitomycin C has been widely used to induce temperate bacteriophages from vaginal *Lactobacillus* spp., other naturally occurring agents are also able to cause the release of *Lactobacillus* bacteriophages, including metabolites of tobacco (Pavlova & Tao 2000), hydrogen peroxide (H_2O_2; Martín *et al*. 2009), antibiotics (Martín *et al*. 2010), and UV radiation (Martín *et al*. 2010).

Benzo[a]pyrene diol epoxide (BPDE; $C_{20}H_{14}O_3$), a metabolite and derivate of benzo[*a*]pyrene found in tobacco smoke, can be detected in vaginal secretions of women who smoke (Melikian *et al*. 1999; Poppe *et al*. 1995), and has been implicated as a co-factor in cervical disease progression (Rubin 2001). This compound was shown to significantly increase bacteriophage induction in vaginal *Lactobacillus* strains at concentrations as low as 16 ng/mL (Pavlova & Tao 2000). In the majority of women who smoke, BPDE concentrations in excess of 1000ng/mL were detected in vaginal fluid (Brotman *et al*. 2014). Previous studies have shown that smokers have lower abundances of vaginal *Lactobacillus* spp. compared to non-smokers (Brotman *et al*. 2014), and smoking is strongly associated with the risk of developing BV (Hellberg *et al*. 2000; Bailey *et al*. 2004). It can therefore be hypothesized that

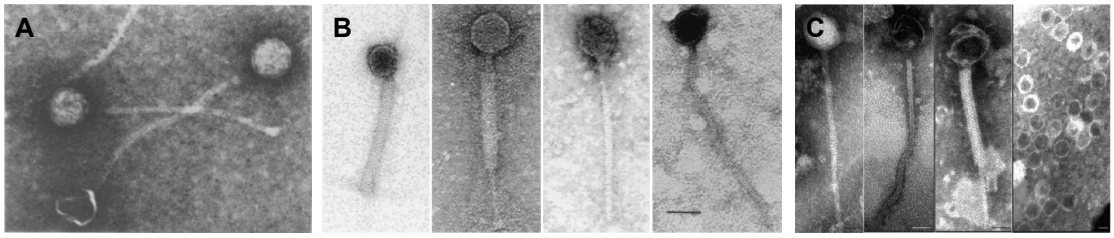


Figure 1.1. Induction of bacteriophages from vaginal *Lactobacillus* strains. Electron micrographs of temperate bacteriophages induced using Mitomycin C from vaginal *Lactobacillus* isolates by Raya *et al.* (1989) [A], Kilic *et al.* (2001) [B] and Damelin *et al.* 2011 [C].

chemicals in cigarette smoke reduce vaginal *Lactobacillus* and/or other vaginal bacterial spp. by promoting the induction of temperate bacteriophages. Smoking has also been associated with the onset, recurrence and progression of Crohn's disease, a gastrointestinal dysbiosis (Parkes *et al*. 2014). Both, the concentration and diversity of bacteriophages in the gastro-intestinal tract (GIT) of patients with Crohn's disease

were >20-fold higher than healthy individuals (Lepage *et al*. 2008; Wagner *et al*. 2013). These studies suggest that bacteriophages are generally involved in dysbioses at other mucosal sites, and that smoking might favour the induction of these bacteriophages, even at the genital mucosa. A common practice in some regions of Sub-Saharan Africa, including South Africa, is the use of tobacco (snuff) as a vaginal drying agent and sex enhancer by women, whereby tobacco is inserted directly into the vagina to dry and tighten it in preparation for sex (Doherty *et al*. 2014; Gafos *et al*. 2010). No studies have been conducted to evaluate how this practice impacts vaginal health or the microbiome, nor what local insertion does to the concentration of BPDE in genital sections.

Martín *et al*. (2009) showed that H_2O_2 (between the concentrations of 0.1-1mM) induced bacteriophages from vaginal *Lactobacillus* isolates, although to a lesser extent than mitomycin C (Martín *et al*. 2009). H_2O_2 has been shown to be a SOS-activating compound (which is induced in response to DNA damage), suggesting that H_2O_2 promotes the selection of bacterial cells harbouring SOS-insensitive, defective prophages (Martín *et al*. 2009). The H_2O_2 concentrations used in these experiments are similar to those found in the FGT, which are typically lower than 100µM (O'Hanlon *et al*. 2011), suggesting that H_2O_2 might be able to induce bacteriophages *in vivo* in the FGT. Martín *et al*. (2010) also demonstrated that the prophage Lv1 could be induced from a vaginal *L. jensenii* isolate using several other SOS-activating agents, including anti-cancer agents (such as mitomycin C and mitramycin), anti-bacterial agents (such as UV light, ampicillin and H_2O_2), and anti-septic agents (like nonoxynol-9, which is widely used spermicide). Madera *et al*. (2009) further described the induction of prophages from *Lactococcus lactis* using bacteriocins (Lactococcin 972 and plantaricin C) and antibiotics (bacitracin and penicillin G). It was therefore hypothesized that various agents that women would be exposed to in "real life" may be capable of inducing bacteriophages *in vivo,* which may affect their vaginal microbiota. This hypothesis is supported with observations that BV is common during chemotherapy for gynaecologic cancers (Von Grueningen *et al*. 2000), and that nonoxynol-9 appears to increase the prevalence of anaerobic gram-negative rods and BV in women in a dose-dependent manner (Jones *et al*. 1994; Schreiber *et al*. 2006), suggesting that *Lactobacillus* bacteriophage induction may occur *in vivo* in a natural setting when women are exposed to environmental stressors.

1.1.3.4.2 Temperate Lactobacillus bacteriophages from other sources

It is also possible that vaginal *Lactobacillus* spp. can be depleted by bacteriophages that are introduced into the FGT from external sources, including probiotics. Kilic *et al.* (1996) showed that *Lactobacillus* strains from half of the yoghurts and acidophilus milks purchased commercially in the US released bacteriophages. The authors found that a bacteriophage was spontaneously released from *L. acidophilus* Y8, isolated from yoghurt, that was capable of killing almost a quarter of the other dairy *Lactobacillus* strains tested, indicating that it has a broad host range. Twenty two *Siphoviridae* bacteriophages that targeted *L. paracasei* were isolated from an industrial plant that manufactured probiotic dairy products (Capra *et al*. 2009). In addition, the bacteriophage Lc-Nu was isolated from the probiotic *L. rhamnosus* strain Lc1/3 (Tuohimaa *et al*. 2006), indicating that bacteriophages might be also contained in probiotic products. Tao *et al.* (1997) screened a wide range of probiotic dairy products containing *Lactobacillus* spp. (including two acidophilus milks, 20 yogurts, three *Lactobacillus* pills, and one vaginal douche mix) from which they isolated 43 *Lactobacillus* strains, from which they were able to induce eleven bacteriophages, and seven of these infected human vaginal *Lactobacillus* strains. These studies suggest that *Lactobacillus* strains used in the manufacture of yoghurts commonly contain temperate bacteriophages, which can be released spontaneously, have broad host ranges, and are capable of infecting *Lactobacillus* species adapted to the FGT.

The consumption of yoghurt and probiotics is common worldwide, and bacteria can reach the FGT from the gut via ano-vaginal transfer (Reid *et al*. 2001). Further, the topical application of yoghurt and probiotics is a common practice to improve vaginal health (Hantoushzadeh *et al*. 2012; Strus *et al*. 2012; Neri *et al*. 1993). It is therefore plausible that bacteriophage-harbouring *Lactobacillus* strains from yoghurts and probiotics can colonize the FGT, where temperate bacteriophages might be released that then could lyse vaginal *Lactobacillus* strains.

1.1.3.4.3 CRISPR loci are present in vaginal Lactobacillus spp.

Evidence that vaginal bacterial species are exposed to virulent bacteriophages comes from the presence of clustered regularly interspaced short palindromic repeat (CRISPR) loci in vaginal bacteria. CRISPR loci are composed of short repeat sequences and hyper variable ‘spacer’ sequences that are usually between 24 and 50 bp in size (Labrie *et al*. 2010; Rath *et al*. 2015). These spacers correspond to

bacteriophage (or plasmid) sequences that have been incorporated into the ancestral bacteria, which survived bacteriophage infection. Four CRISPR loci have been found in vaginal bacterial samples through the Human microbiome project (Rho *et al*. 2012), including LjassL36, LcrisL29, LjensL36, and AlactL29. LjassL36 was found in 33 vaginal samples, in different *Lactobacillus* genomes, including *L. crispatus* and *L. gasseri*. LcrisL29 was found in 31 vaginal samples in *L. crispatus* genomes. LjensL36, which targets the lysogenic Siphoviridae *Lactobacillus* bacteriophage Lv-1, was found in 28 of the vaginal samples screened, in *L. jensenii, L. crispatus* and *L. gasseri* genomes. The least common CRISPR loci was AlactL29, which was found in three vaginal samples, initially identified from the genome of *Anaerococcus lactolyticus,* a Gram-positive anaerobe that is a normal part of the human microbiome but has been found in ovarian abscesses and vaginal discharge (Murphy & Frick 2013). The common identification of these CRISPR loci in vaginal *Lactobacillus* strains suggested that they were frequently infected by bacteriophages. It is still unclear how lytic bacteriophages reach the FGT, but since bacteria (and bacteriophages) may be transferred ano-genitally (Reid *et al*. 2001), it is possible that *Lactobacillus* bacteriophages from the GIT, where they are present (reviewed by Villion and Moineau, 2009), could migrate to the FGT to infect vaginal *Lactobacillus* strains.

Macklaim *et al.* (2013) compared *L. iners* isolates from healthy and dysbiotic women, and identified eight genes encoding the proteins for the CRISPR anti-bacteriophage defence system that were highly expressed in isolates from women with BV but not in those from healthy women. They concluded that *L. iners* was responding to increased bacteriophage load in women with BV, and that bacteriophages influence the bacterial community under dysbiotic conditions. This supports the earlier findings from Pavlova *et al*. (1997) that temperate bacteriophage detection was lower in *Lactobacillus* isolates from healthy women compared to women with BV. Comparative genome analyses of probiotic *Bifidobacterium* (*B. dentium, B. longum, B. adolescentis*) and *Lactobacillus* genomes (*L. plantarum, L. salivarius, L. brevis, L. reuteri, L. fermentum, L. acidophilus, L. helveticus, L. johnsonii, L. gasseri, L. bulgaricus, L. casei, L. sakei*) revealed the presence of several prophage-like elements (indicative of temperate bacteriophages) and CRISPR-related systems (Ventura *et al*.

2009), arguing that horizontal gene transfer by bacteriophages may be important for the ecological niche-specific adaptation in probiotic bacteria.

In summary, several lines of *in vitro*, *in vivo* and sequence based evidences suggests that bacteriophages may play a role in modulating the vaginal microbiota and possibly BV.

1.1.3.5 BV increases genital inflammation

Several studies, including from this laboratory, have shown that genital tract concentrations of pro-inflammatory cytokines are significantly increased in women with BV, even when asymptomatic, such as interleukin (IL)-1α, IL-1β and tumour necrosis factor (TNF)-β (Mlisana *et al*. 2012; Masson *et al*. 2015; Cauci *et al*. 2003; Mitchell & Marrazzo 2014; Campos *et al*. 2012; Hemalatha *et al*. 2012; Alcaide *et al*. 2017; Mitchell *et al*. 2008). This may partly be due to increasing concentrations of bacterial toll-like receptor (TLR) ligands in the FGT under dysbiotic conditions (Anahtar *et al*. 2016). TLRs play a fundamental role in the activation of the innate immune system, as they recognize pathogen-associated molecular patterns (PAMPs) derived from various microorganisms, and mediate the production of cytokines necessary for the induction of effective immunity (Kawasaki & Kawai 2014). However, down-regulation of some chemotactic cytokines (like interferon gamma-induced protein [IP]-10, growth regulated oncogene [GRO], macrophage-derived chemokine [MDC], and macrophage inflammatory protein [MIP]-1α), responsible for recruitment of immune cells, and haematopoietic cytokines (IL-7, granulocyte-macrophage colony-stimulating factor [GM-CSF]) has also been observed in women with BV compared to those with no BV (Masson *et al*. 2014; Ryckman *et al*. 2008). Suppression of chemotactic cytokine responses may result in fewer immune cells being recruited to the FGT, which could contribute to the lack of symptoms in some women with BV (Masson *et al*. 2015; Mlisana *et al*. 2012). Downregulation of genital chemokine responses in women with BV may reflect that the microbes commonly found in BV are commensals rather than pathogens (Danielsson *et al*. 2011). Borgdorff *et al.* (2016) further found significantly higher levels of the pro-inflammatory cytokine IL-36α (a member of the IL-1 family) and its receptor agonist (IL-36RA), complement component C5, glucose-6-phosphate isomerase, and

macrophage migration inhibitory factor (MIF) in women with BV compared to those who did not have BV, by mass spectrometry. Glucose-6-phosphate isomerase, also known as neuroleukin, is a host protein that functions extracellularly, both as a neurotrophic factor that promotes growth and survival of neurons, and as a lymphokine that induces immunoglobin secretion and promotes angiogenesis (Zong *et al*. 2015; Deng *et al*. 2014). MIF is a regulator of innate immunity, as it promotes the pro-inflammatory functions of immune cells during the host´s response to antimicrobial infections and stress. MIF is rapidly released by immune cells that are exposed to microbial products or to pro-inflammatory cytokines, or during antigen-specific activation, and has potent autocrine and paracrine effects that promote cell growth and survival (reviewed by Calandra and Roger, 2003). Treatment of BV with metronidazole was found to reduce genital tract levels of IL-1β, IL-8 and RANTES, and the number of activated cervical CCR5+ CD4+ T-cells (Rebbapragada *et al*. 2008).

The BV-associated bacteria *G. vaginalis* and *A. vaginae* have been shown to modulate inflammatory responses *in vitro*, indicating that these microorganisms might be responsible for the different cytokine profile seen in women with BV. *In vitro* experiments showed that *G. vaginalis* triggered the secretion of pro-inflammatory cytokines (TNF-α, IL-1 β, IL-6 and IL-8) and the activation of the nuclear factor-kappa B (NF-kB) pathway in HeLa cells, a cervical cancer cell line (Santos *et al*. 2018), and increased levels of the inflammatory cytokines IL-1 β and IL-6 in explant models using human chorioamniotic membranes (Zaga-Clavellina *et al*. 2012). The NF-kB pathway plays a complex role in inflammation, and is also activated by TLR signalling. NF-κB is involved in the expression of several pro-inflammatory cytokine, chemokine, and adhesion molecule genes that regulate leucocyte recruitment and cell survival (reviewed by Liu *et al*., 2017). NF-κB may also have anti-apoptotic functions that can both protect against inflammation (based on genetic evidence in mice), by promoting epithelial cell survival and mucosal barrier integrity, and maintain the inflammatory response through persistent leukocyte activation (reviewed by Lawrence, 2009; Hoesel and Schmid, 2013).

In vitro experiments with *A. vaginae* showed that pro-inflammatory cytokines IL-6, IL-8 and the antimicrobial peptide human β-defensin 4 (HBD4) were also induced in

vaginal epithelial cells, mediated by TLR2 via activation of the NF-kB pathway (Libby *et al*. 2008). HBDs are antimicrobial peptides produced by epithelial cells, and have the capacity to kill or inhibit a variety of bacteria and fungi *in vitro* (reviewed by Quayle, 2002; Pazgier *et al.* 2006; Kaushic *et al.* 2010).

1.1.3.6 BV increases the risk of STIs, including HIV infection and transmission

BV increases the risk of acquiring and transmitting HIV (Sewankambo *et al*. 1997; Cohen *et al*. 2012; Martin *et al*. 1999; Taha *et al*. 1998; Myer *et al*. 2005; Van de Wijgert *et al*. 2008; Anahtar *et al*. 2016; Gosmann *et al.* 2017) and other STIs (including *T. vaginalis*, *N. gonorrhoea, C. trachomatis,* human papillomavirus [HPV] and herpes simplex virus type 2 [HSV-2]; Guo *et al*. 2012; Brotman *et al*. 2010; Nagot *et al*. 2007). As STIs and genital inflammation associated with these infections have been associated with an increased risk of HIV infection (Ward & Rönn 2010; McClelland *et al*. 2007), it is difficult to determine whether BV directly or indirectly increases the risk of HIV acquisition.

HIV-infected women with BV have higher concentrations of HIV-1 detected in their cervicovaginal fluid (Wang *et al*. 2001; Sha, Zariffard, *et al*. 2005; Coleman *et al*. 2007), and are more likely to transmit HIV to their male partners than BV negative women (Cohen *et al*. 2012). However, BV treatment with oral metronidazole (400 mg twice daily for 10 days) did not reduce the number of women in whom genital HIV was detected, or the concentration of HIV-1 detected in genital secretions (Wang *et al*. 2001). This may suggest that the mechanism by which HIV-infected women with BV are more infectious to their male partners may not be solely related to the amount of HIV in their genital secretions. BV-associated bacteria, including *G. vaginalis* and *P. bivia,* are capable of directly inducing HIV-1 replication and expression *in vitro* (Hashemi *et al*. 2000; Hashemi *et al*. 1999; Simoes *et al*. 2001), indicating that changes in the composition of vaginal bacterial communities might lead to a higher rate of sexual transmission by increasing HIV infectiousness.

1.1.3.7 BV increases risk of reproductive complications

Untreated BV leads to some severe reproductive complications in women, including premature delivery, miscarriage, intrauterine foetal death, post-operative infections

and pelvic inflammatory disease (PID) (Haggerty *et al*. 2016; Nelson *et al*. 2014; Clark *et al*. 1994). Further, exposure of the infant to BV during pregnancy has been associated with adverse neonatal outcomes, in both full- and pre-term infants, as BV was associated with an increased risk of assisted ventilation/respiratory distress at birth (adjusted risk ratio=1.28), neonatal intensive care unit admission (adjusted risk ratio =1.42) and neonatal sepsis (adjusted risk ratio=1.60) (Dingens *et al*. 2016).

As BV is associated with a variety of adverse outcomes that put women, their partners and their children at risk, it is crucial to have effective and durable treatment strategies for BV.

1.1.3.8 Current management of BV in South Africa

Syndromic management, which intends to treat the signs or symptoms (syndrome) of a group of diseases rather than treating any specific disease, was first conceptualized by public health physicians, particularly those working in Africa in resource-limiting settings, to circumvent the need for an etiological STI diagnosis, and advocated by the WHO in 1991 (Vuylsteke 2004). In South Africa, treatment for BV falls under the vaginal discharge syndrome guidelines, which means if a women presents with vaginal discharge, clinic staff will follow signs and symptoms to identify the underlying disease, rather than offering specific etiologic testing of STIs or Nugent scoring to diagnose BV[1].

Syndromic management was adopted by the South African Department of Health as standard of care in 1996, with the most recent guidelines being published in 2015[1]. Abnormal vaginal discharge, suggestive of BV or vaginal candidiasis, is treated with metronidazole (oral, 2g single dose) and a clotrimazole vaginal pessary (500 mg as single dose) or clotrimazole vaginal cream (inserted every 12 hours for 7 days) according to South African guidelines. If a woman does not respond after completion of the treatment course, it is recommended that a higher dose of oral metronidazole for a longer duration be administered (400 mg, every 12 hours for 7 days). If this still does not resolve the symptoms after 7 days, women are referred to a hospital for further management[1]. Similarly, the current US CDC treatment guideline for BV is

[1] Sexually Transmitted Infections: Management Guidelines 2015, DOH, South Africa.

either oral or vaginal administration of metronidazole (500mg oral tablets twice daily for 7 days, or metronidazole gel 0.75%, 5 g vaginally, once a day for 5 days) or clindamycin (300 mg orally twice daily for 7 days).

Antibiotic treatment of BV, however, only results in a temporary resolution of symptoms, as recurrence rates of microbial dysbiosis are high, with ~30% of cases recurring within three months and ~50% recurring within six months (Barrons & Tassone 2008). While *Lactobacillus* spp. are resistant to metronidazole, BV-associated organisms like *Prevotella* spp. and *G. vaginalis* are thought to largely be susceptible (Hillier 2013; Petrina *et al*. 2017). Both *Prevotella* spp. and *G. vaginalis,* however, commonly develop resistance to metronidazole (Nagaraja 2008; Hillier 2013), which likely contributes to the high recurrence rates of BV. The best-characterized mechanism by which these organisms become resistant to metronidazole is by the inactivation and/or deletion of genes with nitroreductase activity that are essential for metronidazole activation (Shaskolskiy *et al*. 2016). Several other mechanisms have been proposed for the development of metronidazole resistance, which include increased activity of bacterial DNA repair systems (metronidazole inhibits the replication, transcription and repair process of DNA), increased activity of bacterial enzymes that consume oxygen (such as catalase, peroxidase, and superoxide reductase) as this prevents reduction/activation of metronidazole, or accelerated clearance of the drug from the bacterial cell by active efflux (Löfmark *et al*. 2010; Shaskolskiy *et al*. 2016).

As a result of poor BV treatment outcomes and high rates of recurrence, several clinical studies evaluated *Lactobacillus*-containing probiotics as adjunctive treatment to antibiotics for BV. Discussed in more detail in subsequent sections (section 1.3), addition of probiotics with antibiotic treatment for BV has shown significant improvements in BV cure rates, with no major product-related adverse events (AEs).

1.2 Vaginal *Lactobacillus* spp. associated with health

Lactic acid bacteria are a group of micro-aerophilic, Gram-positive organisms that ferment hexose sugars to produce primarily lactic acid, and historically contain several genera, including *Lactobacillus*, *Carnobacterium*, *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Leuconostoc* spp. (reviewed by Stiles

and Holzapfel, 1997). *Lactobacillus* spp. successfully colonise a variety of niches in the human body, including the mouth, GIT, breast milk and the FGT, as well as environmental niches (including plants, soil, water, sewage, manure, silage), and food products (including beer, milk, meat, vegetables) (Klein *et al*. 1998). The evolution of *Lactobacillus* spp. is thought to be strongly influenced by their ecological niches, evidenced by loss of several ancestral genes, and reducing their genome sizes (Makarova *et al*. 2006). While some *Lactobacillus* spp. are highly adapted to specific ecological niches, others (like *L. plantarum*) remained genetically diverse, giving them the advantage of being able to colonize a variety of ecological niches (reviewed by Siezen & Van Hylckama Vlieg 2011). *Lactobacillus* spp. commonly found in the FGT but absent from other niches, including *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* (**Table 1.1**), are highly specialized to their habitat, reflected by their significantly smaller genome size compared to non-vaginal *Lactobacillus* spp. (reviewed by Mendes-Soares *et al*. 2014). In agreement, the genome size and protein encoded by vaginal *Lactobacillus* spp. (including *L. crispatus, L. gasseri, L. jensenii, L. vaginalis, L. mucosae, L. iners*) are significantly smaller than those from probiotic species (including *L. acidophilus, L. casei, L. rhamnosus, L. reuteri*; **Table 1.1**). Such reduction in genome size is commonly associated with a symbiotic lifestyle (reviewed by Mendes-Soares *et al*. 2014), suggesting that vaginal *Lactobacillus* spp. strongly interact with their host and/or other bacteria surrounding them in the FGT to offset their smaller genomes. Within vaginal *Lactobacillus* spp., each genome codes for species-specific gene families. For example, a unique DNA polymerase, gene families related to bacteriocins, toxin-anti-toxin systems and mobile genetic elements are unique to *L. crispatus* genomes; while *L. iners* genomes lack several integral membrane proteins and transcriptional regulators, but cytolysin genes are exclusively present in *L. iners* (reviewed by Mendes-Soares *et al*. 2014). Furthermore, expression of cytolysin (which is capable to lyse host blood or epithelial cells) appears to be 6-fold higher in *L. iners* strains isolated from women with BV than women with no BV (Macklaim *et al*. 2013), which might explain their role in the pathogenesis of BV.

Phylogenetic analysis of vaginal *Lactobacillus* spp. showed that *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* are phylogenetically distinct from *L. vaginalis* and *L. mucosae* (**Figure 1.2**; Zheng *et al*. 2015). While *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* are considered homo-fermenters (fermenting glucose to lactic acid as

their primary metabolic product), *L. vaginalis* and *L. mucosae* are hetero-fermenters (fermenting glucose to either lactic acid, ethanol/acetic acid or CO_2; Zheng *et al*. 2015; **Table 1.1**). Together, these genotypic and phenotypic data suggests that *L. crispatus*, *L. gasseri*, *L. jensenii* and *L. iners* are more similar to each other than to *L. vaginalis* and *L. mucosae,* and therefore might behave in a different biological manner.

The commonly used probiotic species *L. acidophilus* (which are homofermenters) clusters together with *L. crispatus*, while *L. reuteri* (which are heterofermenters) clusters more closely with *L. vaginalis* (**Figure 1.2;** Zheng *et al*. 2015). In contrast, other common probiotic spp., such as *L. rhamnosus* (which are homo-fermenters) and *L. casei* (which are facultative heterofermenters, that produce CO_2 and other by-products only under certain conditions) are located together in a separate phylogenetic cluster (**Figure 1.2;** Zheng *et al*. 2015).

Table 1.1. Genetic and metabolic characteristics of vaginal and probiotic *Lactobacillus* species

Species	Genome size[#] [MB]	Protein count[#]	GC content[#] [%]	Fermentation	Primary metabolites
Vaginal species					
L. crispatus	2.07	1937	36.9	Homofermenter	Lactic acid
L. gasseri	1.93	1754	34.9	Homofermenter	Lactic acid
L. jensenii	1.67	1465	34.3	Homofermenter	Lactic acid
L. iners	1.29	1153	32.7	Homofermenter	Lactic acid
L. vaginalis	1.83	1654	40.7	Heterofermenter	Lactic acid, ethanol/acetic acid, CO_2
L. mucosae	2.07	1699	46.7	Heterofermenter	Lactic acid, ethanol/acetic acid, CO_2
Mean (SD)	1.81 (0.27)*	1610 (247)[#]	37.7 (4.7)		
Probiotic species					
L. acidophilus	1.97	1866	34.6	Homofermenter	Lactic acid
L. rhamnosus	2.95	2780	46.7	Homofermenter	Lactic acid
L. reuteri	2.16	1972	38.6	Heterofermenter	Lactic acid, ethanol/acetic acid, CO_2
L. casei	2.99	2804	46.4	Facultative heterofermenter	Lactic acid, and possibly ethanol/acetic acid, CO_2
Mean (SD)	2.52 (0.46)*	2356 (438)[#]	41.6 (5.2)		

[#]Based on NCBI genome summary statistics, medians are displayed. *p=0.0254; [#]p=0.015

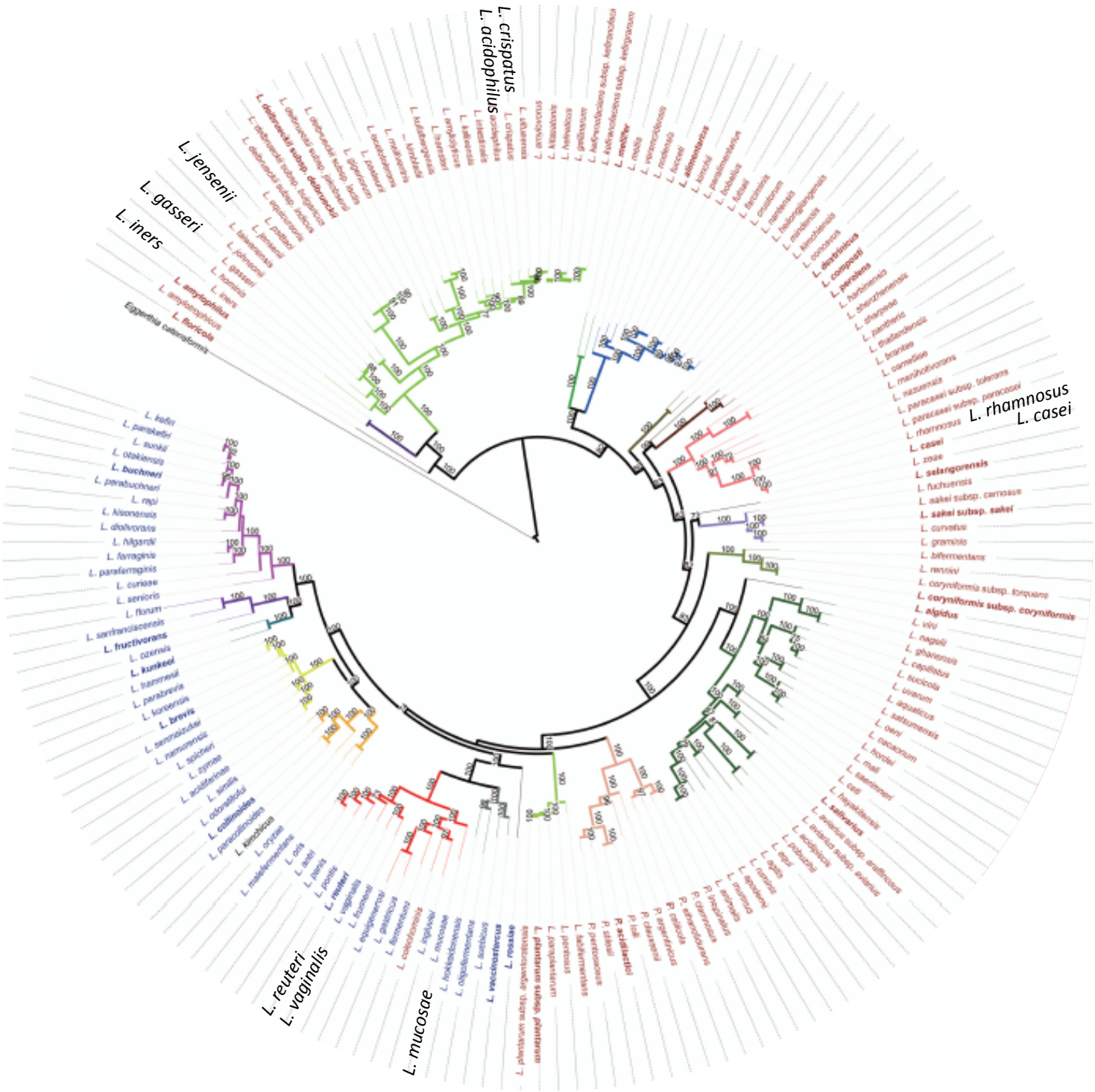


Figure 1.2. Maximum likelihood phylogenetic tree of *Lactobacillus* species, based on core protein sequences. Phylogenomic analysis and basic features of 174 *Lactobacillus* and *Pediococcus* type strains based on the concatenated protein sequences of single-copy core genes. The maximum likelihood tree was inferred by PhyML using the best model predicted by ProtTest. Bootstrap support values were calculated from 1,000 replicates, and only values above 70% are shown in the figure. Members of the same group are indicated by the same color for branches, and the type strain of each group is printed in bold. Names of homofermentative species are printed in red; names of heterofermentative species are printed in blue. Adapted from (Zheng et al. 2015).

1.2.1 Ability of *Lactobacillus* spp. to inhibit BV-associated bacteria

Lactobacillus spp. have been found to inhibit bacterial species that are commonly associated with a dysbiotic vaginal microbiota, such as *G. vaginalis* and *P. bivia,* (Castro et al. 2015a; Teixeira et al. 2012; Joo et al. 2011; Skarin & Sylwan 1986; Atassi et al. 2006; Coudeyras et al. 2008; Mastromarino et al. 2002; Jang et al. 2017; Andreeva et al. 2016) though various biological mechanisms (discussed in detail in section 1.2.3). Atassi *et al.* (2006) showed that *L. acidophilus*, *L. jensenii*, *L. gasseri* and *L. crispatus* isolates all inhibited *G. vaginalis* and *P. bivia* in a highly strain-specific way *in vitro,* by reducing their viability. Furthermore, they also showed that cell-free *L. gasseri* supernatants killed *G. vaginalis* and *P. bivia* that were adherent to HeLa cells, which they determined to be attributable to compounds in the culture supernatant that were resistant to proteolytic enzymes, but not due to low pH and the presence of lactic acid (Atassi *et al*. 2006), which could possibly be a bacteriocin or bacteriolysin. This co-culture of *G. vaginalis* or *P. bivia* with the *L. gasseri* strain also resulted in inhibition of the adhesion of these pathogens to HeLa cells (Atassi *et al*. 2006). Similarly, the probiotic strains *L. rhamnosus* HN001 and *L. acidophilus* La-14 significantly inhibited the adherence of *G. vaginalis* to HeLa cells and growth of *G. vaginalis in vitro* (Jang *et al*. 2017). Coudeyras *et al.* (2008) also found that co-culture of the probiotic strain *L. rhamnosus* Lcr35 with *G. vaginalis* or *P. bivia* decreased the cell division rate of the pathogens (possibly due to H_2O_2, lactic acid, and antibacterial compounds), causing bactericide of these BV-associated species. Interestingly, Castro *et al.* (2015) found that *G. vaginalis* isolated from a BV positive woman had the capacity to displace *L. crispatus* but not *L. iners* in a HeLa cell culture model (Castro et al. 2015b).

Teixeira *et al.* (2012) showed that vaginal *Lactobacillus* strains from healthy women were able to inhibit *G. vaginalis* strains more strongly *in vitro* than *Lactobacillus* strains isolated from women with BV. They also found that *G. vaginalis* strains from women with BV were more resistant to the antagonistic activity of *Lactobacillus* strains, especially when the *Lactobacillus* strain originated from a BV positive women. Metronidazole-resistant *G. vaginalis* strains seem to be less susceptible to the inhibitory activity of *Lactobacillus* strains than metronidazole-susceptible *G. vaginalis* isolates (McLean & McGroarty 1996). These finding suggest that both

Lactobacillus and *G. vaginalis* genotype affect the susceptibility of *Gardnerella* strains to inhibition.

In mice, Teixeira *et al*. (2012) further showed that vaginal colonization with *G. vaginalis* was 10-fold lower and pathology associated with *Gardnerella* colonization were less severe in animals previously colonised with *L. johnsonii*. Also in mice, Joo *et al.* (2011) reported that *L. johnsonii* HY7042 inhibited *G. vaginalis*-induced vaginosis, in addition to inhibiting the expression of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, the regulatory molecules COX-2, and iNOS, and the NF-κB pathway, while increasing the expression of the regulatory cytokine IL-10. Similarly, Jang *et al.* (2017) showed that the probiotic strains *L. rhamnosus* HN001 and *L. acidophilus* Ls-14 inhibited *G. vaginalis*-induced disruption of the mouse genital mucosa, in addition to suppressing myeloperoxidase activity, NF-κB activation, IL-1β, TNF-α, IL-17 and RORγt expression, while increasing IL-10 and Foxp3 expression. These two *Lactobacillus* strains inhibited LPS-induced NF-κB activation in macrophages and the differentiation of splenocytes into Th17 cells *in vitro*, rather favouring their differentiation into Treg cells (Jang *et al*. 2017). Further, *L. acidophilus* has also been shown to produce a bacteriocin that inhibits the growth of several *G. vaginalis* strains (Aroutcheva *et al*. 2001). These findings suggest that vaginal *Lactobacillus* spp. might inhibit and attenuate *G. vaginalis*-induced dysbiosis by regulating both vaginal and systemic immune responses and by regulating activation of the NF-κB pathway, in addition to producing bacteriocins, and directly inhibiting and competing with *G. vaginalis*.

1.2.2 Ability of *Lactobacillus* spp. to inhibit STIs

There is a considerable body of evidence that a *Lactobacillus*-dominated vaginal microbiota also protects women against various viral (such as HIV [section 1.2.2.1], HSV-2 [section 1.2.2.2], and HPV [section 1.2.2.3]), bacterial (such as *C. trachomatis* [section 1.2.2.4] and *N. gonorrhoea* [section 1.2.2.5]), and parasitic STIs (such as *T. vaginalis* [section 1.2.2.6]). Each of these examples will be discussed in more detail in the following sections.

1.2.2.1 Lactobacillus-mediated inhibition of HIV

Lactobacillus spp. have been shown to inhibit HIV-1 infection *in vitro* (Martín *et al*. 2010; Aldunate *et al*. 2013; Palomino *et al*. 2017; Lai *et al*. 2009). Conditioned media from *L. crispatus, L. acidophilus, L. paracasei ssp. casei,* and *L. rhamnosus* were shown to significantly inhibit HIV-1 replication *in vitro*, and co-culture with *L. gasseri* inhibited HIV-1 replication by >60% (Zabihollahi *et al*. 2012). Furthermore, lactic acid inactivated cell-free HIV *in vitro*, and cervicovaginal explant infection with HIV *ex vivo*, at an acidic but not neutral pH (Aldunate *et al*. 2013; Palomino *et al*. 2017), and L-lactic acid inhibited HIV more effectively than D-lactic acid *in vitro* (Aldunate *et al*. 2013). Lai *et al*. (2009) showed that lactic acid abolished the negative surface charge of HIV without lysing the virus membrane at an acidic pH, while HIV maintained its native surface charge at a neutral pH, which resulted in HIV becoming trapped in human cervicovaginal mucus at acidic but not neutral conditions. Lai *et al*. (2009) proposed that negatively charged lactate ions at an acidic pH interfered with HIV surface structures, disrupting the viral envelope and allowing exposure of the capsid. Furthermore, Nunn *et al*. (2015) showed that cervicovaginal mucus with high concentrations of D-lactic acid (>0.3% w/v) from women with *L. crispatus*-dominant microbiota effectively trapped HIV. However, they found that vaginal pH, Nugent scores, or total lactic acid levels did not correlated with mucus trapping of HIV. In humanized mice, Olmsted *et al*. (2005) reported that low vaginal pH decreased HIV target cell chemotaxis, resulting in increased cell death, which suppressed transmission of cell-associated HIV. Together, these studies suggest that low vaginal pH and the presence of lactic acid in vaginal fluid has an inhibitory effect on HIV, in addition to reducing both the number of HIV target cells in this ecological niche, as well as the number of infectious HIV particles.

1.2.2.2 Ability of Lactobacillus spp. to prevent HSV-2 infection

HSV-2 is one of the most prevalent sexually transmitted viruses (Looker *et al*. 2015), whose infection is characterised by periodic symptomatic or asymptomatic vaginal shedding in women, and causing painful genital ulcers (Gupta *et al*. 2007). HSV-2 initially infects the genital mucosa, with several glycoprotein-receptor complexes being implicated in host cell entry (Salameh *et al*. 2012). HSV-2 initially replicates cytolytically in epithelial cells, causing epithelial cell death and detachment, which

manifests as a fluid-filled genital blister that contains cellular debris, inflammatory cells and new virions (reviewed by Gupta, Warren and Wald, 2007). Subsequently, HSV-2 virions penetrate through the dermis and are transported retrogradely to the neural soma in the sensory nerve root ganglia, entering latency. Reactivation of HSV-2 occurs periodically, as frequently as once a month in most individuals, whereby virions are transported in the peripheral nerves back to the mucosal or skin surface (reviewed by Grinde, 2013). Despite this complex infection cycle and HSV-2 latency, several studies have shown that *Lactobacillus* spp. are able to inhibit HSV-2.

Co-incubation of HSV-2 with *Lactobacillus* strains prior to *in vitro* infection decreased the virus titre by 60%, indicating that *L. crispatus* might directly interact with the viral envelope (Mousavi et al. 2017a). Similarly, *L. gasseri* CMUL57 decreased the cytopathic effects induced by HSV-2 in Vero cells, used to propagate HSV-2 *in vitro*, in an envelope-mediated manner (Kassaa *et al*. 2015). This suggests that *Lactobacillus* spp. may possibly inhibit HSV-2 in a manner similar to HIV-1, by lactic acid interference with the negative charges of viral envelopes, causing inactivation (Lai *et al*. 2009). Kassaa *et al.* (2015) also detected HSV-2 DNA within the bacterial pellet, leading them to suggest that *Lactobacillus* spp. may also be able to trap and co-aggregate with HSV-2. In addition, *L. crispatus* was shown to inhibit the initial steps of HSV-2 entry into both Vero and HeLa cells, although they did not affect HSV-2 adsorption and multiplication (Mousavi *et al*. 2017; Zabihollahi *et al*. 2012). Adherence of *L. crispatus* to different HSV-2 target cell lines correlated positively with the protection of the cells against HSV-2 infection, suggesting that *L. crispatus* may physically block HSV-2 entry receptors on target cells (Mousavi et al. 2017a). Goudarzi and Fazeli (2015) showed that the abiotic culture supernatant of the probiotic *L. acidophilus* strain La-5 reduced cytopathic effects of HSV-2 in HeLa cells, which was not dependant on H_2O_2 production, concentrations of H^+ ions or an acidic pH. Conti *et al.* (2009) showed that the inhibitory effect of vaginal probiotic *Lactobacillus* strains (such as *L. brevis* CD2, *L. salivarius* FV2, and *L. plantarum* FV9) at the early phase of HSV-2 infection was dependent on the adhesion of the *Lactobacillus* spp. to Vero cells, while they found that other soluble bacterial by-products were able to interfere with the production of infective virions. They further showed that purified lactic acid and H_2O_2 exerted a dose-dependent virucidal effect against HSV-2. Lactic acid interfered with viral intracellular antigen synthesis, and

both the virucidal activity and the inhibition of replication correlated with an acidic pH (Conti *et al*. 2009). Of the *Lactobacillus* strains tested, Conti *et al.* (2009) found that *L. brevis* CD2 had the strongest HSV-2 inhibitory effect, although it did not produce H_2O_2, indicating that H_2O_2 most likely is not necessary for HSV-2 inhibition. *In vivo* experiments in BALB/c female mice, in which naked and scratched skin was infected with HSV-1 together with a live *L. gasseri* suspension, showed that *L. gasseri* also exerted significant protection against HSV-1 infection (Zabihollahi *et al*. 2012).

1.2.2.3 Lactobacillus spp. protect against HPV infection

HPV is another common viral infection in women (Brianti *et al*. 2017), with certain types (like HPV-16 and -18) increasing the risk of cervical intraepithelial neoplasia and cervical cancer (Munoz *et al*. 2003). Unlike HIV and HSV-2, HPV is a non-enveloped virus (McBride 2017). Mucosal HPV types infect basal epithelial cells of the squamous epithelium of the lower FGT, accessed following mucosal trauma or through micro-abrasions, by interacting with epithelial cell surface receptors. Within infected cells, the high-risk HPV types 16 and 18 will express the oncogenes E6 and E7, which will degrade the tumor suppressor protein p53 and the retinoblastoma protein (which control the host cell cycle), allowing unhindered host cell division to ensure their own replication cycle can commence (reviewed by McBride 2017). This interference with cell cycle processes increases risk of cancer development (reviewed by Brianti, De Flammineis and Mercuri, 2017).

Both *L. crispatus* SJ-3C-US and *L. rhamnosus* GG culture supernatants were shown to significantly decrease the expression of HPV-18 E6 in HeLa cells, indicating that *Lactobacillus* spp. might inhibit non-enveloped viruses like HPV via envelop-independent mechanisms (Motevaseli *et al*. 2014). Suppression of HPV oncogenes has been shown to reverse cervical carcinogenesis in women with integrated HPV infections, suggesting that *Lactobacillus* spp. might also have therapeutic potential for women with cervical cancer (Goodwin and DiMaio 2000). Two clinical trials have shown that *Lactobacillus*-containing probiotics have beneficial effects for women with HPV infections, leading to higher clearance rates of HPV and HPV-related cytological abnormalities (Verhoeven *et al*. 2013; Palma *et al*. 2018).

1.2.2.4 Lactobacillus spp. protect against C. trachomatis infection

C. trachomatis is the most common bacterial STI worldwide (Newman *et al*. 2015), and considered a major cause of female infertility, adverse pregnancy outcomes, PID, and HIV risk (reviewed by Manavi, 2006). Its life cycle consists of two distinct phases: an extracellular infectious phase involving *C. trachomatis* elementary bodies and an intracellular replication phase involving reticulate bodies. During the infectious phase, *C. trachomatis* elementary bodies bind to host cells (which are thought to primarily be non-ciliated columnar cells of the reproductive tract and macrophages) and are then internalized (reviewed by Manavi, 2006). Inside the host cell, *C. trachomatis* elementary bodies germinate and form reticulate bodies that begin to replicate. After replication, the reticulate bodies transition back into elementary bodies that are released from the host cell to start the next cycle of infection (Elwell *et al*. 2016).

Lactobacillus spp. and their metabolic by-products have been shown to inhibit *C. trachomatis* in several ways. Mastromarino *et al.* (2014) showed that probiotic *Lactobacillus* strains (*L. brevis* and *L. salivarius*) inhibited *C. trachomatis* replication in HeLa cells. *L. brevis* inhibited all steps of the infection cycle better than *L. salivarius,* including the extracellular phase (elementary bodies), adhesion to host target cells, and the intracellular phase (reticular bodies; Mastromarino *et al*. 2014). Similarly, vaginal *L. crispatus* strains were more effective at inhibiting *C. trachomatis* than *L. gasseri* or *L. vaginalis* strains (Rizzo et al. 2015a; Nardini et al. 2016). Gong *et al*. (2014) reported that lactic acid produced by *Lactobacillus* spp. was responsible for inhibition of *C. trachomatis* elementary bodies in a time- and concentration-dependant manner, although they did not determine which isomer was mediating this inhibition. However, others showed that D-lactic acid producing *Lactobacillus* spp. (including *L. crispatus* and *L. jensenii*) provided significantly better protection against *C. trachomatis* infection than *L. iners*, which produces predominantly L-lactic acid (Edwards *et al*. 2015). Furthermore, neutralizing the acidic *Lactobacillus* culture media completely abolished the ability of *Lactobacillus* spp. to inhibit *C. trachomatis* (Gong *et al*. 2014; Nardini *et al*. 2016). Gong *et al.* (2014) argued that H_2O_2 was not involved in this inhibition since the concentrations of this metabolite in the culture supernatant, although comparable to those reported for cervicovaginal fluid, were not sufficient to inhibit *C. trachomatis* elementary bodies.

In HeLa (human epithelial cells) and J774 (macrophages) cell lines, the presence of *L. crispatus* increased concentrations of IL-10, while decreasing production of pro-inflammatory IL-6, IL-8 and TNF-α in response to *C. trachomatis* infection, indicating that beneficial vaginal *Lactobacillus* spp. may also act to induce anti-inflammatory responses of host cells, which might mitigate the harmful effects of *C. trachomatis*-induced inflammation (Rizzo et al. 2015b).

1.2.2.5 Ability of Lactobacillus spp. to inhibit N. gonorrhoea infection

N. gonorrhoeae is another bacterial STI, being a gram-negative diplococci that causes PID, infertility and ectopic pregnancies (reviewed by Quillin and Seifert, 2018). During infection, *N. gonorrhoeae* adheres to the mucosal host epithelial cells in the urogenital tract using hair-like pili, rich in sugars and LPS, and forms a biofilm on the cell surface that enables them to compete with other commensal bacterial species for adherence and nutrients (Anderson *et al*. 2016). *N. gonorrhoeae* can also infect non-ciliated epithelial cells via transcytosis, leading to cytokine activation in macrophages and dendritic cells, and recruitment of neutrophils that phagocytose *N. gonorrhoeae*. This influx of neutrophils to the site of infection results in the appearance of a purulent exudate, that further facilitates transmission (reviewed by Quillin and Seifert, 2018).

Lactobacillus spp. have been shown to inhibit *N. gonorrhoea in vitro* and *ex vivo* in porcine vaginal explants (Amant *et al*. 2002; Spurbeck and Arvidson 2008; Graver and Wade 2011; Spurbeck and Arvidson 2010; Foschi *et al*. 2017; Ruiz *et al*. 2015; Breshears *et al*. 2015). Colonization of endometrial epithelial cells with vaginal *Lactobacillus* spp. prior to introduction of *N. gonorrhoea* reduced their adherence to epithelial cells by ~50%, postulated to be through the actions of surface-associated proteins of *Lactobacillus* isolates or biosurfactant production (Spurbeck and Arvidson 2010). Furthermore, invasion of epithelial cells by *N. gonorrhoea* was reduced by more than 60%, indicating that *Lactobacillus* spp. inhibit steps during the initial infection (Spurbeck and Arvidson 2008). *N. gonorrhoea* has been shown to co-aggregate with adherent *Lactobacillus* spp., and this complex frequently detached from the epithelial cell surface, which resulted in reduced colonisation with *N. gonorrhoea* (Vielfort *et al*. 2008). Vielfort *et al*. (2008) further showed that several

Lactobacillus spp. (including *L. crispatus*, *L. gasseri* and *L. reuteri*) were capable of reducing gonococcal adherence, but also that purified *N. gonorrhoea* pili were capable of removing adherent *Lactobacillus* spp. from the cell surface, suggesting that the beneficial *Lactobacillus* spp. and *N. gonorrhoea* compete for adherence to the host cells.

The ability of *Lactobacillus* spp. to inhibit *N. gonorrhoeae* appears to depend on low pH, as *Lactobacillus* isolates (like *L. crispatus* and *L. gasseri*) that significantly lowered the pH in culture were the best able to reduce growth of *N. gonorrhoeae*, while other isolates (like *L. jensenii*) that did not acidify as effectively also did not inhibit *N. gonorrhoeae* (Graver and Wade, 2011). Several studies have shown that the presence of lactic acid was crucial for anti-gonococcal activity (Breshears et al. 2015b; Foschi et al. 2017). In contrast, the ability of H_2O_2 to inhibit *N. gonorrhoeae* is more controversial. Amant *et al*. (2002) found that *L. crispatus* and *L. jensenii* had anti-gonococcal activity at both low and neutral pH, which lead to them concluding that H_2O_2 was the primary mediator of inhibition, however, others were not able to detect any H_2O_2 during these experiments (Spurbeck and Arvidson 2008; Graver and Wade 2011).

Lactobacillus bacteriocins (Ruiz *et al*. 2015) and biosurfactants (Foschi *et al*. 2017) have recently been shown to inhibit *N. gonorrhoea*. Ruiz *et al.* (2015) described two bacteriocin-like inhibitory compounds, called L23 and L60, which contributed more than 80% to the inhibitory effect of *Lactobacillus* strains against *N. gonorrhoea.* Foschi *et al.* (2017) further found that surface components released by *Lactobacillus* strains had biosurfactant properties that facilitated co-aggregation of *Lactobacillus* strains with *N. gonorrhoea*, thereby inhibiting the adhesion of the pathogen.

1.2.2.6 Lactobacillus spp. inhibit T. vaginalis infection

Trichomoniasis is a common cause of vaginitis in women, which increases the risks of adverse pregnancy outcomes, and acquisition and transmission of HIV and HSV-2 (reviewed by Meites 2015). It is caused by *T. vaginalis,* an obligate protozoon with five flagella, which primarily adheres to the squamous epithelium of the genital tract, where it replicates by binary fission. Unlike other intracellular protozoon parasites, *T.*

vaginalis only has a single stage in its life cycle - the trophozoite stage (taken from Greek: trophē=nourishment and zoon=animal), which is the activated, intracellular feeding stage, during which *T. vaginalis* can be found in vaginal secretions and urine of women (reviewed by Kissinger, 2015). No cystic stage has been observed for *T. vaginalis*.

A few studies have suggested that *Lactobacillus* spp. may also play a protective role against *T. vaginalis* infection (Phukan *et al*. 2013; Valadkhani *et al*. 2016). Using a well-characterized panel of *T. vaginalis* strains with either low or high adhesion properties, Phukan *et al.* (2013) showed that *Lactobacillus* spp. inhibited *T. vaginalis* adhesion to ectocervical cells in a dose-dependent manner, which required direct contact, with *L. gasseri* exhibiting the highest level of inhibition. Valadkhani *et al.* (2016) also reported that binding of *T. vaginalis* to vaginal epithelial cells was inhibited by cell-free *L. acidophilus* supernatants *in vitro,* suggesting that a secreted *Lactobacillus* factor was causing the inhibition.

T. vaginalis has also been shown to inhibit the growth and adherence of vaginal *Lactobacillus* strains to a range of human epithelial cell lines *in vitro* (McGrory *et al*. 1994; Fichorova *et al*. 2013). In addition, women with trichomoniasis tend to have lower levels of vaginal *Lactobacillus* colonization (Brotman *et al*. 2012), although it is not possible to determine whether the absence of vaginal *Lactobacillus* spp. allow *T. vaginalis* to colonise, or whether *T. vaginalis* infection causes depletion of vaginal *Lactobacillus* spp. without longitudinal sampling.

1.2.3 Key protective mechanisms of *Lactobacillus* spp. in the FGT

The protective effects of *Lactobacillus* spp. (described in section 1.2.1 and 1.2.2) have been attributed to several different metabolites they produce or properties they confer to their host, which will be discussed in more detail in the following sections. These include their ability to: (i) lower vaginal pH (section 1.2.3.1); (ii) produce microbicidal compounds (including L- and D- lactic acid and H_2O_2; section 12.3.1), biosurfactants (section 1.2.3.2), and bacteriocins (section 1.2.3.3); (iii) competitively exclude other bacterial species through competition for adhesion (section 1.2.3.4), and form biofilms (sections 1.2.3.5).

1.2.3.1 Lactobacillus spp. lower vaginal pH, produce H_2O_2 and lactic acid

The physiological genital pH associated with reproductive health is typically considered to be <4.5 (Boskey *et al*. 1999; A. Aroutcheva *et al*. 2001), forming one of the clinical criteria used in Amsel's criteria to diagnose BV (Amsel *et al*. 1983). One of the mechanisms by which the lower FGT is acidified is through the production of organic acid metabolites, such as alpha hydroxy acid, lactic acid and short chain fatty acids by vaginal epithelial cells, released in genital secretions (Preti & Huggins 1975; Boris & Barbés 2000; Boskey *et al*. 2001). In addition, *Lactobacillus* spp. contribute to lowering the vaginal pH by secretion of their own organic acids, predominantly lactic acid (Barrons & Tassone 2008; Boris & Barbés 2000). Vaginal short chain fatty acids and other organic acids, such as acetic acid (which has a dissociation constant [pKa] of 4.76), propionic acid (pKa 4.87), butyric acid (pKa 4.82) and succinic acid (pK_{a1} 4.16 and pK_{a2} 5.61) are all weaker acids than lactic acid (pKa 3.89), suggesting that lactic acid is the major acidifier of the FGT *in vivo* (Tachedjian et al. 2017a). Together, the low vaginal pH and lactic acid produced by *Lactobacillus* spp. and vaginal epithelial cells inhibit the growth and proliferation of viral, bacterial and fungal pathogens, including those causing STIs (section 2). The organic acid metabolic profiles of vaginal microbiota dominated by *Lactobacillus* spp. differ drastically from those typical of BV, with a strong decrease in the amount of lactic acid produced (~110 mM to <20 mM), and an increase in short chain fatty acids, including acetic acid (0–4 to <120 mM), propionic acid and butyric acid (<1 to <4 mM), and succinic acid (<1 to <20 mM) (Gajer et al. 2012; Mirmonsef et al. 2011; Al-Mushrif et al. 2000; Tachedjian et al. 2017b). Together, these changes in acid metabolites may contribute to the increase of vaginal pH observed in women with BV. The ability of vaginal *Lactobacillus* spp. to lower pH *in vitro* has been shown to correlate well with the rate and extent to which they acidify the lower FGT *in vivo* (Boskey *et al*. 1999; María Silvina Juárez Tomás *et al*. 2003).

In the 1990s, the ability of *Lactobacillus* spp. to produce H_2O_2 was thought to be the primary mechanism contributing to lowering vaginal pH and being protective in female genital health (Hawes *et al*. 1996; Hillier *et al*. 1993; Cherpes *et al*. 2008). However, more recent studies have suggested that the contribution of H_2O_2 to vaginal health *in vivo* may be less important (Tachedjian *et al.* 2018). This is because large quantities of oxygen are required by *Lactobacillus* strains to achieve H_2O_2

concentrations considered antimicrobial (>1000mM required to inactivate BV-associated bacteria), while the vaginal environment is microaerobic and therefore has low oxygen availability (Ocana *et al*. 1999; O'Hanlon *et al*. 2011; Martín & Suarez 2010). Furthermore, H_2O_2 also inhibited the growth of *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners* at these high concentrations (O'Hanlon *et al*. 2011). Under aerobic conditions, *Lactobacillus* spp. produce high levels of H_2O_2 (3.3 to 4.4mM; Tomás, Bru and Nader-Macías, 2003) but little or no H_2O_2 under hypoxic or anaerobic conditions (<100μM; O'Hanlon *et al*. 2011; O'Hanlon *et al*. 2010; María Silvin Juárez Tomás *et al*. 2003; Shalev *et al*. 1996). Further, semen inhibited the microbicidal activity of H_2O_2 produced by vaginal *Lactobacillus* spp. (O'Hanlon *et al*. 2010). Overall, $H_2O_2$is quite unlikely to be produced in any quantities that are antimicrobial *in vivo* (Tachedjian *et al.* 2018).

While the majority of vaginal *L. crispatus*, *L. jensenii* and *L. gasseri* are able to produce some H_2O_2 *in vitro*, less than 10% of *L. iners* strains appear able to do so (Antonio *et al*. 1999). Tomás *et al*. (2005) noted that vaginal *Lactobacillus* strains that produced H_2O_2 *in vitro* also produced considerably more lactic acid than strains producing no H_2O_2. While the protective role of H_2O_2 *in vivo* in the vagina might be questionable, the finding by Tomás *et al*. (2005) indicates that *in vitro* H_2O_2 production might be a good surrogate measure for metabolic capacity and beneficial properties of vaginal *Lactobacillus* strains.

Lactic acid produced by *Lactobacillus* spp. has also been shown to inhibit the growth of BV-associated pathogens (section 1.2.1). However, secreted lactic acid does not inhibit *Lactobacillus* spp., suggesting that healthy vaginal microbiota is not affected by the production of these bactericidal compounds (O'Hanlon *et al*. 2011). The antimicrobial effect of lactic acid seems to be associated with vaginal pH, with the antimicrobial activity of lactic acid being lower at higher pHs (~7.0) (O'Hanlon *et al*. 2011). Environmental factors that elevate the physiological vaginal pH might therefore also influence the protective effect of lactic acid. For instance, semen is alkaline (pH~8.2; Harraway, Berger and Dubin, 2000), and studies have suggested that semen influences the pH of vaginal fluid in a dose-dependent manner. While Bouvet *et al*. (1997) reported that vaginal fluid containing no semen had a pH of 3.7; samples with low semen content had an increased pH of 4.0, while vaginal fluid with

high semen content had a pH of 6.1. This suggests that semen may be able to inhibit the antimicrobial action of lactic acid, by neutralizing vaginal pH. Furthermore, blood has a pH of ~7.4 (Mehta & Emmett 2014), which might also increase vaginal pH during menses, and thus alter the antimicrobial potential of lactic acid.

The protonated form of lactic acid (predominantly present at pH<3.9) and the lactate anion (charged, unprotonated, predominantly present at pH>3.9) are thought to exert their action by distinct mechanisms. Only the protonated form of lactic acid, and not the lactate anion, has been shown to have antimicrobial and immunomodulatory activity (O'Hanlon *et al*. 2011; Aldunate *et al*. 2013). In contrast, the unprotonated form of lactic acid is thought to be membrane-permeable and therefore able to enter bacterial cells without the use of transporters or receptors, and acidifies the cytosol of pathogenic bacteria. In addition to permeabilising bacterial membranes, this acidification has been shown to inhibit various intracellular functions and ultimately leads to bacterial cell death (Kashket 1987; Ahmed *et al*. 2009; Kuchiiwa *et al*. 2011; Alakomi *et al*. 2005).

Lactic acid exists as both D- and L-isomers (**Figure 1.3**), with L-lactic acid found to inhibit HIV more effectively than D-lactic acid (Aldunate *et al*. 2013), and D-lactic acid found to inhibit bacterial pathogens like *C. trachomatis* more effectively than L-lactic acid (Valore *et al*. 2002; Graver & Wade 2011; Nardini *et al*. 2016). There are differences in the capacity of *Lactobacillus* spp. to produce lactic acid, with *L. gasseri* strains producing significantly more lactic acid than *L. jensenii* and *L. crispatus* strains (Hutt *et al*. 2016). Notably, the D-lactic acid isomer is produced by *L. crispatus*, *L. jensenii* and *L. gasseri*, but not *L. iners*, which only produces the L-isomer (Witkin *et al*. 2013; France *et al*. 2016). Similarly, vaginal epithelial cells only produce L- but no D-lactic acid (Tachedjian *et al.* 2018). Despite producing different amounts and isomers of lactic acid, however, Hutt *et al.* (2016) found that there was no significant difference between *Lactobacillus* spp. in their ability to antagonize BV-associated spp., including *G. vaginalis*. This latter finding suggests that a single antimicrobial factor alone does not explain the complete spectrum of protection offered by *Lactobacillus* spp. Interestingly, concentrations of vaginal L-lactic acid measured in women with a *Lactobacillus*-dominated microbiota tended to be higher than in women with BV, and D-lactic acid concentrations were higher in samples

containing *L. crispatus* than in those dominated by *L. iners* or *Gardnerella* spp. (Witkin *et al*. 2013).

Due to the different inhibitory activities of the lactic acid isomers, *Lactobacillus*-containing probiotics for vaginal health would need to produce both D- and L-lactic acid and acidify the vagina to a pH <3.9 (pKa of lactic acid) to maximise the inhibitory properties of lactic acid, as the protonated form of lactic acid dominates at this low pH (Tachedjian et al. 2017b).

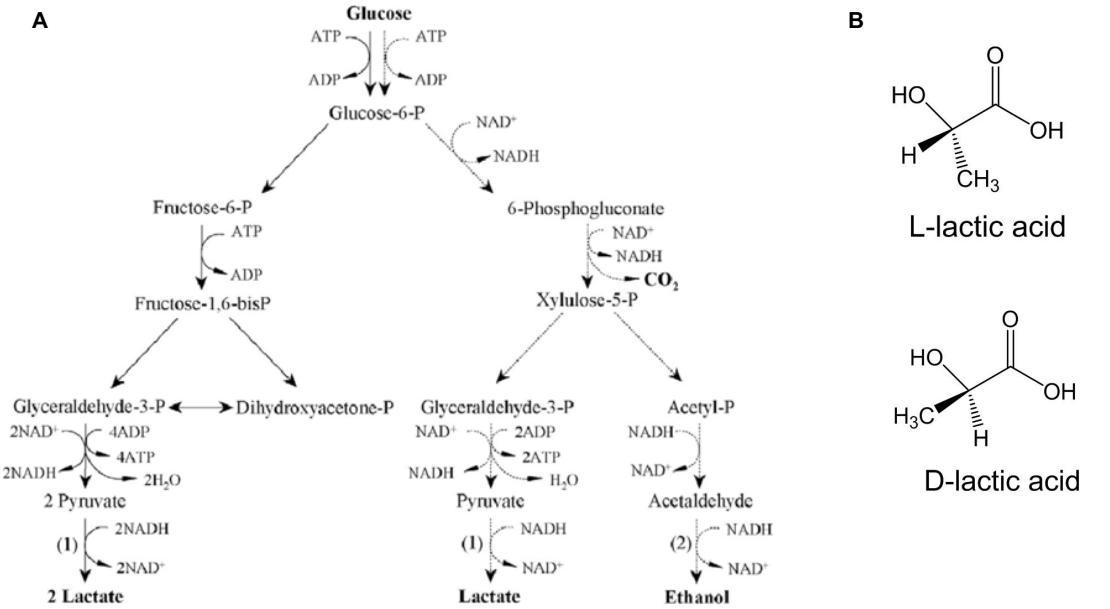


Figure 1.3. Metabolic pathways of Lactic acid bacteria to metabolize lactic acid from glucose. (A) Homofermentative (production of lactate only, solid line) and heterofermentative (production of lactate and ethanol, dotted line) *Lactobacillus* spp. produce lactic acid (lactate). (B) Lactic acid exists as L- and D-isomer. P, phosphate; ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; NAD + , nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced form); (1), lactate dehydrogenase; (2), alcohol dehydrogenase. Taken from (Wee et al. 2006).

1.2.3.2 Ability of Lactobacillus spp. to produce biosurfactants

Biosurfactants are a diverse group of surface active molecules, including glycolipids, lipopeptides, lipoproteins and fatty acids synthesized by microorganisms (Desai and Banat 1997), which reduce surface tension and facilitate the uptake of water-immiscible substrates by microbes (Gudina *et al*. 2010). Diverse metabolic pathways are involved in the synthesis of biosurfactants, and depended on the main carbon source in the culture medium (reviewed by Santos *et al*. 2016). When a hydrocarbon is used as carbon source, the lipolytic pathway and gluconeogenesis is the primary mechanism by which biosurfactants are produced, but when carbohydrates are the

primary carbon source, the lipogenic and glycolytic pathways are supressed, and glucose is degraded and oxidised to form biosurfactant precursors (Santos *et al*. 2016). Biosurfactants produced by *Lactobacillus* spp. have been shown to inhibit the adhesion of pathogens commonly found in the urogenital tract (including *E. faecalis*, *E. coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus agalactiae, Pseudomonas aeruginosa* and *Candida albicans*) *in vitro* to various cell lines and materials, like glass, rubber, and polymer surfaces (Velraeds *et al*. 1996; Velraeds *et al*. 1998; Heinemann *et al*. 2000; Gudina *et al*. 2010; Reid 2000). Several biosurfactants from *Lactobacillus* spp. have been isolated and characterized (Heinemann *et al*. 2000; Rodrigues *et al*. 2006; Rodrigues *et al*. 2006; Gudina *et al*. 2010), and appear to be both heat and pH stable, be produced early (within the first 4 hours of *in vitro* culture; Rodrigues *et al*. 2006), and have antimicrobial and anti-adhesive activities against a variety of pathogenic bacteria (including *E. coli, S. agalactiae* and *Streptococcus pyogenes*; Gudina *et al*. 2010).

1.2.3.3 Ability of Lactobacillus spp. to produce bacteriocins

Bacteriocins are ribosomally-synthesized peptides produced by microbes that have antimicrobial activity (Cotter *et al*. 2005; Turovsky *et al*. 2009). Bacteriocins are thought to contribute to the maintenance of stable bacterial populations and decrease the number of competitors in order to obtain more nutrients and living space in different environmental niches (reviewed by Yang *et al*. 2014). Among Gram-positive bacteria, lactic acid bacteria are the main producers of bacteriocins, which vary in size, structure, chemical properties and antimicrobial activity (reviewed by Yang *et al*. 2014), and are classified as either class I, class II or class III bacteriocins.

Class I bacteriocins have generally a broad spectrum, are post-translationally modified, linear or globular peptides that contain lanthionine, β-methyl lanthionine and dehydrated amino acids (reviewed by Bastos *et al*. 2015), and form pores in bacterial cell walls or inhibit cell wall synthesis (Islam *et al*. 2012). Nisin A and Z produced by *Lactococcus lactis subsp. lactis* (Mulders *et al*. 1991; Field *et al*. 2012); and Labyrinthopeptin A2 produced by an *Actinomadura* strain (Meindl *et al*. 2010) are some examples of class I bacteriocins.

Class II bacteriocins are a more heterogeneous class of small peptides, generally with a narrow spectrum, which are heat stable, not post-translationally modified, and non-lanthionine-containing (reviewed by Bastos *et al*. 2015). Class II bacteriocins permeabilise the bacterial cell membrane, causing an efflux of ATP molecules and dissipation of the proton motive force, which result in complete arrest of microbial biosynthesis (Nissen-Meyer *et al*. 2009). Bacteriocins belonging to this group include Lactin F produced by *L. johnsonii* VPI11088 (Fremaux *et al*. 1993), lactococcin A produced by *L. lactis subsp. cremoris* LMG 2130 (Holo *et al*. 1991) and ABP-118 from the human probiotic strain *L. salivarius subsp. salivarius* UCC118 (Flynn *et al*. 2002). Lactocin 160, produced by healthy vaginal *L. rhamnosus* strain, has been shown to completely block biosynthesis of *G. vaginalis* (Turovsky *et al*. 2009).

Class III bacteriocins are large, heat-labile proteins, and include caseicin 80 purified from *L. casei* B80 (Müller and Radler 1993) and Helveticin J produced by *L. helveticus* 481 (Joerger and Klaenhammerl 1990).

While bacteriocin-producing strains generally are not sensitive to the bacteriocin they produce (Bastos *et al*. 2015), bacteriocins produced by one *Lactobacillus* strain can inhibit different strains of the same species (Holo *et al*. 1991), as well as pathogenic species, like *G. vaginalis* (Aroutcheva *et al*. 2001). Pathogenic bacteria can acquire resistance to bacteriocins, as was demonstrated by *Listeria monocytogenes,* which developed resistance to the bacteriocin nisin (Gravesen *et al*. 2004).

1.2.3.4 Ability of Lactobacillus spp. to adhere to vaginal epithelial cells

Several studies have shown that the adhesion of vaginal *Lactobacillus* spp. to vaginal cells can competitively prevent the attachment of pathogens (Boris *et al*. 1998; Reid *et al*., 2000; Osset *et al*. 2001; Atassi *et al*. 2006; Coudeyras *et al*. 2008; Mastromarino *et al*. 2002; Zarate and Nader-Macías 2006; McLean *et al*., 2000). Adhesion of *Lactobacillus* spp. to vaginal cells *in vitro* is highly strain-specific, with the adhesion strength correlating with the ability of *Lactobacillus* strains to auto-aggregate (Otero and Nader-Macías 2007; Tomás *et al*. 2005; Malik *et al*. 2013). The ability of vaginal *Lactobacillus* spp. to auto-aggregate has been suggested to allow for the formation of biofilms (Ventolini et al. 2015a). *Lactobacillus* spp. also seem to be

able to co-aggregate with pathogens like *G. vaginalis*, thereby inhibiting pathogen adhesion (Ocaña & Nader-macías 2002; De Gregorio *et al*. 2014; Boris *et al*. 1998; Mastromarino *et al*. 2002). However, Machado *et al*. (2013) found that some BV-associated bacteria were able to adhere to inert surfaces (chamber glass slides) that had been pre-coated with *L. crispatus*. They reported that *G. vaginalis* was more adherent to *L. crispatus* than the other anaerobic BV-associated bacteria, followed by *Fusobacterium nucleatum* and *P. bivia* (Machado *et al*. 2013).

1.2.3.5 Ability of Lactobacillus spp. to form biofilms

Some *Lactobacillus* genotypes produce biofilms that protect against pathogens (Martín et al. 2008; Terraf et al. 2016; Ventolini et al. 2015b). Biofilm formation facilitates colonization and long-term persistence of beneficial *Lactobacillus* strains in the FGT, by maintaining a stable ecosystem that inhibits the ability of pathogens to invade this ecological niche (Jones and Versalovic 2009; Ventolini *et al*. 2015; Terraf *et al*. 2016). *Lactobacillus* strains used, the culture medium in which they are grown, the inoculum concentration and their growth kinetics all appear to influence the ability of strains to form biofilms *in vitro* (Terraf *et al*. 2012; Terraf *et al*. 2016). However, *Lactobacillus* spp. like *L. jensenii,* tend to form complex and highly-structured biofilms with a large amount of extracellular matrix being produced (Martín *et al*. 2008; Ventolini *et al*. 2015). *Lactobacillus* spp. biofilms have also been observed in fresh vaginal wet mount preparations, with *L. jensenii* being the most common species associated with biofilm formation (Ventolini et al. 2015b). Therefore, biofilm formation by *Lactobacillus* spp. might hinder *G. vaginalis* to lay down its own biofilm *in vivo*, and not allow other pathogenic bacteria to colonize the FGT.

In conclusion, there are several potential mechanisms whereby *Lactobacillus* spp. may confer health benefits to their hosts, and many of these are *Lactobacillus* spp.- and strain-specific. Therefore, the composition of *Lactobacillus* spp. and strains in the FGT is likely to determine the overall protective effect towards vaginal health in women.

1.3 Harnessing probiotics for vaginal health

The WHO defines probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill *et al*. 2014). The Russian immunologist and Nobel laureate Eli Metchnikoff first proposed the use of live intestinal microorganisms “to modify the flora in our bodies and to replace the harmful microbes by useful microbes" more than 100 years ago (Anukam and Reid 2007). Since then, probiotics have been administered in many disease settings to improve microbial dysbioses, defined as a functional alteration of the microbiota that result from an imbalance between protective and pathogenic bacteria (reviewed by Chan 2013). It is, however, challenging to define “healthy” or “normal” microbiota in any human niche, because its composition varies not only between body sites, but also between geographic area and environment and by gender, age, and diet within one body site. Taking this into account, it has been argued that microbial health can instead be characterized by its diversity, stability, resistance and resilience (Lozupone *et al*. 2012; Levy *et al*. 2017). On the other hand, microbial dysbiosis can be characterized by overgrowth of pathobionts (which are commensals that have the potential to cause pathology in the wrong site or setting), a loss of commensals, or alteration in microbial diversity (Levy *et al.* 2017; Chow and Masmanian 2010).

Probiotics have been explored to treat a wide range of conditions associated with a disrupted microbiome, including those that target the (i) intestinal tract [like irritable bowel syndrome (Zhang *et al*. 2016; Distrutti *et al*. 2013); diarrhoea (Sazawal *et al*. 2006); obesity (Kang *et al*. 2013; Sáez-Lara *et al*. 2016)], (ii) skin [like eczema (Nylund *et al*. 2013); *S. aureus* infections (Prince *et al*. 2012)], (iii) FGT [like BV, (K. Anukam et al. 2006; Reid et al. 2001; Hummelen et al. 2010; Petricevic & Witt 2008)], (iv) respiratory tract [including allergies, (Ciprandi *et al*. 2005); respiratory tract infections (Marseglia *et al*. 2007)], and the (v) oral cavity [like gingivitis, (Iniesta *et al*. 2012)]. McFarland (2014) suggested that probiotics might also be beneficially administered to prevent or support microbial health in individuals who initially have a healthy, undisturbed microbiota, but who’s microbiota is at risk of becoming disrupted as a result of various treatments (such as antibiotics, chemotherapy or infection with pathogens); or to treat or improve an already disrupted microbiota, due to a pre-existing chronic condition or an active disease.

In the case of BV, probiotics have been administered to women for both preventive and therapeutic purposes. A total of 37 clinical trials were identified and reviewed for this dissertation (from searches of GoogleScholar and PubMed), conducted using *Lactobacillus*-containing probiotics in treatment of BV, either alone or in combination with antibiotics. Details of each of these trials are summarized in Appendix I. Almost all of these trials reported some although not always statistically significant improvements in BV cure and recurrence rates. However, these trials had some important differences in how they were conducted; including (i) differences in the probiotic species that were tested (including *L. gasseri, L. rhamnosus, L. reuteri* and *L. plantarum*); (ii) differences in the doses given (ranging from 10^4 to 10^{10} CFU/dose); (iii) differing durations of treatment (ranging for 6 days to 6 months); (iv) differences in the route by which probiotics were administered (oral vs. vaginal); and (v) differences in the populations in which the trials were performed (European, South American, Asian, African). Furthermore, not all were placebo-controlled; and not all gave probiotics as adjuct to antibiotics. While only 25/37 trials explicitly included reporting of adverse events (AEs), no serious AEs were reported in the probiotic intervention arm, even in immunocompromised individuals (Appendix I, Happel *et al*. 2018). In those trials that included AE reporting, candidiasis, vaginal discharge, odour, itching, bleeding, swollen vulva, vulvovaginitis, diarrhoea, nausea and headaches were the most common (Appendix I), although most of these were considered not to be probiotic-related.

Two meta-analyses have been performed to evaluate whether probiotics improve BV cure or recurrence rates (Huang *et al*. 2014; Tan *et al*. 2017). Huang *et al*. (2014) included 1304 patients from twelve randomized clinical trials (RCTs) and concluded that probiotics significantly improved BV cure rates in adult women, with a relative risk (RR) of 1.53 (95% confidence interval [CI] 1.19–1.97). In contrast, Tan *et al*. (2017) included 1186 patients from five RCTs, comparing metronidazole in combination with probiotics to metronidazole alone for the treatment of BV, and found no significant improvement overall (RR 1.03, 95%CI 0.96 – 1.09). Tan *et al*. (2017) went further to show that this was consistent irrespective of how BV was diagnosed (Amsel criteria 1.04 [95%CI 0.96–1.13] or Nugent scores 1.02 [95%CI 0.94–1.11]); irrespective of what the duration of the effect was (short-term 1.01

[95%CI 0.93–1.10] or long-term effect 1.06 [95%CI 0.98–1.14]); and independent of the ancestry of the participants (European 1.06 [95%CI 0.95–1.19] or non-European women 0.99 [95%CI 0.94–1.05]). The disagreement between these meta-analyses and the huge variation in how the trials were performed argue strongly for better-designed studies to confirm the efficacy of adjunctive probiotics for the treatment of BV.

Thus, there appears to be clinical equipoise around the treatment of BV with probiotics. The latest Cochrane review including RCTs comparing probiotics with placebo, probiotics used in conjunction with antibiotics compared with placebo or probiotics alone compared with antibiotics, regardless of diagnostic method, probiotic preparation (single or multiple strains) and type, dosage and route of administration used, states that probiotics may improve antibiotic treatment in BV, although there is currently not enough evidence to definitively support this as the preferred method of treatment[2]. However, sub-analyses in this Cochrane review suggested a beneficial outcome of microbiological cure with regimens of metronidazole/probiotic (Odds Ration [OR] 0.09; 95%CI 0.03–0.26) when compared to metronidazole/placebo, while probiotics alone vs. metronidazole (OR 0.27, 95%CI 0.07–1.10) and probiotic versus placebo (after open treatment with clindamycin ovules; OR 1.21; 95%CI 0.67–2.19) showed smaller effect sizes[2]. Interestingly, probiotic/estriol preparations have also been shown to have some beneficial effects on BV cure (OR 0.02, 95%CI 0.00–0.47). Overall, this data provides a rational to use probiotics in adjunct to antibiotics to treat BV rather than using probiotics alone.

As the composition of what would be considered healthy vaginal microbiota likely depends on several factors, including geographic location and race (Anahtar et al. 2015a; Ravel et al. 2011; Gajer et al. 2012), it is important to perform RCTs in different geographic locations, to allow evaluation of adjunctive probiotics under relevant local conditions. Further, it may be important to administer probiotics that contain vaginal *Lactobacillus* spp. (rather than gastro-intestinal species). Since the abundance of vaginal bacterial communities differ by ethnicity and/or region (Ravel et al. 2011; Zhou et al. 2007; Anahtar et al. 2015a; Lennard et al. 2017), it may also be important to develop probiotics that contain locally-derived vaginal *Lactobacillus*

[2] https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD006289.pub2/media/CDSR/CD006289/CD006289.pdf [13 August 2018]

strains. Despite incredibly high BV rates (Kenyon *et al*. 2013) and the highest HIV incidence in the world [3], no RCT has yet been conducted in South Africa that compares metronidazole treatment to a combination of probiotics with metronidazole in the management of BV.

1.3.1 Regulation of probiotic versus live biotherapeutic products

Because of increasing appreciation of the importance of the microbiome in human health and interest in probiotics, there has been rapid growth in the probiotic market for pharmaceutical use, called 'next-generation' probiotics in Europe and the United Kingdom and live biotheraputics in the US. This has created the need for more effective legislative regulations of the probiotic industry.

1.3.1.1 Regulation of probiotic products in South Africa

The most recent guidelines from the South African Health Product Regulatory Authority (SAHPRA), that regulates medicines, probiotics and other health supplements in South Africa, classifies probiotic-containing products as medicines when the products make any medicinal claims or contain $\geq 10^9$ CFU per dose, or as complementary medicines/health supplements if these criteria are not met[4]. To date, however, no probiotic product has been registered with the SAHPRA, and all probiotics are sold exclusively as over-the-counter (OTC) products.

1.3.1.2 Regulation of probiotic versus live biotherapeutic products in the US

The The US Food and Drug Administration (FDA) defines live biotherapeutic products (LBP) as biological products containing live organisms, which are intended to be used to prevent, treat, or cure a human disease/condition, but are not a vaccine[5]. The FDA states that probiotics may be regulated as drugs or as dietary supplements, conventional food or meal replacement, depending on their intended use[6]. However, under FDA guidelines, vaginally delivered LBP are never considered a dietary

[3] http://www.unaids.org/en/regionscountries/countries/southafrica [13 August 2018]
[4] http://www.mccza.com/Publications [05 March 2018]
[5] http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/General/UCM292704.pdf; [13 July 2018])
[6] http://www.fda.gov/regulatoryinformation/guidances/ucm144657.htm [05 March 2018]

supplement but always a drug, as opposed to orally delivered LBP, which can be either based on intend to use[6].

1.3.1.3 Regulation of probiotic products in the European Union (EU)

For probiotics sold as nutritional supplements in the EU, the European Food and Safety Authority (EFSA) assesses all health claims made by the manufacturer, and each European country has its own country-specific rules regulating these products. Despite >300 requests from different manufacturers for products including >200 probiotic stains and combinations of these, with >60 beneficial effects being claimed, the EFSA has rejected all submitted health claims for probiotic products to date[7]. Therefore, labels on probiotic products in Europe typically contain little or no description of what benefits can be expected from consumption of the probiotics. In some European countries, products are not allowed to put the word 'probiotic' on the label. Some of the stated reasons that the EFSA has rejected all of the health claims submitted to date are, (i) that bacterial strains in formulation have not been sufficiently characterised; (ii) that products have poorly defined claims or claims are not beneficial; (iii) that there is no direct evidence of health outcomes from clinical trials; and/or (iv) that the human studies were considered low quality[7]. However, until recently vaginally delivered LBP could be sold in pharmacies as medical devices, but now, vaginally delivered LBP are considered as drug and required to follow the drug pathway, which EFSA does not regulate.

1.3.2 Good Manufacturing Practice (GMP) considerations for probiotics

Regulation of probiotic products is a rapidly evolving concept in South Africa and worldwide, as is regulation of probiotic manufacturing and GMP conditions. Considering GMP for local probiotics would ensure high standards of quality assurance in the development, manufacturing and control of products, and is the basis for a successful registration of a product making beneficial claims with regulatory authorities elsewhere in the world. According to the SAHPRA, GMP requires that the holder of a manufacturing licence must manufacture products in such a way that: (i) they are fit for their intended use; (ii) comply with the requirements of the medicine

[7] https://www.probiotaamericas.com/wp-content/uploads/2016/06/IPAWCPBA-Dr-Valeriu-Curtui.pdf [29 March 2018]

registration; and (iii) do not place consumers at risk due to inadequate safety, quality or efficacy[8]. GMP includes both production and quality control to ensure that products are consistently produced and controlled to the quality standards appropriate to their intended use, and as required by the medicine registration. Basic GMP requirements to maintain a licence to manufacture in South Africa are: (i) that all manufacturing processes are clearly defined, systematically reviewed and validated; (ii) that all necessary facilities for GMP are provided (including appropriately qualified and trained personnel, adequate premises and space, suitable equipment, correct materials, containers and labels, approved procedures and instructions, and suitable storage and transport); (iii) that instructions and procedures are written in clear and unambiguous language; (iv) that records are made during manufacture, which demonstrate that all the steps required were followed and that the quantity and quality of the product was as expected; (v) that records (including distribution) with the complete history of a batch are made; (vi) that a system is available to recall any product batch from sale or supply (if necessary); and (vii) that all complaints about marketed products are examined, the causes of quality defects investigated and appropriate measures taken to prevent re-occurrence[8].

In addition, the SAHPRA stipulates that the starting and packaging materials, intermediate, bulk, and finished products have to be sampled, inspected and tested using validated methods as part of quality control[8]. Finished products containing active ingredients have to comply with the qualitative and quantitative composition of the medicine registration, be of the purity required, enclosed within their proper containers and correctly labelled. Therefore, no batch of products can be released for sale or supply prior to certification, and sufficient reference samples of starting materials and products have to be retained to permit future examination of the batch if necessary. Although regulation of the manufacture of probiotics is being developed by SAHPRA, only one of the probiotic products currently available OTC in South Africa explicitly stated that it was manufactured under GMP conditions, which is in contrast to many products available in the EU (discussed in more detail in Chapter 2).

[8] http://www.mccza.com/documents/e9c0424e16.01_Licence_to_manufacture_Feb04_v1.pdf [07 March 2018]

1.4 Rationale for this study

BV is highly prevalent and recurrent in South Africa (Kenyon *et al*. 2013; Johnson *et al*. 2005; Barnabas *et al*. 2017) and places women at higher risk for HIV infection (Sewankambo *et al*. 1997; Cohen *et al*. 2012; Martin *et al*. 1999; Taha *et al*. 1998; Myer *et al*. 2005; Van de Wijgert *et al*. 2008; Anahtar *et al*. 2016). It is a condition that is often asymptomatic and therefore not treated using the syndromic management algorithm practiced in South Africa, although it is known to cause significant inflammation whether asymptomatic or symptomatic (Mlisana *et al*. 2012; Masson *et al*. 2014; Masson *et al*. 2015). BV is also difficult to treat with metronidazole in the long-term, with six-month recurrence rates of 50% (Barrons & Tassone 2008). There is therefore an urgent need to re-evaluate how BV is managed, which characteristics of *Lactobacillus* strains are necessary to ensure an improvement of vaginal health, and whether these organisms can be harnessed in conjunction with metronidazole to treat BV and mitigate inflammatory processes that may influence HIV risk in South African women.

1.4.1 Aims and objectives

The aim of this dissertation is to evaluate *Lactobacillus*-containing probiotics for vaginal health available on the South African market, to compare properties of these probiotic *Lactobacillus* strains with vaginal *Lactobacillus* isolates from South African women, and to explore the use of probiotics as adjunctive treatment to antibiotics for BV in South African women. A minor aim was to evaluate the contribution of bacteriophages to the etiology of BV and *Lactobacillus* spp. depletion from the FGT.

These aims will be evaluated with the following specific objectives:

Specific objective 1: To survey OTC probiotic products available in South Africa

Rationale: To evaluate the availability and suitability of probiotics marketed for vaginal health in South Africa for the treatment of BV.

Hypothesis: *There is a lack of locally designed probiotics for vaginal health in South Africa. Although it is not clear whether these were manufactured under GMP conditions in South Africa, probiotic products marketed for vaginal health will be of good quality, contain the species stated on the packaging at the stated dose, with minimal inter- and intra-lot variations in dose and microbial characteristics. Probiotic isolates from some products will perform better than those from others in characteristics considered important for probiotic function (including their ability to grow well in culture, lower culture pH, produce lactic acid, and adhere to genital epithelial cells).*

Specific objective 2: To determine the efficacy of a South African OTC probiotic product marketed for vaginal health for the treatment of BV in South African women in a single-blind, randomised clinical trial approved by SAHPRA

Rationale: No probiotic product in South Africa has yet been registered with the SAHPRA. The regulatory landscape for probiotics development and testing needs to be developed in South Africa. No RCT testing the effect of adjunctive probiotics together with metronidazole treatment on BV treatment in South African women has yet been performed.

Hypothesis: *BV cure rates will be higher in women taking metronidazole with adjunctive probiotics compared to SOC treatment with metronidazole alone. Women being treated with a combination of probiotics with metronidazole will have increased vaginal colonization with beneficial Lactobacillus spp. and decreased colonization with BV-associated bacteria compared to women taking metronidazole alone. Further, genital inflammation (measured by using cytokine biomarkers) will be lower in the intervention compared to the control group.*

Specific objective 3: To characterize vaginal *Lactobacillus* isolates from South African women for the development of geographically relevant probiotic products for vaginal health in Africa

Rationale: None of the OTC probiotics currently marketed for vaginal health in South Africa contain *Lactobacillus* spp. that commonly colonize to the FGT of South

African women. There is therefore an urgent need to screen vaginal *Lactobacillus* isolates from South African women for properties considered beneficial for a vaginal probiotic.

Hypothesis: Vaginal Lactobacillus isolates from healthy South African women will possess strain-specific characteristics with an overall better probiotic profile than the currently commercially available Lactobacillus strains, which make them more relevant probiotic candidates for the South African market.

Specific objective 4: To evaluate the role of bacteriophages in the etiology of BV

Rationale: Bacteriophages play a major role in manipulating the GIT microbiome. Many *Lactobacillus* isolates from commercial probiotic and diary products, as well as clinical sources (including the lower FGT) have temperate bacteriophages within their genomes that can be induced under various stress conditions. The mechanisms causing the rapid shift in bacterial communities during the onset of BV remain unknown, and bacteriophages may contribute.

Hypothesis: Prophage sequences (as measure for temperate bacteriophages) and CRISPR loci (as measure for previous attacks by virulent bacteriophages) are common in the genomes of vaginal Lactobacillus strains. Depletion of Lactobacillus spp. during the onset of BV is caused by temperate bacteriophages induced under environmental stress condition, or by virulent bacteriophages present in FGT secretions.

Chapter 2

The South African probiotics market

2. What is on retailer's shelves?

2.1 Introduction

The concept to use microorganisms "to modify the flora in our bodies and to replace the harmful microbes by useful microbes" was introduced by Eli Metchnikoff >100 years ago (Anukam & Reid 2007). Since 1997, the interest in probiotics has grown exponentially, demonstrated by the increasing reference to these in the public media, including scientific articles, published reviews, conference abstracts, and other editorial material and letters (<100 in 1997 to >3000 in 2017, source: Wed of Science). Similarly, the number of patents filed that contain the word probiotics or probiotic in their titles (source: GooglePatents) rose from <200 in 1997 to >3000 in 2017.

The Asia-Pacific region remains the largest global probiotic consumer, but the probiotic market is expected to improve penetration in the US and Europe because of supportive political landscapes and favourable regulatory frameworks[9]. According to the United Nations Development Programme report (2010), many developing countries in Africa, Southeast Asia and the Middle East have a gross income and quality of life index that is below average (Monachese *et al*. 2011). In these regions, diarrhoea account for more than 45% of deaths among children <5 years (Nahaisi *et al*. 2014), with generally poor nutritional status, and high prevalence of HIV (Monachese *et al*. 2011). Although probiotics are recognized as a strategy to improve overall human health in these regions, the probiotic market in less developed, and sparse data are available for market share of probiotics in Africa and other developing countries. Fermentation of milk mainly occurs in northern West and East Africa, the Sahara and the Savannah (Franz *et al*. 2014), and South Africa appears to have a relatively well-established probiotics market (Brink *et al*. 2005; Franz *et al*. 2014). While in 2004 the probiotic industry in South Africa was worth ~ZAR 45 million (3.87 million USD) per year with >11 million doses taken annually[10], the probiotic industry in South Africa was predicted to be worth over ZAR 527 million (45.29 million USD) for 2017[11]. More recent data showed that the probiotic supplements

[9] https://www.gminsights.com/industry-analysis/probiotics-market [27 March 2018]
[10] https://www.feednavigator.com/Article/2004/02/25/Probiotics-fail-to-match-label-claims-in-S.Africa-study [15 March 2018]
[11] http://www.bizcommunity.com/Article/196/162/167701.html [15 March 2018]

market grew by a very significant compound annual growth rate (CAGR) of 21.6% between 2012 and 2017 in South Africa, and is predicted to continue to grow at a robust CAGR of 15.7% for the period 2018-2022, confirming that this sector is growing tremendously [12]. However, there has been no study published describing the availability of probiotics on the South African retail market, their health claims and bacterial composition.

Thus, this Chapter aimed to evaluate the availability of OTC probiotics on the South African retail market, including their bacterial composition, beneficial claims made, formulation, storage, mode of administration, and price. Some of the findings of this Chapter were published in BMC Women's Health (Happel *et al*. 2017).

[12] http://www.bizcommunity.com/Article/196/323/183048.html [26 October 2018]

2.2 Methods

2.2.1 Assessment of the probiotics market in South Africa 2015/2016

To review probiotics available on the South African retail market, a cross-sectional survey by means of a two-stage cluster sampling was conducted in Durban and Cape Town, South Africa, between September 2015 and January 2016. The primary clusters were geographically demarcated and consisted of the seven and eight districts constituting the Durban and Cape Town metropoles, respectively. The secondary clusters were the major shopping malls in each of the respective districts. Details on all probiotic products in every store retailing probiotics, including pharmacies, health stores and supermarkets in the major shopping mall servicing each of the seven districts making up the Durban metropole [Central (The Workshop, City Centre), Outer West (Hillcrest Centre, Hillcrest), South (Galleria Centre, Amanzimtoti), South Central (Chatsworth Centre, Chatsworth), Inner West (The Pavillion, Westville), North Central (Bluff Centre, Bluff) and North (Gateway Centre, Mt. Edgecombe)] and the eight suburbs making up the Cape Town metropole [South (Blue Route Centre, Tokai), South Peninsula (Long Beach Mall, Fishhoek/Kommetjie), Atlantic Seaboard (Cape Quarter, Greenpoint), City Bowl (V&A, Waterfront), West Coast (Bayside Mall, Bloubergstrand), North (Canal Walk Mall, Century City), Cape Flats (Liberty Promenade, Mitchells Plain) and Helderberg (Waterstone Centre, Somerset West)] were collected. The following characteristics were captured: formulation (tablet, power or liquid/spray), composition (strains, number of viable cells/CFU, presence/absence of prebiotics, additional ingredients), target population (general, women, infants), storage (room temperature, refrigerated), price, expiry date, recommended mode of administration, medicinal claims and manufacturer's details. The shelf life of products was estimated by calculating the time difference between time of survey and expiry date of the product.

2.2.2 Data capture and analysis

Product characteristics were electronically captured in a spreadsheet. The shelf life of products was estimated by calculating the time difference between time of survey and expiry date of the product. Statistical analysis was conducted using STATA^{TM} version 11.0 (StataCorp, College Station, TX, USA). Descriptive measures (mean, median, frequencies and percentages) were used to summarize the data. The Mann-Whitney U

test was used to compare two groups of non-parametric continuous variables, and for three or more groups, the Kruskal-Wallis one-way analysis of variance was used. 95% confidence intervals and p values of <0.05 were used to assess statistical significance. GraphPad Prism6® (GraphPad Software, USA) was used to generate graphs.

2.3 Results

2.3.1 Availability of probiotics in South Africa

A total of 104 unique OTC probiotic products were surveyed from South Africa, with 94 available in Cape Town, and 59 in Durban. Forty-five products were only found in Cape Town, 10 were only available in Durban, and 49 products were common to both cities (**Figure 2.1**). Probiotic product range differed by district, with the eight Cape Town districts averaging 37 distinct products per district [highest (64) in the Northern suburbs and the lowest (13) in the Atlantic Seaboard], and the seven districts of Durban averaged 16 distinct products per district [highest (30) in the Outer West district to the lowest (5) in the Southern district] (**Figure 2.1**).

Medicinal claims of probiotics in both cities were highly similar between Cape Town and Durban (**Figure 2.1**). Of the 104 probiotics identified, about half (51%) had GIT health claims for adults, and 17% were indicated for both GIT and skin conditions in infants. Less than 10% of probiotic products had claims in multiple health categories, and less than 3% were marketed exclusively for improvement of the immune system. Less than 4% of probiotics (4/104; including Provacare® Probiotic Vaginal Care [manufactured in Canada], UltraFlora® Women's [manufactured in US], and Reuterina™ Femme and Vagiforte® PLUS [manufactured in South Africa]) in both cities in South Africa were explicitly marketed for vaginal health. While all four products were available in Cape Town, only Reuterina™ Femme was marketed in Durban. After completion of the study, the distribution of Provacare® Probiotic Vaginal Care was discontinued on the South African market, while a new product, Vagiforte® PLUS Combo Pack, was launched. Of the 104 products reviewed, only one of them (QuatroFlora™, Beta Pharmaceuticals, South Africa) stated on the manufacturer's brochure that it was manufactured according to European GMP guidelines for pharmaceuticals.

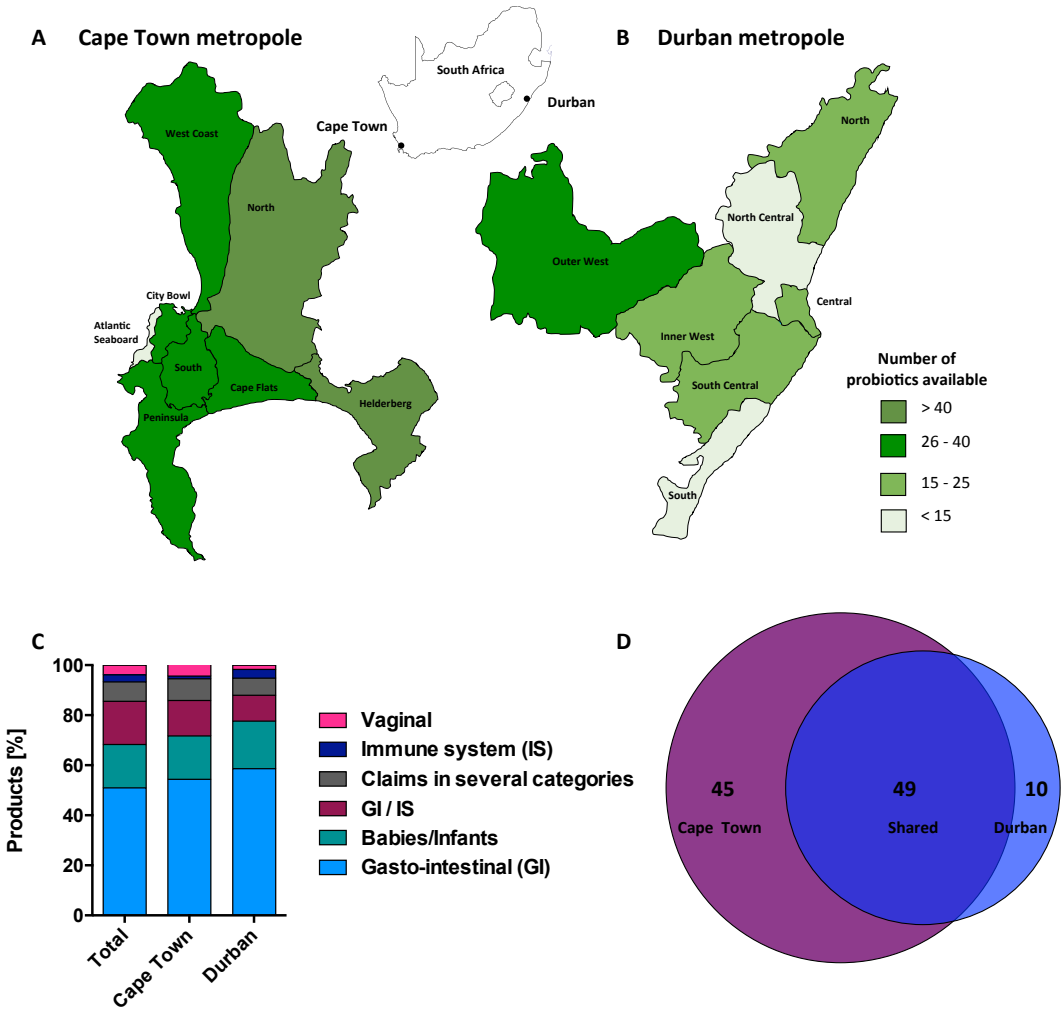


Figure 2.1. Availability of probiotics by districts in the Cape Town (A) and Durban (B) metropoles in South Africa. Cape Town consists of eight districts, while the Durban metropole is made up of seven districts. A colour scale (increasing number of products from from light to dark green) was used to denote variation in the number of distinct probiotics available for each suburb. (C) Categories of marketed probiotics. Each product was grouped into one of six categories, according to their health claim (v = vaginal health, IS = immune system, GI = gastro-intestinal health); and the overall or city-specific distribution is depicted by the stacked bars. (D) Market share Cape Town vs. Durban. The area proportional Venn diagram represents the number of probiotics marketed exclusively in Cape Town (purple) or Durban (light blue) and those common to both cities (blue).

2.3.2 Claims of probiotics for vaginal health

Four probiotics made explicit claims on the package insert for vaginal health, of which none had SAHPRA or FDA approval, and none was manufactured under GMP conditions. Reuterina™ Femme claims to help maintain a healthy vaginal flora and prevent urogenital infections and BV. Its dosage can be increased from one to two oral capsules daily and there are no known symptoms when overdosed, but no references were provided to support this statement. The package insert seems to contradict the claims on the outer packing as it states that this product is not intended

to treat, cure or prevent disease. Provacare® Probiotic Vaginal Care is classified as nutritional substance and claims to restore vaginal flora balance and maintain the ideal pH. It also claims to assist in the relief of symptoms of burning, stinging and vaginal discharge. One capsule of this product should be administered vaginally twice a day. Bioflora Vagiforte® Plus claims to restore and maintain the normal vaginal flora by controlling the overgrowth of pathogenic microorganisms in the GIT and preventing their transfer to the urogenital tract. High dose treatment is stated to prevent the development of BV and candidiasis. For Vagiforte® Plus, the dose is one oral capsule daily for five days and then one tablet vaginally daily for the next five days, while for the Vagiforte® Plus Combo pack, one capsule should be administered orally once a day for 5 days, followed by once oral capsule and two squirts of vaginal spray twice a day for the next 10 days, or until the spray bottle is finished. UltraFlora® Women's is taken orally once to twice daily and is claimed to restore and maintain a healthy vaginal microbiome and reportedly reduces pathogenic bacteria and yeasts. Further, UltraFlora® Women's claims to assist treatment of BV in conjunction with antibiotics when taken twice daily for at least 7 days 2–3 hours before or after the antibiotics.

2.3.3 Bacterial species contained in probiotic formulations

While the manufacturers made health claims for all 104 products marketed, only for 101 probiotics the bacterial species contained in the formulation was available on the package leaflet, and only 38 of 101 products (37.6%) provided information about the specific strains. The most common bacterial species found in the probiotics belonged to the genera *Lactobacillus* [15 species; most commonly *L. acidophilus* (in 53.5% of the recorded products), *L. rhamnosus* (37.6%), and *L. plantarum* (19.8%)] and *Bifidobacterium* [8 species; most commonly *B. longum ssp. longum* (35.6%), *B. animalis ssp. lactis* (33.7%), and *B. bifidum* (26.7%)] (**Figure 2.2**). The most common combinations of species were *L. acidophilus* with *B. longum ssp. longum* (in 24 products of 101 product, 23.8%), *L. acidophilus* with *B. bifidum* (23/101; 22.8%), *B. longum ssp. longum* with *B. bifidum* (23/101; 22.8%), *L. acidophilus* with *L. rhamnosus* (19/101; 18.8%), and *L. rhamnosus* with *B. longum ssp. longum* (18/101; 17.8%; **Figure 2.3**; Appendix II). Other less common organisms contained in probiotics were *Saccharomyces boulardii* and *S. cerevisiae*, *Streptococcus thermophilus*,

Enterococcus mundtii, *Lactococcus lactis* and *Propionobacterium shermanii* (**Figure 2.2**). The number of bacterial species contained per product ranged from one (25.0 %, 26/104) to 12 (2.9 %, 3/104), with a median of three (IQR 2-4).

Interestingly, there was no association between the species contained in the formulation or the combination of species and the category (GIT, immune system, babies/infants, or vaginal health) for which the product was marketed (**Figure 2.3**). The probiotics marketed for vaginal health contained either a combination of four species (*L. acidophilus, L. rhamnosus GG, B. longum ssp. longum* and *B. bifidum*; Bioflora Vagiforte® PLUS Combo Pack; only launched after the completion of the survey), three species (*L. acidophilus, B. longum ssp. longum and B. bifidum*; Bioflora Vagiforte® PLUS), two species (*L. rhamnosus* and *L. reuteri*; Reuterina™ Femme and UltraFlora® Women's), or the single bacterial species *L. rhamnosus* (Provacare® Probiotic Vaginal Care; discontinued after completion of the survey).

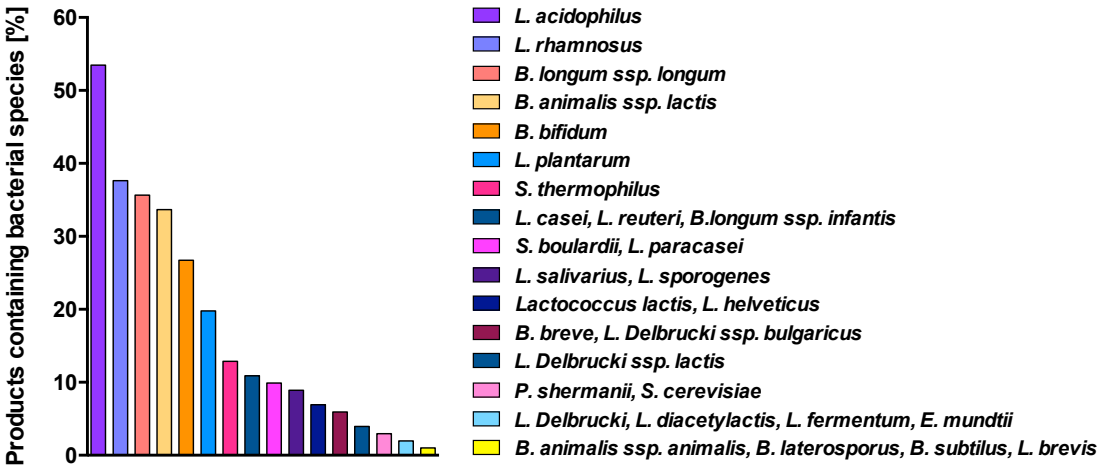


Figure 2.2. Constituting organisms of probiotics in South Africa. Bacterial species contained in probiotics marketed in South Africa belonged to the genera *Lactobacillus* with 15 species, *Bifidobacterium* with 8 species, *Streptococcus thermophilus, Enterococcus mundtii* and *Propionibacterium shermanii*. The yeast *Sacchaeromyces* was represented with two species. The colour-coding is based on percentgae of products containing a specific species. Species that are contained in the same percentage of products share the colour.

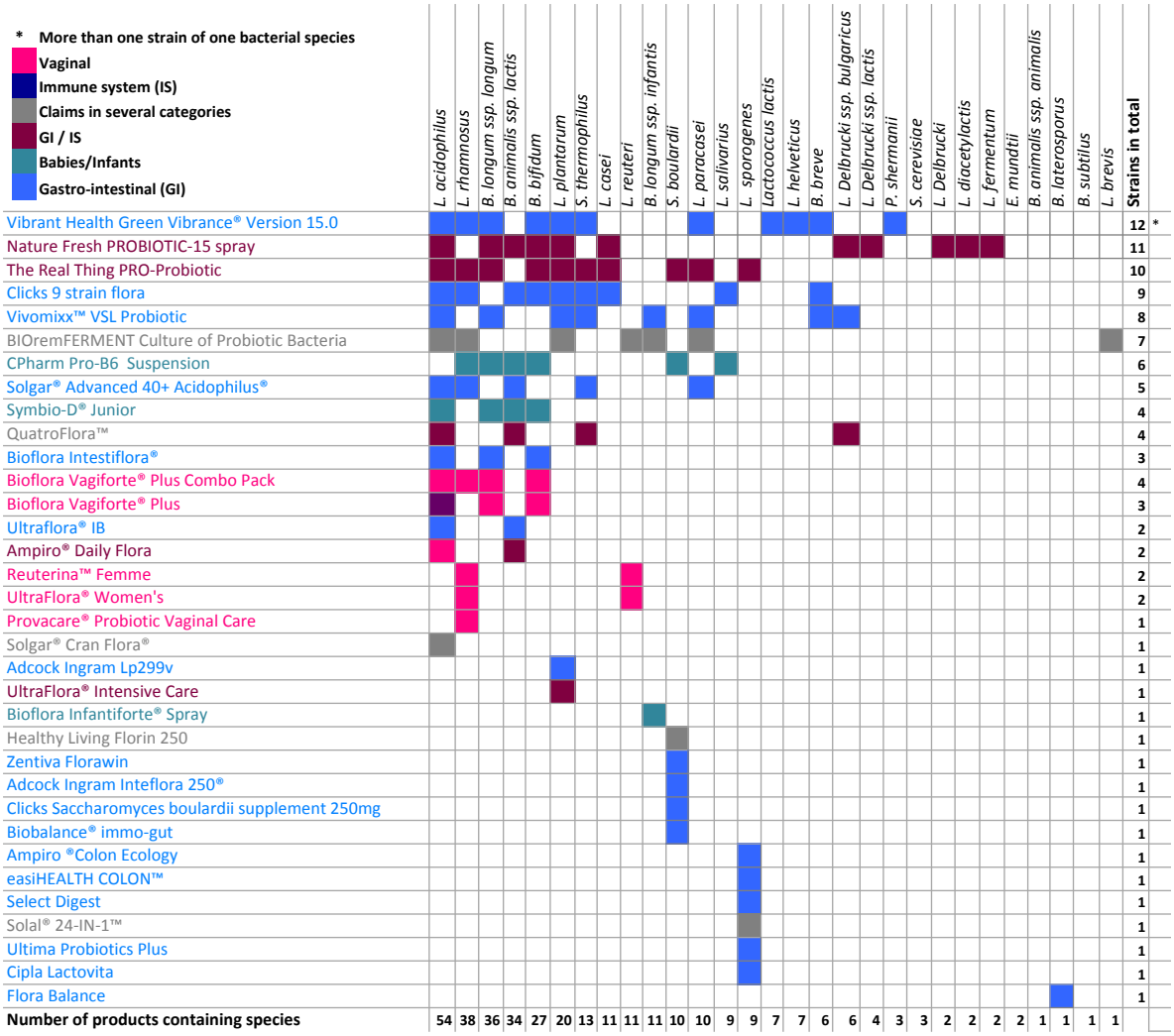


Figure 2.3. Health claims and association with bacterial composition. Each product was grouped into one health category (v = vaginal, IS = immune system, GI = gasto-intestinal) as described above and bacterial strains were listed. The complete Figure is shown in Appendix II.

2.3.4 Dose, formulation and storage

Overall, probiotic products claimed to contain a median of $1.5x10^9$ viable organisms (colony-forming units, CFU) ranging from $4x10^7$ to $9x10^{11}$ CFU per dose, with daily dosage recommended. The daily dose (capsules per day) of products depended on therapeutic indication: prophylaxis (chronic), treatment (acute) or as an adjunct to antibiotic treatment. However, organisms included in these different probiotic preparations did not differ according to indication. Some products formulated with the same bacterial species and CFU were marketed by different manufacturers under both, similar and different health claims (Appendix II). Other products contained the same combination of bacterial species, but different doses of each species in different products (e.g. two species combination *L. acidophilus* and *B. animalis ssp. lactis*:

range $5x10^8$ to $15x10^9$ CFU or single species *L. sporogenes*: range $4x10^7$ to $6x10^9$ CFU). In some instances, the same manufacturer produced a range of probiotic products with identical bacterial species content and dose, while marketing them as distinct products with different health claims (Appendix II).

The most common formulation was capsules (59.8%), with some products available as liquids (18.7%), tablets (13.1%) or powder (8.4%). The majority of probiotics (82.7%, 86/104) were stored on the shelf (room temperature), while 17.3% (18/104) were stored in the refrigerator (4^{o}C). Probiotics maintained at 4 ^{o}C tended to be from the same manufacturer (10/18 produced by Metagenetics® and 5/18 produced by Bioflora), but did not appear to be systematically different in formulation, probiotic composition, or expiry date. Of the four products marketed for vaginal health, only UltraFlora® Women's (manufactured by Metagenetics®) required to be stored in the refrigerator. The average shelf life time was 14 months (from time of survey to recorded product expiry date) for both, products stored at room temperature and those stored at 4^{o}C. There was no correlation between shelf life and formulation of the product. Probiotics were predominantly recommended for oral administration (97.1 %, 101/104). Of the four vaginal products available, two were intended for oral administration, one was for both oral and a vaginal administration and one for vaginal dosing only.

Two-thirds (64.4%, 67/104) of the surveyed products were manufactured in South Africa [88.1 % (59/67) of shelf products versus 11.9 % (8/67) refrigerated products]. The total of 104 products was produced by 39 manufacturers, of which 27 (69.2%) were based in South Africa, mainly in Johannesburg (12/27), Pretoria (6/27) and Cape Town (6/27). Of the four vaginal products, two were manufactured in South Africa (Reuterina™ Femme, Bioflora Vagiforte® PLUS), one in Canada (Provacare® Probiotic Vaginal Care) and one in the US (UltraFlora® Women's).

Aside from bacteria, most products contained additional ingredients, such as vitamins, minerals, enzymes or fruit extracts. Common enzymes included were digestive enzymes, such as amylase, protease, invertase, malt diastase, lipase, cellulose or lactase. A few products (8/104, 7.7%) contained folic acid (vitamin B9), which is important for nucleotide synthesis in humans and bacteria but cannot be synthesized

by humans (reviewed by Greenberg *et al*. 2011). Some bacteria synthesize folate, including many *Bifidobacterium* spp. but only few *Lactobacillus* spp. and thus, it needs to be ingested (Rossi *et al*. 2011). A similar percentage of products contained thiamine (vitamin B1), a coenzyme in the metabolism of sugars and amino acids, and riboflavin (vitamin B2) or nicotinamide (vitamin B3) that are both needed for oxidation-reduction reactions (Tataru & Boca 2015). Those products also included pantothenic acid (vitamin B5), which is a cofactor for the synthesis and metabolism of proteins, carbohydrates and fats, vitamin B6, a coenzyme for amino acid, glucose and lipid metabolism, and biotin (vitamin B7) that is a coenzyme for carboxylase enzymes needed for the synthesis of some amino acids, fatty acids and gluconeogenesis (reviewed by Tataru & Boca 2015).

The majority of products (67.3%) did not contain prebiotics. In those that did, the most common were fructo-oligosaccharides and inulin, which are both indigestible carbohydrates that reach the intestine intact where they encounter *Lactobacillus* and *Bifidobacterium* spp., which are able to metabolize and transport these compounds (Goh & Klaenhammer 2015).

2.3.5 Cost of probiotics

The median cost per probiotic capsule or tablet in this study was USD 0.42 [currently equivalent to 6.30 South African Rand (ZAR)], ranging from USD 0.12 - 3.04 (ZAR 1.80 - 46.00). Probiotic prices did not differ between Cape Town and Durban (mean of ZAR 6.50 versus ZAR 6.00), nor when comparing probiotics manufactured locally to those that were imported, or probiotics stored on the shelf to those requiring refrigeration (data not shown). Instead, prices of products differed according to manufacturer, with products from Metagenetics®, Bioflora, Viridian and Vibrant Health being more expensive (mean ZAR 22.40, USD 1.48 per capsule/tablet) when compared to those from other manufacturers evaluated in this study (mean: ZAR 4.60, USD 0.30 per capsule/tablet; $p<0.0001$). Given that >60% of households of Cape Town and Durban metropoles earned <USD 422.00 (ZAR 6367) per month in 2011[13], the cost of one treatment course per individual of any of the vaginal probiotics publically available in South Africa would use up 1.6 - 4.9% of monthly income.

[13] Census 2011, http://www.statssa.gov.za [02 January 2018]

2.4. Discussion

The aim of this Chapter was to determine the availability of vaginal probiotics, which may improve BV treatment in adjunct with antibiotics, in South Africa, a region with high BV and HIV burden in young women. Overall, 104 OTC products were identified in Cape Town and Durban, with more products marketed in Cape Town than in Durban. Although there were fewer stores in Durban selling probiotics, these two major cities do not differ in population size, gross domestic product (GDP), average income, unemployment rates or medical aid coverage[13], proxies for social-economic status and general health in both cities. Factors contributing to this discrepancy in probiotic availability are likely to be complex, but may include cultural and ethnic differences in the populations, and differences in traditional, complementary and alternative medicine use (Peltzer 2009).

The predominant indication for probiotics in South Africa is for GIT health with only four products being marketed for vaginal health, illustrating a huge discrepancy in product availability given BV rates of up to 58% in South Africa (Kenyon *et al*. 2013). Most of the bacterial strains contained in the vaginal probiotics identified in this study were not common colonizers of the lower reproductive tract. For instance, *B. longum ssp. longum* and *B. bifidum* are commensal in the GIT (Turroni *et al*. 2014), while *L. acidophilus, L. reuteri and L. rhamnosus* primarily colonize the GIT (Walter 2008), but also have been isolated from the FGT (Cribby *et al*. 2008).

While the efficacy of probiotics on BV cure and recurrence still needs to be confirmed in clinical trials, the development of vaginal probiotics should, as a reasonable starting point, contain species that are frequently commensals of the healthy vaginal tract, such as *L. crispatus, L. gasseri, L. jensenii, L. vaginalis* and *L. mucosae*. Further, the efficacy of probiotic formulations containing various combinations of vaginal commensal bacterial species and strains, along with adjunctive antibiotic use, to treat BV and prevent recurrence needs to be evaluated.

The most common bacterial species found overall in probiotics on the South African market were *L. acidophilus*, *L. rhamnosus*, *B. longum ssp. longum*, *B. animalis ssp.*

[13] Census 2011, http://www.statssa.gov.za [02 January 2018]

lactis, *B. bifidum* and *L. plantarum*. Some reasons cited for the use of *L. acidophilus* strains in probiotic formulations include that it is stable in products, resistant to GIT bile, tolerant to low pH, and adherent to human colonocytes in cell culture (Azcarate-peril *et al*. 2004; Buck *et al*. 2005; Pfeiler & Klaenhammer 2009; Khaleghi *et al*. 2010). In addition, *L. acidophilus* produces antimicrobial substances and contains lactase activity, meeting the criteria needed for an effective probiotic (Bull *et al*. 2013). Similar characteristics have been described for all the above-mentioned *Lactobacillus* and *Bifidobacterium* spp. commonly found in probiotics (Jungersen *et al*. 2014; Millette *et al*. 2008; Toscano *et al*. 2015). Additionally, these probiotic species do not compete with each other for essential nutrients and therefore, can be combined in products (Millette *et al*. 2008). *Bifidobacterium* spp. are able to produce acetic acid that reduces yeast growth (Picard *et al*. 2005), which may be a reason why it is contained in some vaginal probiotics, although these species are much less common in the FGT than *Lactobacillus* spp. (Redondo-Lopez *et al*. 1990; Lennard *et al*. 2017).

In addition to *Lactobacillus* and *Bifidobacterium* spp., other species were less commonly found in probiotics in South Africa, including *S. boulardii, S. cerevisiae* and *E. mundtii*. The non-pathogenic yeast strains *S. boulardii* and *S. cerevisiae* may regulate intestinal microbial homeostasis (Buts 2009), interfere with the ability of pathogens to colonize and infect the mucosa (Chen *et al*. 2006; Mumy *et al*. 2008; Wu *et al*. 2008), modulate immune responses (Machado Caetano *et al*. 1986; Dalmasso *et al*. 2006; Zanello *et al*. 2009), stabilize the GI barrier function and induce absorption of nutrients (Kelesidis & Pothoulakis 2011). The inclusion of *E. mundtii* in probiotics is controversial, as it is thought to be marginally virulent, with reports of endophthalmitis (inflammation of the intraocular cavities) published (Higashide *et al*. 2005; Repizo *et al*. 2014), and it is not considered safe by the FDA (Repizo *et al*. 2014).

In the US, the FDA applies a complex framework of regulation to validate manufacturers' claims for the products they market (Turner *et al*. 2005). In this framework, a health claim is "any claim made on the label or in labelling of a food, including a dietary supplement, that expressly or by implication, including 'third party' references, written statements, symbols, or vignettes, characterises the

relationship of any substance to a disease or health related condition" (U.S. Department of Health and Human Services 2006). In the case of vaginal probiotics, this could possibly be a reduction in the risk of incurring BV in a healthy population. While regulatory bodies (including the FDA and the SAHPRA) are more concerned with product safety than misleading claims, sound scientific approaches need to be used to demonstrate the health benefits of probiotics, such as dose-response relationships (reviewed by Heimbach 2008). Previous studies have shown poor correlation between label claims and actual probiotics content in South Africa and world-wide (Elliott & Teversham 2004; Weese 2003), so post-market surveillance should be mandatory to demonstrate health benefits related to probiotics.

This survey had some limitations. The exact shelf lifetime could not be determined because only the expiry date and date of survey, but not the date of production were captured. Only Cape Town and Durban but no other cities in the country, and only one centre per district within these cities were surveyed.

Conclusion

This evaluation of the South African probiotics market showed that currently only four products for vaginal health are retailed, and these products contain GI bacterial species while FGT commensals were absent, and thus the development of probiotics for vaginal health containing bacterial species that are commensals in the FGT is urgent. Further, none of these probiotics were manufacturer under GMP conditions, therefore batch testing of these off-the-shelf products would be necessary to evaluate quality.

Chapter 3

The potential of South African probiotics to improve vaginal health

3. Evaluating commercial probiotic formulations for vaginal health *in vitro*

3.1 Introduction

Of the 104 OTC probiotics that are currently commercially available in South Africa, only four were explicitly marketed for vaginal health (Provacare™ Probiotic Vaginal Care, Vagiforte® PLUS and Vagiforte® PLUS Combo Pack and Reuterina™ Femme®; Chapter 2). While species data was available for all products, some strain data was only available for Provacare™ and Vagiforte® PLUS Combo Pack. Provacare™ contained *L. casei rhamnosus* Lcr35, which was isolated from the GIT of a child (Forestier *et al*. 2001). Vagiforte® PLUS Combo Pack was advertised to contain *L. rhamnosus* GG, which was isolated from the GIT of a healthy human in the 1980's (Gorbach and Goldin in 1983; Gorbach & Goldin 1989), while Vagiforte® PLUS did not contain *L. rhamnosus*. Both Vagiforte® products contained *L. acidophilus*, *B. bifidum* and *B. longum*, and Reuterina™ Femme® contained *L. reuteri* and *L. rhamnosus* but no strain information was available. A contentious South African survey conducted by Elliott and Teversham (2004) suggested that the viability, dose and microbial contents of locally produced South African products need to be carefully evaluated, as they identified some discrepancies between advertised content and cultivable bacteria in laboratory studies, although they failed to declare a conflict of interest in this study. Thus, two European products, Gynophilus® from France (contained *L. casei rhamnosus* Lcr35) and Muvagyn® from Spain (contained *L. gasseri* and *L. rhamnosus*) were included as reference products in this Chapter.

Various characteristics of *Lactobacillus* spp. are considered important for FGT health; including to lower vaginal pH (Barrons & Tassone 2008; Boris & Barbés 2000), produce microbicidal compounds, such as L- and D-lactate (Aldunate *et al*. 2013; O'Hanlon *et al*. 2011; Hutt *et al*. 2016) and H_2O_2 (Hawes *et al*. 1996; Hillier *et al*. 1993; Cherpes *et al*. 2008), biosurfactants (Heinemann *et al*. 2000; Gudina *et al*. 2010; Reid 2000) and bacteriocins (Bastos *et al*. 2015; Holo *et al*. 1991), and to competitively exclude pathogenic bacterial species through competition for adhesion and biofilm formation.

In South Africa, probiotics are regulated as "health supplements" by the SAHPRA

(Medicines Control Council 2016), which suggests that they require less strict manufacturing and monitoring criteria than medicines. Therefore, this Chapter aimed (1) to evaluate if the currently in South Africa available probiotics for vaginal health contained the bacterial species and dosing that the manufacturer claimed on the packaging insert; (2) to assess batch-to-batch variation of the same probiotic product available commercially; and (3) to investigate whether *Lactobacillus* isolates from different probiotic lots have similar phenotypic probiotic characteristics. To evaluate *Lactobacillus* isolates purified from the various probiotic products for characteristics considered important for their potential to improve vaginal health, their growth kinetics under various environmental pHs, ability to lower culture pH, susceptibility to common antibiotics, ability to adhere to cervical cells and to produce lactic acid and H_2O_2 under anaerobic conditions, as well as their ability to inhibit growth of BV-associated *G. vaginalis* and *P. bivia* and Group B *Streptococcus* (GBS; associated with bacterial infections in newborns) strains were evaluated. This information on the quality and probiotic performance of locally sourced probiotic products was critical to inform the choice of the most suitable of the South African probiotics to use in a small pilot clinical trial assessing the effect of adjunctive probiotics on BV cure rates in South African women (Chapter 4).

3.2 Methods

3.2.1 Selection of probiotics for vaginal health

The four commercial OTC probiotics marketed in South Africa for vaginal health that were evaluated in this Chapter included: (1) Reuterina™ Femme capsules, intended to be given orally (containing *L. reuteri* and *L. rhamnosus*; manufactured by Ikacia, Isando, South Africa; now Ascendis Pharma, South Africa; cost one treatment course = ZAR195 [currently equivalent to 13.25 USD], storage at room temperature); (2) Provacare® Probiotic Vaginal Care capsules, intended for vaginal use (*L. casei rhamnosus* Lcr35, manufactured by Provacare®, Canada; ZAR250 [USD17.12], storage at room temperature); (3) Vagiforte® Plus oral capsules and vaginal tablets (*L. acidophilus*, *B. bifidum* and *B. longum*, manufactured by Bioflora CC, Pretoria, South Africa, ZAR97 [USD 6.64], storage at room temperature); and (4) Vagiforte® Plus Combo Pack oral capsules and vaginal spray (*L. rhamnosus* GG, *L. acidophilus*, *B. bifidum* and *B. longum*, manufactured by Bioflora CC, Pretoria, South Africa; ZAR195 [USD13.35], storage at room temperature). UltraFlora® Women's, which is also available in South Africa (Chapter 2) was not evaluated in this study due to its storage requirements (refrigerator) and high cost (one treatment course=ZAR615 [currently equivalent to 42.12 USD]), which would make the use of this product in a trial in a South African setting unfeasible. Between March and June 2016, three different lot numbers of Reuterina™ Femme (Lot numbers Z1006 [expiry date January 2017], Z1058 [expiry date August 2017], and Z0159 [expiry date October 2017]) and Vagiforte® PLUS products (Lot numbers 20868 [expiry date September 2017], 20531 [expiry date April 2017], and 21134 [expiry date January 2018]) were purchased for this study. The distribution of Provacare™ Probiotic Vaginal Care was discontinued in South Africa in April 2016; therefore only one lot number of this product was available for this study (lot number 0154, expiry date May 2016). The two European probiotics included as reference products in this study were: Muvagyn® vaginal pessary (including *L. gasseri* and *L. rhamnosus*, lot number 7447212, expiry date November 2016, manufactured by Hälsa Pharma GmbH, Germany; distributed in Spain) and Gynophilus® vaginal pessary (including *L. casei rhamnosus* Lcr35, lot number 0200, expiry date October 2017, manufactured by Probinov (now called biose), distributed in France; kindly provided by Dr Remy Froissart, CNRS, Montpellier, France). Only a single lot number per European product was available.

All products were stored according to the manufacturer's instructions, and all data provided on the package leaflet (including concentration of live organisms per dose (colony-forming units, CFU), recommended route of administration, dosage instructions, treatment duration, and storage conditions) were captured (**Table 3.1**)

Table 3.1. Details of selected probiotic products for vaginal use

Product	Lot number	Application	Species contained	Concentration per dose (CFU or mg)	Dosage	Storage	Expiry date
Provacare™	0154	Vaginal capsules	*L. casei rhamnosus Lcr35*	3.4 x 10^8 CFU	1 capsule twice daily; 5 days	< 25ºC	05/16
Reuterina™ Femme®	Z1006	Oral capsules	*L. reuteri* *L. rhamnosus*	5 x 10^9 CFU	1 capsule 1-2 times daily; 5 days	< 25ºC	01/17
	Z1058						08/17
	Z1159						10/17
Vagiforte® PLUS	20868	Oral capsules	*B. bifidum* *B. longum* *L. acidophilus*	3 x 10^9 CFU	1 tablet and capsule once daily; 5 days	< 25ºC	09/17
		Vaginal tablets		1 x 10^9 CFU			
Vagiforte® PLUS Combo Pack	20531	Vaginal spray Oral capsules	*L. acidophilus* *L. rhamnosus GG* *B. bifidum* *B. longum*	Spray and capsule each 2 x 10^9 CFU	1 oral capsule once daily; 15 days 2 squirts vaginal spray daily;10 days [starting at day 6 of treatment course]	< 25ºC	04/17
	21134						01/18
Muvagyn®	7447212	Vaginal capsules	*L. gasseri* *L. rhamnosus*	1 x 10^8 CFU	1 capsule once daily; 8 days	< 25ºC	11/16
Gynophilus®	0200	Vaginal capsules	*L. casei rhamnosus Lcr35*	341 mg protein	1 capsule once daily; 14 days	< 25ºC	10/17

3.2.2 Determining bacterial concentration per dose

From each product, one full dose (including the total contents of a probiotic capsule or tablet) was dissolved in 40 mL of either Man-Rogosa-Sharpe broth (MRS [69966, Sigma-Aldrich®, USA], supplemented with 0.05g/L L-Cystein [30089, Sigma-Aldrich®, USA] and 0.1% Tween80 [P8074, Sigma-Aldrich®, USA]; used when products only contained *Lactobacillus* spp.) or supplemented Brain Heart Infusion broth (sBHI [53286, Sigma-Aldrich®, USA], supplemented with 0.1% starch [33615, Sigma-Aldrich®, USA] and 1% yeast [51475, Sigma-Aldrich®, USA], used when products contained both *Lactobacillus* and *Bifidobacterium* spp.). For the Vagiforte® PLUS probiotic spray (contained in a spray bottle), the contents of one squirt was transferred into a 1.5ml Eppendorf tube, and processed in the same way as the capsules and tablet. Dissolved probiotics were serially diluted (10^0-10^{-4}), and 10 µL of

each dilution was plated into triplicate wells onto 6-well agar plates (each well contained 3 mL of MRS or sBHI), using sterile glass beads. Beads were removed with sterile forceps and the plates were closed and incubated at 37ºC for 48 hours, under anaerobic conditions (using Oxoid™ AnaeroGen™ 2.5L Sachets, Thermo Fisher Scientific Inc., USA). Wells containing abiotic MRS or sBHI agar were included as negative controls. The CFU per well were counted and the average concentration per dose for each probiotic and lot number was calculated by averaging the mean of the triplicates of each dilution. Experiments were repeated a total of three times.

3.2.3 Isolation of pure bacterial strains

To isolate *Lactobacillus* strains from each probiotic product, a sterile loop was dipped into MRS broth and then into the probiotic powder contained in capsules, crushed tablets (which were crushed using a sterilised pill crusher) or spray before streaking onto corresponding MRS agar plates. Similarly, to isolate *Bifidobacterium* strains, a sterile loop was dipped into sBHI broth and then into the probiotic powder or spray before streaking onto sBHI plates. Once dry, the plates were inverted and incubated under anaerobic conditions using Oxoid™ AnaeroGen™ 2.5L Sachets (Thermo Fisher Scientific Inc., USA) at 37ºC for 48 hours. Single colonies with distinct morphologies were picked with a sterile loop, re-streaked onto new agar plates, and incubated under the same conditions until clearly homogenous colonies were obtained. From these plates, a single colony was collected and transferred into a 1.5ml tube (Eppendorf AG, Germany) containing 1.4 mL of MRS or sBHI broth, incubated at 37ºC for 48 hours, centrifuged at 589g (Microcentrifuge, Labnet Inc., USA), and the pellet resuspended with 300 µL of 60 % glycerol (20% v/v final) in MRS or sBHI before storing at –80 ºC as the primary stock. The bacterial species were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), using a MALDI Biotyper (Bruker Daltonik, USA) by Dr Rémy Froissart (at the University of Montpellier, France).

3.2.4 Gram staining

Gram staining was performed to determine Gram staining characteristics, as well as bacterial shape and size. Single colonies from each purified bacterial isolate were smeared onto microscopic slides (GLAS4S22M1000F, Lasec®, South Africa), heat-

fixed and then stained with crystal violet solution (77730, Sigma-Aldrich®, USA) for one minute, and rinsed with water. Subsequently, iodine solution (77730, Sigma-Aldrich®, USA) was added for one minute and rinsed off with water. Finally, the slide was flooded with decolorizer (77730, Sigma-Aldrich®, USA) for five seconds, rinsed with water, and safranin (77730, Sigma-Aldrich®, USA) was added for one minute, and then rinsed off with water. Slides were air dried, and coverslips (CLS284518, Sigma-Aldrich®, USA) attached using DPX Mountant (Merck Pty Ltd, South Africa). Slides were visualised using a Leica DM500 light microscope (Leica microsystems, Germany), under a 40x objective lens. Images of each slide were captured using Zeiss colour digital camera and ZEN software (Carl Zeiss AG, Germany). The size of at least 100 bacterial rods per probiotic isolate was measured using the ImageJ software (ImageJ, Version 2.0.0-rc-43/1.50e, USA).

3.2.5 Determining bacterial counts at a standardized optical density (OD)

To enable large-scale experiments to be performed in which multiple isolates could be compared simultaneously, absorbance (optical density $[OD]_{600nm}$) was used to monitor growth kinetics (Koch 1970; Sutton 2011) rather than determining CFUs, which is more labour intensive. Since bacterial size, even within the same genus, may vary by species (De Vos *et al*. 2011; Whitman *et al*. 2012), variation in estimated bacterial concentrations at an adjusted OD was verified by plating and quantifying *Lactobacillus* or *Bifidobacterium* isolates [adjusted to OD_{600nm} 0.1(±0.01)] on MRS or sBHI agar plates, respectively. $OD600_{nm}$ was determined using a VersaMAX microplate reader (Molecular Devices, USA). From the bacterial cultures of each isolate at OD_{600nm} 0.1, 10 µL of serial 1:100 dilutions (10^0, 10^{-2} and 10^{-4} dilutions) were plated using glass beads in triplicates on 6-well plates (each well contained 3 mL of MRS agar for *Lactobacillus* spp. and sBHI for *Bifidobacterium* spp.), and incubated anaerobically at 37ºC for 48 hours. Colonies were counted and the CFU per mL at OD_{600nm} 0.1 (±0.01) for each isolate was calculated. Experiments were repeated a total of three times.

3.2.6 Bacterial growth kinetics at different environmental pHs

To investigate the ability of the various probiotic strains to grow at different pHs (relating to their ability to survive at low pHs in the vagina), overnight cultures were

standardized to an OD_{600nm} of 0.1 (±0.01) in MRS broth (for *Lactobacillus* spp.) or sBHI broth (for *Bifidobacterium* spp.) that was adjusted to either pH 3.5, 4.0 and 4.5 or 6.0 using hydrochloric acid and incubated in triplicate wells under anaerobic condition in a sealed 96-well plate at 37ºC for 24 hours. At 0, 3, 6, 12, 18, and 24 hours, the absorbance (OD_{600nm}) was measured using a VersaMAX microplate reader (Molecular Devices, USA) using SOFTmax PRO software (Version 5). In addition to determining growth kinetics at OD_{600nm}, plating of serial dilutions (section 3.2.5) at each time point allowed assessment of the viability and the CFU/mL of the bacteria at varying time points and pHs. Experiments were repeated a total of three times.

3.2.7 Influence of bacterial growth on culture pH

Bacterial isolates were standardised to an absorbance of OD_{600nm} 0.1 (±0.01) in 15 mL MRS (for *Lactobacillus* spp.) or sBHI (*Bifidobacterium* spp.), normalized to pH 6.0 and 7.0, respectively, using a VersaMAX microplate reader (Molecular Devices, USA). Cultures were incubated anaerobically at 37ºC for 3, 6, 12, 24, and 48 hours. The pH of the culture supernatant at each time point was measured using a calibrated Jenway Bench pH Meter 2510 (Bibby Scientific, UK).

3.2.8 Antibiotic susceptibility testing

3.2.8.1 Determining antibiotic susceptibility using the disc diffusion assay

The double-layered disc diffusion method was used to determine the susceptibility of purified probiotic isolates to commonly used antibiotics, including: metronidazole (5 µg/disc), clindamycin (2 µg/disc), penicillin (2 µg/disc) and amoxicillin (10 µg/disc; Davies Diagnostics (Pty)Ltd, South Africa). Two hundred µL of each isolate, standardised to an absorbance of OD_{600nm} 0.1 (±0.01), were added to 3 mL liquid sloppy (0.6% agar) MRS (for *Lactobacillus* spp.) or sBHI (for *Bifidobacterium* spp.), and then poured onto MRS or sBHI plates (1.2% agar), respectively. All four antibiotics were tested on the same plate. Once dry, the antibiotic discs were placed into the centre of each quadrant of the plate; and the plates were incubated under anaerobic conditions at 37ºC for 24 hours, and the diameter of the zone of inhibition of each antibiotic disc was measured. These experiments were performed in duplicate for each purified isolate.

3.2.8.2 Determining antibiotic susceptibility using the minimal inhibitory concentration (MIC) assay

For rifampicin and isoniazid (R3501 and I3377, Sigma-Aldrich®, USA), commonly used in treatment of tuberculosis (TB) in South Africa, the MIC was determined using two-fold serial dilutions in 96-well plates. Overnight cultures of the purified bacterial isolates were standardised to $5x10^5$ CFU/mL, as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The first row of the 96-well plate was filled with a final concentration of 1000µg/mL rifampicin (100 mg/mL, R3501, Sigma-Aldrich®, USA) or 10 µg/mL Isoniazid (1 mg/mL, I3377, Sigma-Aldrich®, USA) in 100 µL MRS (for *Lactobacillus* spp.) or sBHI (for *Bifidobacterium* spp.). To the rest of the wells, 50 µL of MRS or sBHI alone was added. Serial 1:2 dilutions of the antibiotics were then done in the wells containing 50 µL MRS or sBHI, excluding the last row, which was used as an abiotic culture control. Fifty µL of adjusted bacterial isolates was added to each well in duplicate. The sealed plates were then incubated under anaerobic conditions in the dark (as these antibiotics are light-sensitive) at 37ºC for 48 hours. The lowest concentration of antibiotic preventing visible growth in wells was considered to be the MIC for a particular isolate, according to the guidelines provided by EUCAST. All experiments were performed in duplicates.

3.2.9 Adhesion to human ectocervical epithelial (Ca Ski) cells

Human ectocervical epithelial cells (Ca Ski, ATCC® CRL-1550™) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich®, USA), containing 10% fetal calf serum (FCS, F2442, Sigma-Aldrich®, USA) and 1% penicillin and streptomycin, glutamine and fungin (PSFL, ThermoFisher Scientific, USA). CaSki cells were sub-cultured at 80% confluence in T25 tissue culture flasks, using 0.25% Trypsin/EDTA (T0409, Sigma-Aldrich®, USA) to lift the cells. Cells were counted and viability assessed using 1:1 dilution with Trypan Blue (10 µL cells:10 µL Trypan; T8154, Sigma-Aldrich®, USA), using a haemocytometer ($TC20^{TM}$, Bio-Rad, USA).

To measure the adhesion of the probiotic *Lactobacillus* isolates to human cervical cells, Ca Ski cells were seeded into 24-well tissue culture plates ($1x10^5$ cells/well) in antibiotic-free DMEM supplemented with 10% FCS and grown for 24 hours at 37°C and 5%CO_2 to 80-90% confluence. Bacterial overnight cultures were adjusted to

$4.2x10^6$ CFU per 500 µL (Fichorova *et al*. 2011) in antibiotic-free DMEM supplemented with 10% FCS, added in triplicates to the cell monolayer and incubated for three hours at 37 °C and 5% CO_2. The culture medium was removed and the plate washed twice with 1 mL PBS using a P1000 micropipette to remove non-adherent bacteria. To lyse the CaSki cell monolayer, 250µL of 0.1% Triton™ X-100 (T8787, Sigma-Aldrich®, USA) was added to each well. The cell lysate was then serial diluted in MRS to achieve dilutions of 10^0, 10^{-2} and 10^{-4}. Ten µL of each dilution was plated on MRS agar plates in triplicates, and then incubated anaerobically at 37 °C for 48 hours. Colonies were counted, and the percentage of bacteria that adhered to the CaSki cell monolayer was calculated. Experiments were repeated a total of three times.

3.2.10 Measurement of bacterial metabolites and ability to inhibit BV-associated organisms and GBS

To obtain abiotic supernatants, purified probiotic isolates were standardised to OD_{600nm} 0.1 (±0.01) in 15 mL MRS and incubated for 24 hours anaerobically at 37ºC as previously described. Cultures were centrifuged at 200g (Eppendorf centrifuge 5810, Eppendorf AG, Germany) to pellet the bacterial cells, and culture supernatants were filtered first through a 0.4 µM and then a 0.2 µM syringe filter to remove any residual bacteria (Millex®, Sigma-Aldrich®, USA). This supernatant (*Lactobacillus*-conditioned media) was stored at -80°C for measurement of lactate (Section 3.2.10.1), hydrogen peroxide (H_2O_2) (Section 3.2.10.2), or stored at 4ºC until measuring the ability to inhibit the growth of *G. vaginalis*, *P. bivia* and growth the next day (Section 3.2.10.3).

3.2.10.1 Measurement of lactate

The concentrations of D- and L-isomers of lactate were measured using D- and L-lactate colorimetric assays (MAK058 and MAK064, Sigma-Aldrich®, USA), according to the manufacturers recommendations. To prepare the standards for the lactate colorimetric assay, 10 µL of 100 mM D- or L-Lactate standard solution (provided with the kit) was diluted with 990 µL of assay buffer (provided with the kit) to prepare a 1 mM standard solution. Then, 0, 2, 4, 6, 8, 10 µL of the 1 mM standard solution was added into a 96-well plate. Assay buffer was added to bring the volume in each well to 50 µL. For abiotic culture supernatant samples from purified probiotic

isolates, 25 µL was mixed with 25 µL of assay buffer (final volume 50 µL) and added to the 96-well plate in duplicate. A master mix was prepared, which included 46 µL assay buffer, 2 µL enzyme mix and 2 µL substrate per sample; or 48 µL assay buffer and 2 µL substrate for the blank [plain MRS], and then 50 µL was added to each of the wells. The samples were mixed using a horizontal shaker (Orbit^{TM} Digital Microtube and Microplate Shaker, Labnet Inc., USA), and the plate was incubated for 30 min at room temperature. The plate was protected from light during the incubation, and the absorbance was measured at 450 nm using a VersaMAX microplate reader (Molecular Devices, USA) and SOFTmax PRO software (Version 5). The lower limit of detection was defined as half of the lowest concentration measured: 2.2 ng/µL for L-lactate, and 0.6 ng/µL for D-lactate. The upper limit of detection was 34.6 ng/µL for L- and 25.7 ng/µL for D-lactate, and levels above that were extrapolated. All experiments were performed in duplicate.

3.2.10.2 Measurement of H_2O_2 concentrations

H_2O_2 concentrations were measured in anaerobically grown abiotic culture supernatants using a H_2O_2 fluorimetric kit (MAK165, Sigma-Aldrich®, USA), according to the manufacturer's instructions. In brief, 22.7 µL of a 3% H_2O_2 solution (provided with the kit) was added to 977 µL of assay buffer (provided with the kit) to prepare a 20 mM H_2O_2 stock solution. Of this 20 mM stock solution, 1 µL was added to 1999 µL of assay buffer to make a 10 µM working solution. This was then serially diluted to prepare 10, 5, 2.5, 1.25, 0.625, 0.33125, 0.156, and 0.078 µM standards in a total volume of 50 µL. Equal volumes of the abiotic culture supernatant (25 µL) and assay buffer (25 µL) was added to the 96-well plate in duplicate. For the blank, plain MRS (25 µL) was added to assay buffer (25 µL). A master reaction mix was prepared, containing 50 µL red peroxidase substrate stock (provided with the kit), 200 µL peroxidase stock (20 units/mL, provided with the kit) and 4.75 mL assay buffer, and 50 µL was added to each of the wells. The sealed plates were mixed well using a horizontal shaker (Orbit^{TM} Digital Microtube and Microplate Shaker, Labnet Inc., USA), and incubated for 30 minutes at room temperature. The plate was protected from light during the incubation, and the fluorescence intensity at λ_{ex}=540/λ_{em}=590 nm was measured using a GloMax®-Multi fluorescence plate reader (Promega, USA). The lower limit of detection was defined as half of the lowest concentration

measured (1.9 µM), while the upper limit of detection was 11.6 µM, and levels above that were extrapolated. All experiments were performed in duplicate.

3.2.10.3 Ability to inhibit G. vaginalis, P. bivia and GBS

The ability of abiotic *Lactobacillus* culture supernatants to inhibit a panel of clinical and ATCC *G. vaginalis* and *P. bivia*, and clinical GBS strains was determined. Five clinical *G. vaginalis* (all vaginal) and *P. bivia* (four vaginal and one semen-derived) strains were kindly provided by Dr Rémy Froissart (CNRS, France), while six clinical GBS (all vaginal) strains were kindly provided by Dr Shameem Jaumdally (UCT). In addition, ATCC strains of *G. vaginalis* (ATCC® 14018™) and *P. bivia* (ATCC® 29303™) were included in each experiment. Clinical and ATCC *G. vaginalis*, *P. bivia* and GBS strains were stored in 20% glycerol stocks at -80°C. To culture *G. vaginalis* and *P. bivia*, a sample of the frozen stocks was streaked onto BHI agar plates supplemented with 5% horse blood using inoculation loops, and incubated anaerobically (using Oxoid™ AnaeroGen™ 2.5L Sachets, Thermo Fisher Scientific Inc., USA) at 37ºC for 48 hours. Colonies were picked and inoculated into BHI broth supplemented with 5% horse blood, and incubated anaerobically at 37ºC for 48 hours. GBS strains were cultured aerobically in Tryptic Soy (TS) medium, using the same approach as described above. *G. vaginalis*, *P. bivia* and GBS isolates were standardised to an absorbance of OD_{600nm} 0.4 (±0.01) (as recommended by Dr Rémy Froissart, CNRS, France) in BHI supplemented with 5% horse blood or TS, respectively, and 100 µL was added in triplicates to a 96-well plate. Then, 100 µL of the *Lactobacillus*-conditioned MRS were added to each well, and the plate was incubated at 37ºC (under anaerobic conditions for *G. vaginalis* and *P. bivia* strains; aerobically for GBS strains) for 48 hours. The absorbance (OD_{600nm}) was measured using a VersaMAX microplate reader (Molecular Devices, USA) with the SOFTmax PRO software (Version 5) at 0, 3, 6, 12, 24, 32 and 48 hours. As controls, the growth of *G. vaginalis*, *P. bivia* and GBS in the presence of plain MRS, added at the same ratio as abiotic culture supernatants, was measured. The area under the curve (AUC) for each growth curve was used to compare the relative growth of *G. vaginalis, P. bivia* and GBS isolates in the presence of plain MRS (control) and *Lactobacillus*-conditioned MRS, as a measure of inhibition. Experiments were repeated a total of three times.

3.2.11 Statistical analyses

GraphPad Prism6® (GraphPad Software, USA) was used for statistical analysis and to generate graphs. Kruskal-Wallis tests and post-hoc Dunn's multiple comparison test corrections were used to determine differences between bacterial strains or between lot numbers of the same product. The non-parametric Mann-Whitney U test was used to compare bacterial loads for orally and vaginally administered probiotics. Wilcoxon matched-pairs signed rank test was used to determine differences between two groups with matched data. Adjusted p-values are reported. P-values<0.05 and 95% confidence intervals were used to assess statistical significance.

3.3 Results

3.3.1 Evaluating the bacterial contents

The majority of probiotic products tested contained live *Lactobacillus* spp. as stated on the product packaging, with 17/20 listed *Lactobacillus* isolates being purified from Provacare™ (1/1, n=1 *L. casei rhamnosus*), Reuterina™ Femme® lot numbers Z1006 (2/2, n=1 *L. reuteri*, n=1 *L. rhamnosus*), Z1058 (2/2, n=1 *L. reuteri*, n=1 *L. rhamnosus*) and Z1059 (2/2, n=1 *L. reuteri*, n=1 *L. rhamnosus*), Vagiforte® PLUS lot 20868 (2/2, n=2 *L. acidophilus*), Vagiforte® PLUS Combo Pack lot 20531 (2/4, n=1 *L. rhamnosus GG*, n=1 *L. acidophilus*; isolated from vaginal spray only) and 21134 (4/4, n=2 *L. rhamnosus GG*, n=2 *L. acidophilus*; isolated from oral capsules and vaginal spray), Gynophilus® (1/1, n=1 *L. casei rhamnosus*), and Muvagyn® (1/2, n=1 *L. rhamnosus*, n=0 *L. gasseri*). No bacteria could be cultured from Vagiforte® PLUS Combo oral capsules from lot #20531, although the other two Vagiforte® lots tested (Lot #20868 and #21134) contained the *Lactobacillus* species as claimed by the manufacturer. For the European products, Muvagyn® probiótico vaginal claimed to contain *L. rhamnosus* and *L. gasseri*, although the latter could not be purified during this study despite numerous attempts within the product's expiry date. Only Vagiforte® PLUS products contained *Bifidobacterium* spp., and two lots of this product (Lot #20868 and #21134) contained culturable *B. bifidum* (n=1, isolated from #21134 vaginal spray) and *B. longum* (n=2, isolated from #20868 oral capsules and vaginal spray). The detailed product information is described in detail in **Table 3.1**.

3.3.2 Estimating probiotic concentration per dose

The concentration of each probiotic dose unit was compared to the concentrations listed on the package insert for each product (**Figure 3.1** and **Table 3.1 Table 3.1**). The advertised probiotic concentration for each of the products varied considerably, with the lowest concentration listed being $1x10^8$ CFU per dose for Muvagyn® Probiótico vaginal capsules, and the highest listed concentration being $5x10^9$ CFU per dose unit for Reuterina™ Femme® oral capsules (**Table 3.1**). The recommended dose of probiotics intended for vaginal application (ranging from $1x10^8$ CFU/dose for Muvagyn® to $2x10^9$ CFU/dose for Vagiforte® PLUS Combo Pack) tended to be slightly lower than those intended for oral application (ranging from $2x10^9$ CFU/dose for Vagiforte® PLUS Combo Pack to $5x10^9$ CFU/dose for Reuterina™ Femme®).

3.3.2.1 Reuterina™ Femme® (manufactured and distributed in South Africa)
Both *L. rhamnosus* and *L. reuteri* were isolated from Reuterina™ Femme®, which were contained in white capsules that the manufacturer claimed contained $5x10^9$ CFUs per capsule. This study found that Reuterina™ Femme® capsules contained almost 2-times that dose, with bacterial concentrations ranging from 9.0-9.4 $x10^9$ CFU per capsule across three lots (**Figure 3.1**A).

3.3.2.2 Provacare™ (manufactured in Canada, distributed in South Africa)
L. casei rhamnosus was isolated from the vaginal capsules that the manufacturer claimed contained $3.4x10^8$ CFU per capsule, which was in agreement with the median dose that was determined for this product in this study (range 2.4 $x10^8$ – $4.2x10^8$ CFU/dose) (**Figure 3.1**B). Since the product was discontinued during the study, a comparison between multiple lot numbers could not be performed.

3.3.2.3 Vagiforte® PLUS and PLUS Combo pack (manufactured and distributed in South Africa)
The two Vagiforte® products (Vagiforte® PLUS and PLUS Combo Pack) were formulated to be delivered both orally and vaginally. Initially, the vaginal dosing of Vagiforte® PLUS was delivered as a vaginal tablet, which was subsequently changed to a vaginal probiotic spray in Vagiforte® PLUS Combo Pack.

From Vagiforte® PLUS and PLUS Combo Pack oral capsules, *L. acidophilus*, *L. rhamnosus* (contained in Vagiforte® PLUS Combo Pack only) and *B. bifidum* were isolated, supporting claims by the manufacturer. However, *B. longum* could not be isolated from oral capsules, although were reportedly present (**Table 3.1**). The Vagiforte® PLUS oral capsules contained a median of $2.7x10^8$ CFU (range 2.3–$3.2x10^8$ CFU/dose), which was ~10-fold lower than the package claim of $3x10^9$ CFU per dose (**Figure 3.1**C). While the manufacturer insert stated that Vagiforte® PLUS Combo Pack oral capsules contained $2x10^9$ CFU per dose, a median concentration of $4.2x10^7$ (range 0.3 - 4.1 $x10^{10}$) CFU per dose was determined, ~40-fold lower than reported by the manufacturer (**Figure 3.1**D). Furthermore, several oral capsules from a second lot number (Lot #20531; **Table 3.1**) of the Vagiforte® PLUS Combo Pack failed to yield any bacterial growth at all, suggesting some heterogeneity in concentration in the oral formulation of this product.

From the Vagiforte® PLUS and PLUS Combo vaginal formulations, *L. acidophilus,*

L. rhamnosus (contained in Vagiforte® PLUS Combo Pack only), *B. longum* and *B. bifidum* were isolated, although not across all lot numbers (section 3.3.1). Each Vagiforte® PLUS vaginal tablet contained a median of $2.7x10^7$ CFU/dose (range $0.35–7.1\ x10^7$), which was ~35-fold lower than the stated dose of $1x10^9$ CFU (**Figure 3.1**C). In contrast, Vagiforte® PLUS Combo vaginal spray contained a median of $1.6x10^{10}$ CFU/metered dose (range $3.1\text{-}4.1\ x10^{10}$), which was 8-fold higher than stated by the manufacturer (**Figure 3.1**D).

3.3.2.4 Gynophilus® (manufactured and distributed in France)

Gynophilus® was confirmed to contain *L. casei rhamnosus* in white capsules, with a median concentration of $6.6x10^8$ CFU per capsule (range $4.8 – 7.7\ x10^8$ CFU/dose) (**Figure 3.1**E). Since the manufacturer reported capsule contents as protein weight (341mg lyophilized bacteria per dose) rather than CFU, it was not possible to compare doses.

3.3.2.5 Muvagyn® (manufactured in Germany, distributed in Spain)

Muvagyn® vaginal capsules were reported to contain $1x10^8$ CFU of *L. gasseri* and *L. rhamnosus*. When streaking the content of a whole Muvagyn® capsule onto MRS agar, only three colonies grew, despite several attempts, using several capsules and the product being within the expiry date. All three colonies were determined to be *L. rhamnosus*. *L. gasseri* could not be isolated from this product.

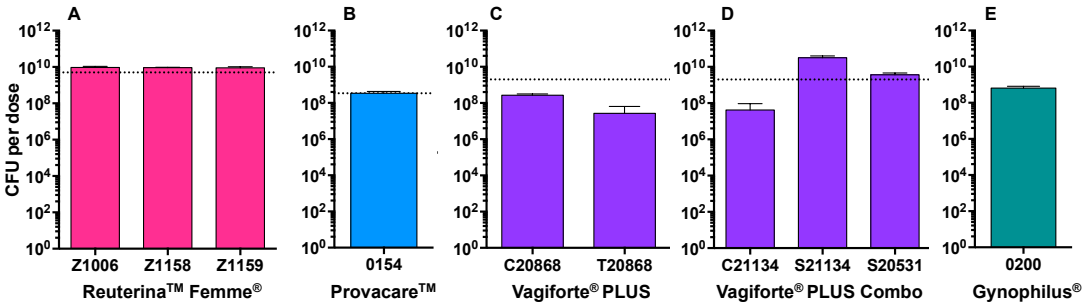


Figure 3.1. Bacterial concentration per probiotic dose unit from different lot numbers of products that were available in South Africa and Europe. The CFU per dose for Reuterina™ Femme® (A), Provacare™ (B), Vagiforte® PLUS (C; C = capsule, T = tablet) and Vagiforte® PLUS Combo Pack (D; S = spray) and Gynophilus® (E) was determined in the laboratory and compared to the manufacturer's claim (dotted line). The manufacturer of Gynophilus® did not state the CFU per dose but rather protein cintent (341 mg).

3.3.3 Phenotypic characterization of probiotic isolates

To evaluate whether bacterial isolates from different probiotic lot numbers had similar characteristics, their relative size (section 3.3.3.2), growth kinetics at various culture

pHs (to reflect different vaginal pHs, section 3.3.3.3), ability to lower pH *in vitro* (section 3.3.3.4), and susceptibility to antibiotics (section 3.3.3.5) was evaluated. For representative isolates of each species of each product, a wider characterisation of their probiotic characteristics was conducted, including adhesion to ectocervical epithelial cells (section 3.3.3.6), production of L- and D-lactate (section 3.3.3.7) and H_2O_2 (section 3.3.3.8), and inhibition of FGT pathogens (section 3.3.3.9).

3.3.3.1 Comparing relative bacterial concentrations at a standardized OD_{600nm}

Absorbance of bacterial cultures at OD_{600nm} was used to monitor growth kinetics; to enable more large scale simultaneous comparison of all of the 20 purified probiotic isolates (n=17 *Lactobacillus* and n=3 *Bifidobacterium* spp.). Because bacteria can vary in size both within and between species (Whitman *et al*. 2012), the extent of variance in concentrations (measured as CFUs/mL) of purified bacterial isolates at a standardized OD_{600nm} of 0.1 (±0.01) was evaluated (**Figure 3.2**). Of all *Lactobacillus* spp. included, *L. rhamnosus* demonstrated the highest concentration [median $3.3x10^7$ CFU/mL; interquartile range (IQR): 2.2–$6.0x10^7$; **Figure 3.2**A], while *L. acidophilus* had the lowest concentration (median $6.0x10^6$ CFU/mL; IQR 4.8–$9.0x10^6$) at OD_{600nm} of 0.1 (**Figure 3.2**C). In comparison, the concentration of the *Bifidobacteria* spp. tested was almost one Log lower than the *Lactobacillus* spp. at the lowest concentrations, with *B. bifidum* (n=2) and *B. longum* (n=1) reaching concentrations of $2.4x10^7$ and $2.4x10^5$ CFU/mL, respectively (**Figure 3.2**D).

Furthermore, some variation was noted in concentrations at a standardized OD_{600nm} within a single species isolated from different products. *L. rhamnosus* was isolated from all three South African products and both European products. The concentration of these *L. rhamnosus* isolates at OD_{600nm} of 0.1 had a coefficient of variation (CV) of 65.5%, with bacterial concentrations ranging from $2.0x10^7$ CFU/mL for *L. rhamnosus* from Reuterina™ Femme® (Lot #Z1159) to $1.1x10^8$ CFU/mL for *L. rhamnosus* from Vagiforte® PLUS Combo (Lot #C21134; **Figure 3.2**A). Furthermore, *L. rhamnosus* isolated from different lot numbers of the same product showed differing extents of variation. For example, the three *L. rhamnosus* isolates from Reuterina™ Femme® had a CV of only 25.2%, while the three isolates from Vagiforte® PLUS had a CV of 58.0%, indicating that the *L. rhamnosus* isolates from different batches of Reuterina™ Femme® might be less variable than those from different Vagiforte®

PLUS lots. *L. acidophilus* and *B. bifidum*, isolated from different lots of Vagiforte® PLUS and PLUS Combo, had CVs of 33.4% and 138.4%, respectively, suggesting that the *L. acidophilus* was less variable than *B. bifidum* isolates from Vagiforte® products. *L. casei rhamnosus* from Provacare™ (Lot #0154) and Gynophilus® (Lot #0200), which contain the same strain (Lcr35), had highly similar concentrations ($5.9x10^7$ and $5.6x10^7$ CFU/mL, respectively), indicating that variation seen in CFU/mL within species might be due to strain variations.

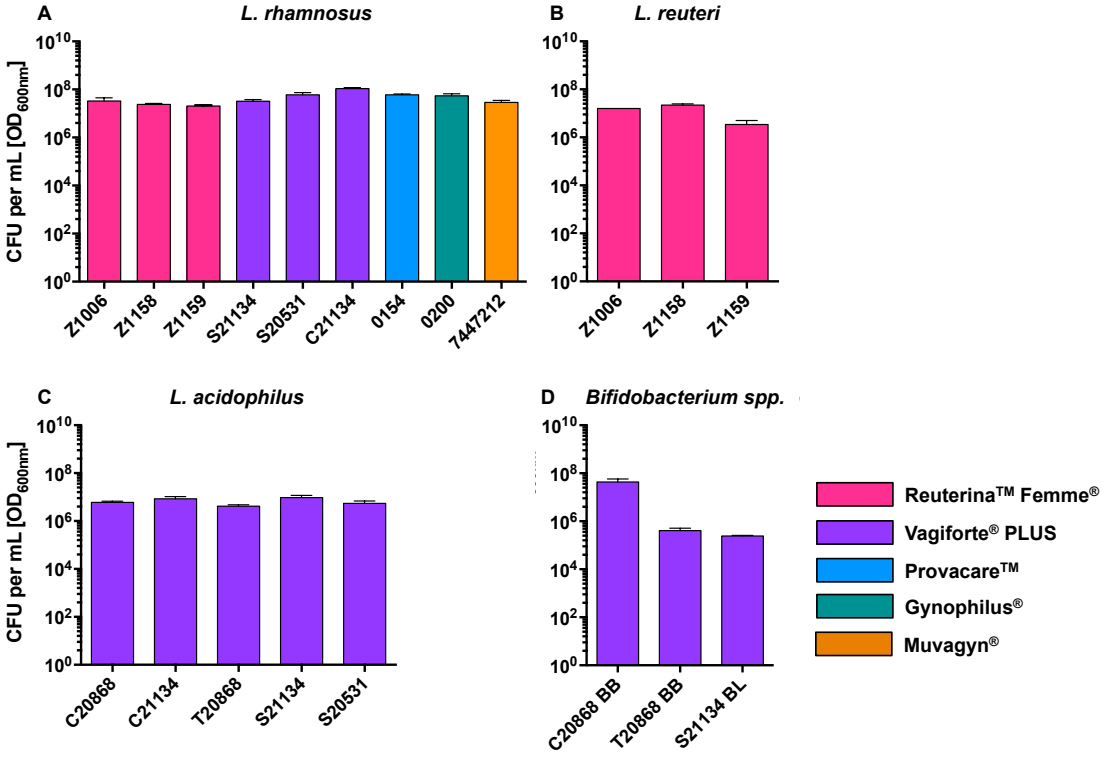


Figure 3.2. Variation in bacterial concentrations at OD_{600nm} 0.1 (±0.01) between probiotic products and species. The CFU per mL at OD_{600nm} 0.1 (±0.01) of *L. rhamnosus* (A), *L. reuteri* (B), *L. acidophilus* (C) and *Bifidobacterium* spp. (D) isolates was determined by plating serial dilutions. Bars are color-coded based on the product the species was isolated from (pink = Reuterina™ Femme®, purple = Vagiforte® PLUS, blue = Provacare™, green = Gynophilus®, orange = Muvagun®) and labelled with the lot number. In graph D, BB = *B. bifidum* and BL = *B. longum*.

3.3.3.2 Gram stain properties of probiotic isolates

As previously described (De Vos *et al*. 2011; Whitman *et al*. 2012), all *Lactobacillus* and *Bifidobacterium* spp. isolated from probiotic products were confirmed to be Gram-positive rod-shaped bacilli. Viewing the Gram-stained bacilli under oil-emersion microscopy, however, some variation in the clumping properties of the *Lactobacillus* spp. was noted. *L. reuteri* isolates appeared as single rods or double rods in a chain-like pattern, whereas *L. rhamnosus* and *L. acidophilus* strains were typically arranged in clusters and chains (**Figure 3.3**). The arrangement of these

organisms tended to be consistent for different strains of the same *Lactobacillus* spp. (exemplary isolates shown in **Figure 3.3**).

Length of the purified *Lactobacillus* bacilli varied by species, with *L. reuteri* tending to be the longest (mean 2.03 µm, range 1.08–3.05 µm), while *L. rhamnosus* tended to be the shortest (mean 1.23 µm, range: 0.80–1.83 µm). However, no significant difference in size within *L. rhamnosus* isolates from differing products or differing lot numbers of the same product was noted, with e.g. *L. rhamnosus* isolates from Gynophilus® having mean rod sizes of 1.22 µm for and *L. rhamnosus* isolates from Reuterina™ Femme® (Lot #Z1006) having similar mean rod size of 1.31 µm.

Bifidobacterium spp. were generally longer than the *Lactobacillus* isolates, with *B. bifidum* tending to be longer than *B. longum* [mean 3.72 µm (± 1.32) vs. 2.62 µm (± 0.63); p=0.0793]. Despite differences in bacterial concentrations noted at a standardized OD, bacterial size did not significantly influence the concentration of each strain at OD_{600nm} 0.1 (Spearman Rho=-0.23, p=0.55).

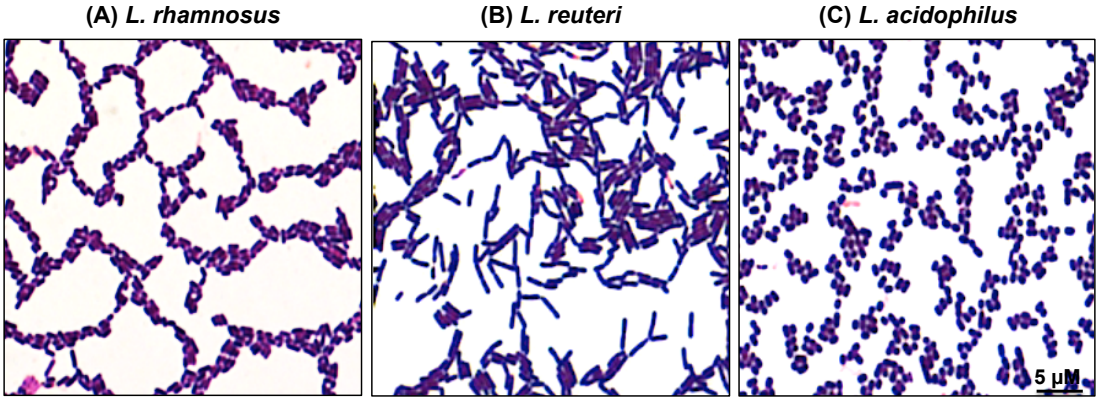


Figure 3.3. Representative images of Gram straining properties of the three probiotic *Lactobacillus* strains. Smears of single (A) *L. rhamnosus* (isolated from Vagiforte® PLUS Combo Pack), (B) *L. reuteri* (Reuterina™ Femme®) and (C) *L. acidophilus* (Vagiforte® PLUS Combo Pack) colonies on microscopic slides were Gram strained. Slides were visualised using a Leica DM500 light microscope, under a 40x objective lens. Images of each slide were captured using Zeiss colour digital camera and ZEN software, and the size of at least 100 bacterial rods per probiotic isolate was measured using ImageJ software (ImageJ, Version 2.0.0-rc-43/1.50e, USA) to determine rod sizes. Magnification scale in C.

3.3.3.3 Growth kinetics of probiotic isolates at varying environmental pHs

In vivo, a vaginal pH ≤ 4.5 is considered healthy, while a pH > 4.5 is indicative of BV according to Amsel's criteria for diagnosing BV (Amsel *et al*. 1983; Sha *et al*. 2005; Bala *et al*. 2017). To investigate whether environmental pH influences probiotic growth, and to test the ability of these strains to tolerate low pHs that tend to be bactericidal to other non-*Lactobacillus* spp. (Saunders *et al*. 2007; Pybus & Onderdonk 1996; Borges *et al*. 2012; Yang *et al*. 2012), the growth kinetics of each isolate was measured in culture medium adjusted to pHs 3.5 - 4.0 (resembling pHs of a healthy FGT) or pHs 4.5 - 6.0 (at which most bacterial strains grow optimally *in vitro*, resembling conditions of BV). It was hypothesized that the probiotic strains should be able to tolerate a wide range of pHs, since they grow well once the FGT has reached a healthy pH of ≤ 4.5.

At pH 6.0, the kinetics of the different *Lactobacillus* and *Bifidobacterium* isolates was comparable over 24 hours, with growth being exponential until 12 hours (log phase), after which the stationary phase was reached (**Figure 3.4**, top panel). The maximum OD_{600nm} for *Lactobacillus* spp. ranged from 1.0–1.55 (with median concentrations of $4.1x10^8$ CFU/ml; IQR $5.2x10^7$ – $1.6\ x10^9$) and varied slightly by species. The identical *L. casei rhamnosus* strain Lcr35 isolated from Provacare™ and Gynophilus® showed similar growth kinetics during the exponential growth phase, and both reached the same maximal OD_{600nm} of 1.4 at 24 hours ($7.8x10^8$ and $7.4x10^8$ CFU/mL, respectively). The *Bifidobacterium* isolates grew slower than *Lactobacillus* strains, reaching a maximum OD_{600nm} of 0.57 (range 0.5–0.7; median concentration of $7.9x10^7$ CFU/ml; range $1.0\ x\ 10^6$ – $2.7x10^8$) within 24 hours.

Growth of probiotic isolates tended to be compromised at lower pHs, and varied greatly between isolates of the same species, with *L. acidophilus* and *Bifidobacterium* isolates tending to be the most vulnerable to lower pHs. *L. rhamnosus* and *L. reuteri* strains tended to be more pH tolerant, although they were not able to grow well at the lowest pH of 3.5 (**Figure 3.4**, bottom panel).

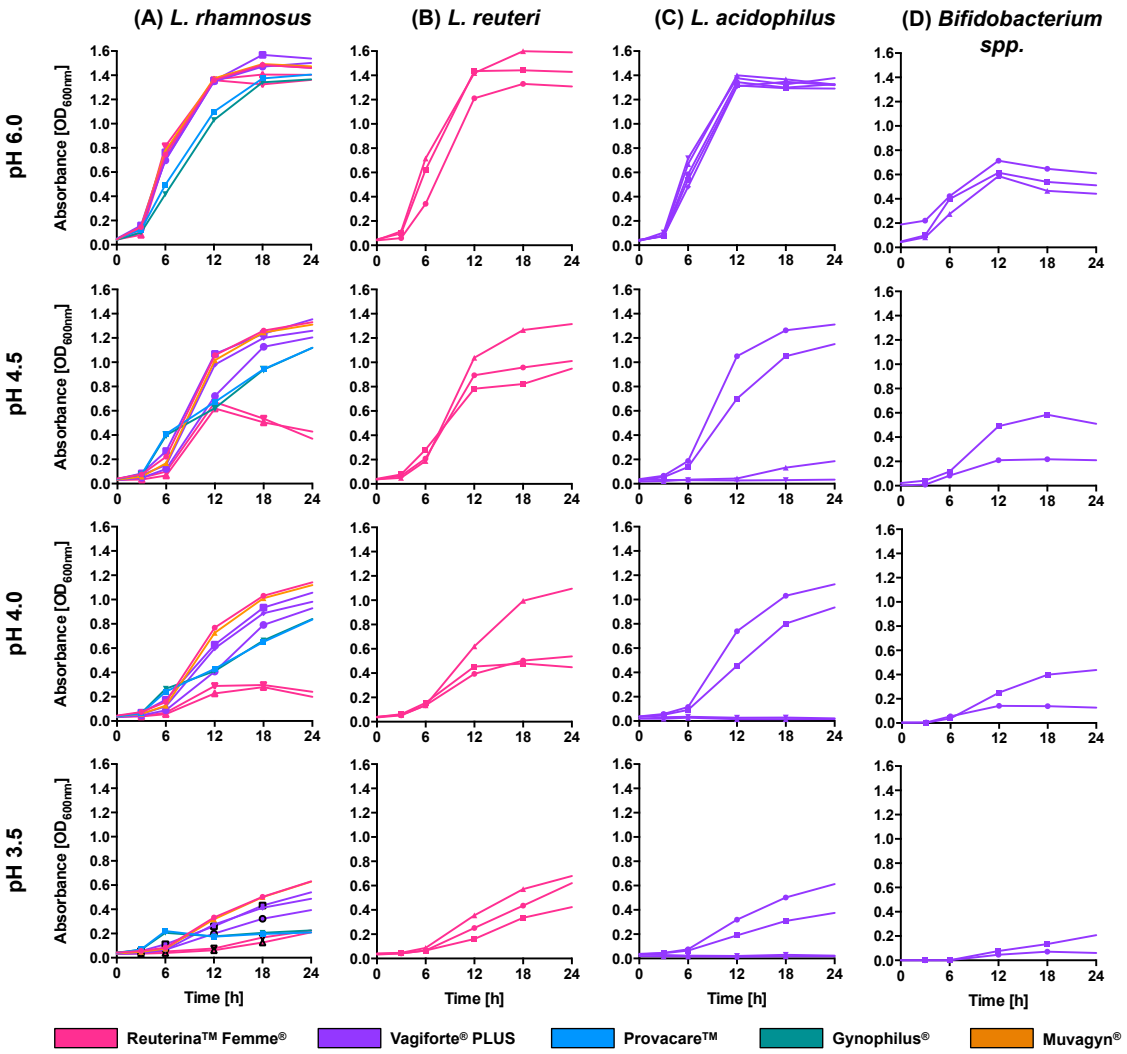


Figure 3.4. Growth kinetics of probiotic *Lactobacillus* and *Bifidobacteria* isolates at varying environmental pHs. Overnight cultures of *L. rhamnosus* (A), *L. reuteri* (B), *L. acidophilus* (C) and *Bifidobacterium* (D) spp. isolates were adjusted to an OD_{600nm} 0.1 (±0.01) in MRS or sBHI broth adjusted to a pH of 6.0, 4.5, 4.0 and 3.5 (fromtop to bottom row), and their absorbance (OD_{600nm}) was measured at 0, 3, 6, 12, 18 and 24 hours. The growth curves show the average of the measured absorbance [OD_{600nm}] of each isolate at eacht time point. The growth curves were color-coded based on the product the species was isolated from (pink = Reuterina™ Femme®, purple = Vagiforte® PLUS, blue = Provacare®, green = Gynophilus®, orange = Muvagun®).

3.3.3.4 Influence of Lactobacillus and Bifidobacterium spp. growth on culture pH

A *Lactobacillus*-dominated microbiota in the lower FGT is thought to be responsible for acidifying the pH < 4.5, which is considered a major protective characteristic of this genera in the FGT (Barrons & Tassone 2008; Boris & Barbés 2000; O'Hanlon *et al*. 2010; Nardini *et al*. 2016). Boskey *et al*. (1999) showed that clinical *Lactobacillus* isolates were able to lower and maintain the culture pH to 3.2–4.8 *in vitro*. To assess

if the isolates from commercially available probiotics achieve similar results, the culture pH was measured over 48 hours (**Figure 3.5**).

For *Lactobacillus* spp., the initial pH of MRS medium was pH 6.2, which did not change over 48 hours in the absence of bacteria (control). All probiotic *Lactobacillus* isolates lowered the culture pH similarly over 12 hours to pH 4.4 (± 0.2), which was maintained until 48 hours (**Figure 3.5**A-C). For *Bifidobacterium* spp., the initial pH of sBHI medium was pH 7.3, which did not change over 48 hours in the abiotic state (control). *B. bifidum* and *B. longum* (**Figure 3.5**D), both isolated from Vagiforte®, did not lower the culture pH as dramatically as the *Lactobacillus* isolates, achieving a more modest pH of 6.0 within 3 hours, which was maintained over the 48 hour experiment, with minimal variation between strains. The ability of probiotic isolates to lower pH (at 24 hours) correlated negatively with their growth at 24 hours (measured by absorbance at $OD_{600nm;}$ Spearman Rho=-0.57, p=0.0185). This suggested that the ability of bacterial strains to lower pH was directly related to their growth kinetics, with those growing well having the highest capacity to acidify. Both *L. casei rhamnosus* Lcr35 strains, isolated from Provacare™ and Gynophilus®, lowered the pH to the same extent (**Figure 3.5**A).

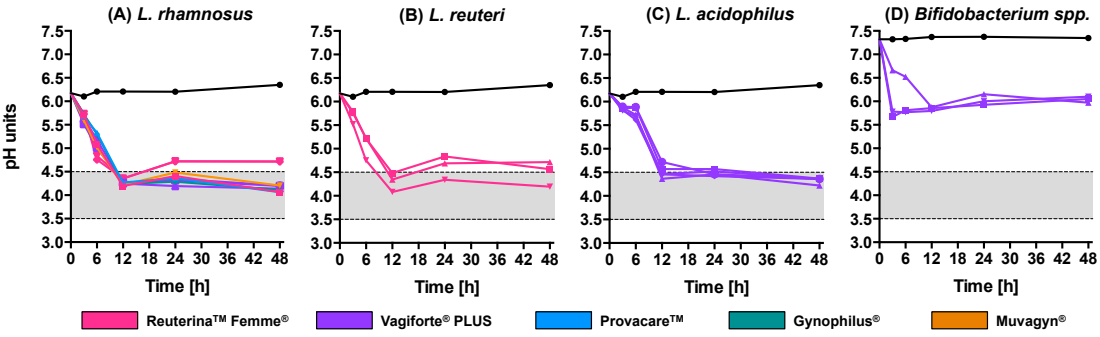


Figure 3.5. Comparison of the ability of *Lactobacillus* and *Bifidobacterium* probiotic isolates to lower culture pH. Overnight cultures of (A) L. rhamnosus (n=9), (B) *L. reuteri* (n=3), (C) *L. acidophilus* (n=5), and (D) *Bifidobacterium* (n=3) isolates were adjusted to an OD_{600nm} 0.1 (±0.01) in MRS or sBHI broth, and the pH of their cultures was measured at 0, 3, 6, 12, 24 and 48 hours. As control, the pH of abiotic media was measured. The lines were color-coded based on the product the species was isolated from (pink = Reuterina™ Femme®, purple = Vagiforte® PLUS, blue = Provacare®, green = Gynophilus®, orange = Muvagun®), and the control (abiotic media) is shown in black. The physiological vaginal pH of healthy women (ranging from pH 3.5 to 4.5) is shaded it grey. Experiments were performed in duplicates. No variation was observed between duplicates.

3.3.3.5 Susceptibility of probiotic strains to antibiotics

Probiotic strains were tested for their sensitivity to antibiotics commonly used to treat BV (including metronidazole and clindamycin), respiratory infections (including penicillin and amoxicillin;**Figure 3.6**); and in the long-term treatment for tuberculosis (TB, including rifampicin and ioniazid; **Figure 3.7**). *Lactobacillus* and *Bifidobacterium* spp. have been shown to be resistant to metronidazole but susceptible to clindamycin, penicillin, and amoxicillin (De Backer *et al*. 2006). For *Lactobacillus* spp., clinical MIC breakpoints for antibiotic resistance have only been established for a small number of antibiotics: benzylpenicillin (considered sensitive if MIC ≤0.25 μg/mL and resistent if MIC >5 μg/mL); ampicillin and amoxicillin (considered sensitive if MIC ≤4 μg/mL, and resistant if MIC >8 μg/mL); piperacillin and tiracillin (considered sensitive if MIC ≤8 μg/mL, and resistant if MIC >16 μg/mL); doripenem and ertapenem (considered sensitive if MIC ≤1 μg/mL, and resistant if MIC >1 μg/mL); imipenem meropenem (considered sensitive if MIC ≤ 2 μg/mL, and resistant if MIC > 8 μg/mL); chloramphenicol (considered sensitive if MIC ≤ 8 μg/mL, and resistant if MIC > 8 μg/mL); clindamycin and metronidazole (considered sensitive if MIC ≤4 μg/mL, and resistant if MIC >4 μg/mL; European Committee on Antimicrobial Susceptibility Testing [EUCAST], 2018). No zone diameter breakpoints have been established for the EUCAST standardised disk diffusion method (EUCAST, 2018). For this reason, this study reports on sensitivity of *Lactobacillus* isolates to the antibiotics tested instead of using the term resistance.

Using the disc diffusion assay, all of the probiotic *Lactobacillus* and *Bifidobacterium* isolates were resistant to metronidazole (data not shown), but the majority showed some sensitivity to clindamycin (18/20), penicillin (18/20) and amoxicillin (20/20). All five *L. acidophilus* isolates were more sensitive to penicillin (median zone of inhibition [ZOI] of 40 mm; IQR 36–41.5) and amoxicillin (median ZOI of 38 mm; IQR 32–39.5) than clindamycin (median ZOI of 17 mm; IQR 16.5–18.5; p=0.0054 and p=0.1641, respectively; **Figure 3.6**). Of the probiotic *Lactobacillus* spp. tested, *L. acidophilus* isolates were the least sensitive to clindamycin, but the most sensitive to amoxicillin. In contrast, the three *L. reuteri* isolates tended to be less sensitive to penicillin (median ZOI of 8 mm; IQR 0–28 mm) than clindamycin (median ZOI of 26 mm; IQR 25–26 mm) and amoxicillin (median ZOI of 20 mm, IQR 19–26 mm), although not significantly. The same pattern was seen for the three *Bifidobacterium*

isolates (**Figure 3.6**). All nine *L. rhamnosus* isolates had a similar susceptibility to all antibiotics tested, with a median ZOI of 23 mm (IQR 10–26 mm) for clindamycin, 26 mm (IQR 1 –31.5 mm) for penicillin and 27 mm (IQR 22–29 mm) for amoxicillin.

Interestingly, the same species of *Lactobacillus* and *Bifidobacterium* isolates from different lot numbers of Reuterina™ Femme® and Vagiforte® products showed some variability in their sensitivity to antibiotics, depending on from which batch they were isolated from (**Figure 3.6**B). The diameter of the inhibition zone of the three Vagiforte® *Bifidobacterium* isolates ranged considerably from 0 to 29 mm for penicillin, indicating that some were resistant while others were sensitive. Interestingly, the three *L. rhamnosus* and *L. reuteri* strains from Reuterina™ Femme® showed little variation in sensitivity to clindamycin, and the variation between isolates was moderate regarding their susceptibility towards amoxicillin but the susceptibility towards penicillin was highly variable. Importantly, the *L. rhamnosus* Lcr35 strains isolated from Provacare™ (Lot #0154) and Gynophilus® (Lot #0200) were resistant to clindamycin, commonly used to treat BV, while all other *Lactobacillus* strains were susceptible (ZOI >17 mm). There was no apparent difference between the probiotic strains isolated from European and South African products regarding antibiotic susceptibility profiles.

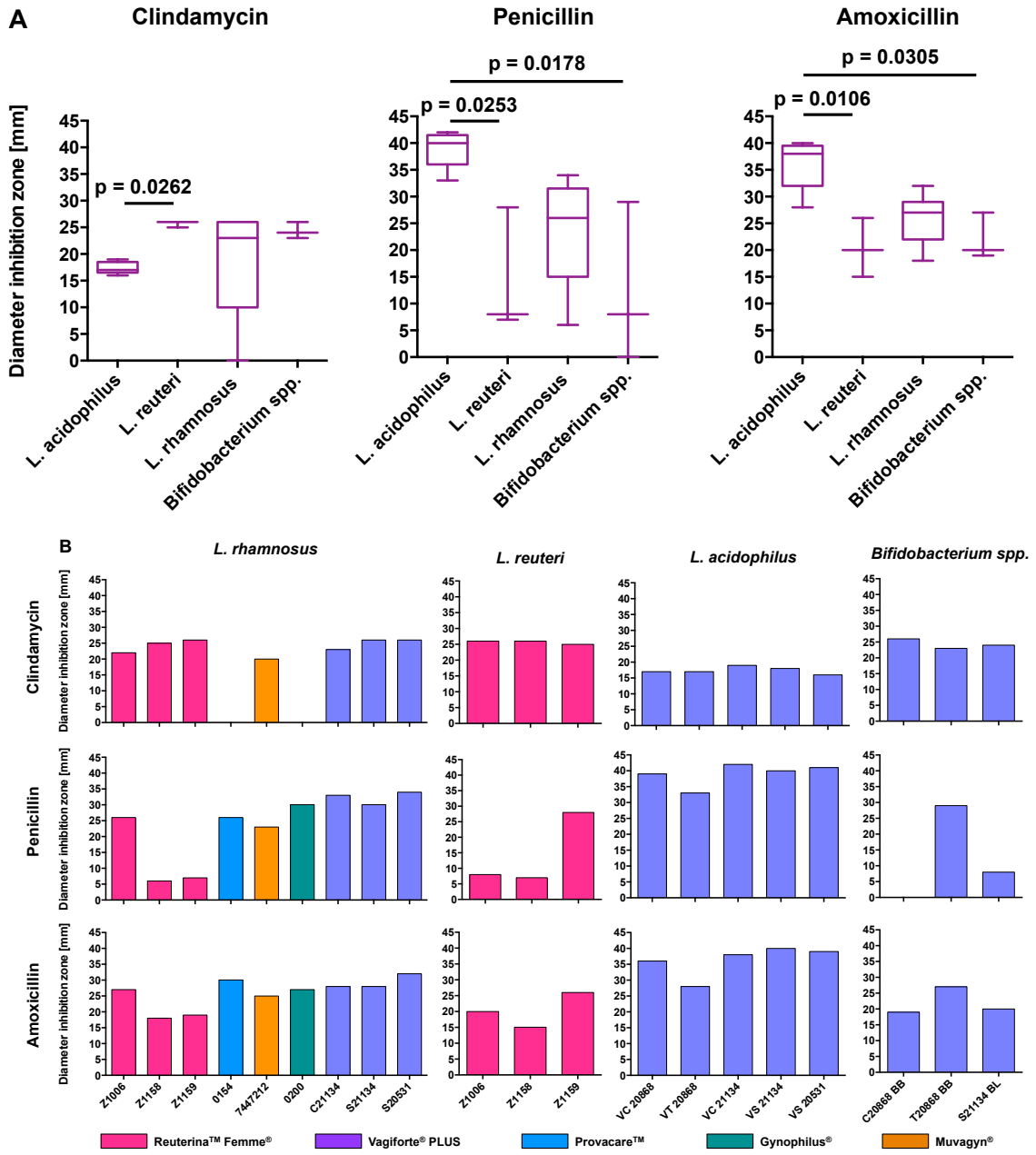


Figure 3.6. Susceptibility of probiotic *Lactobacillus* and *Bifidobacterium* isolates to clindamycin, penicillin and amoxicillin. (A) Overnight cultures of probiotic *L. acidophilus* (n=5), *L. rhamnosus* (n=9), *L. reuteri* (n=3) and *Bifidobacterium* (n=3) strains were used to test their antibiotic susceptibility to clindamycin [2 µg], penicillin [2 µg] and amoxicillin [10 µg] using the disc diffuson method. The Kruskal-Wallis test was used to determine differences between groups. P-values were adjusted for multiple comparisons, using the post-hoc Dunn's test. (B) The bars were color-coded based on the product the species and strains was isolated from (pink = Reuterina™ Femme®, purple = Vagiforte® PLUS, blue = Provacare™, green = Gynophilus®, orange = Muvagun®). Experiments were performed in duplicates, which gave identical results.

Since TB is a very common infection in South Africa (estimated 1000 cases per 100,000 persons) and many other countries in sub-Saharan Africa, Asia and South America (World Health Organization 2018), and treating uncomplicated TB typically

involves six months with a combination of rifampicin and isoniazid (Nahid *et al*. 2016), it was important to evaluate the sensitivity of probiotic strains to these commonly administered antibiotics. In this study, all 17 probiotic *Lactobacillus* isolates were highly susceptible to rifampicin. However, they were not sensitive to isoniazid, even at concentrations that were higher than those reported in plasma of individuals receiving TB treatment (>5 µg/mL; Moussa *et al*. 2016; Burhan *et al*. 2013). All *Lactobacillus* isolates were susceptible to much lower concentrations of rifampicin than are typically found in plasma or brain tissue levels from patients being treated with this antibiotic (~6 µg/mL and ~0.3 µg/mL, respectively; Mindermann *et al*. 1998). There was some variability between *Lactobacillus* isolates in their susceptibility to rifampicin, with MICs ranging from 0.06 µg/mL for *L. reuteri* to 0.12 µg/mL for *L. acidophilus* (**Figure 3.7**). The MICs of the *L. rhamnosus* strains Lcr35 isolated from Provacare™ (Lot #0154) and Gynophilus® (Lot #0200) were identical (MIC 0.03 µg/mL; **Figure 3.7**B). Similarly, there was little variation in rifampicin susceptibility of *L. reuteri* isolates from different batches of Reuterina™ Femme® (**Figure 3.7**A), although there was some variation for *L. rhamnosus* strains from different batch numbers of the same product, as well as between *L. acidophilus* strains that originated from Vagiforte® products (**Figure 3.7**C). The susceptibility of *Bifidobacterium* spp. is not shown because the growth of the *Bifidobacterium* strains in the control wells without any antibiotic was poor.

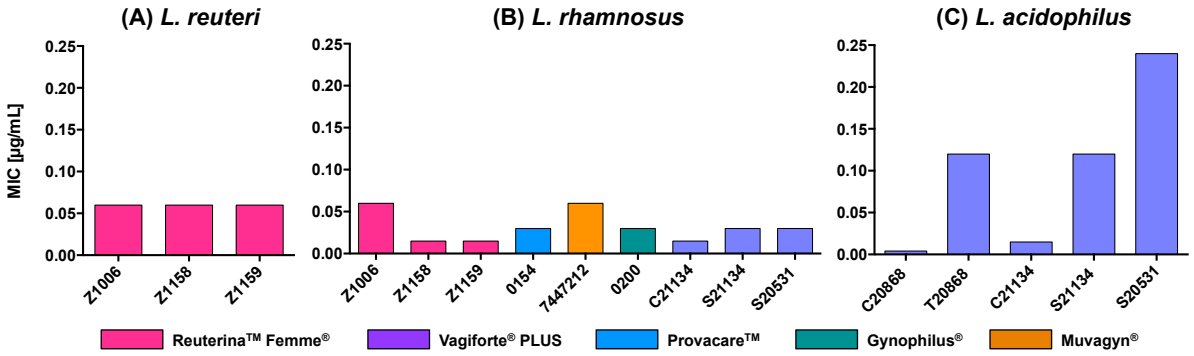


Figure 3.7. Susceptibility of probiotic *Lactobacillus* isolates to rifampicin. Overnight cultures of *L. rhamnosus* (A), *L. reuteri* (B), and *L. acidophilus* (C) strains were adjusted to $1x10^5$ CFU/mL and the MIC to rifampicin (1000 – 0.004 µg/mL) was determined using serial dilutions. The bars were color-coded based on the product the species and strains was isolated from (pink = Reuterina™ Femme®, purple = Vagiforte® PLUS, blue = Provacare™, green = Gynophilus®, orange = Muvagun®). Experiments were performed in duplicates, which gave identical results.

3.3.3.6 Ability of probiotic Lactobacillus strains to adhere to epithelial cells

From each product, a total of 10/17 of the best-performing *Lactobacillus* strains representing each species were chosen for detailed investigation into their ability to adhere to cervical epithelial cells, including one *L. reuteri* isolate (Reuterina™ Femme® Lot# Z1006), six *L. rhamnosus* isolates (Reuterina™ Femme® Lot #Z1006, Provacare™, Gynophilus®, Muvagun®, Vagiforte® Lot #21134 oral capsule and vaginal spray), and three *L. acidophilus* isolates (Vagiforte® Lot #21134 oral capsule and vaginal spray, #20868 vaginal tablet). *Bifidobacterium* isolates were excluded because they are not commonly considered healthy commensals in the lower FGT (Lennard *et al*. 2017; Rosenstein *et al*. 1996), did not grow well and did not lower the culture pH as efficiently as the *Lactobacillus* isolates.

The first critical step for probiotic organisms intended to colonize the lower FGT is their ability to adhere to vaginal and/or cervical epithelial cells, as this increases the likelihood that they will remain at the site of action, and allows them to compete with FGT pathogens for adhesion and nutrients (Atassi *et al*. 2006; Coudeyras *et al*. 2008; Mastromarino *et al*. 2002; Zarate and Nader-Macías 2006; McLean *et al*., 2000). Thus, the ability of the probiotic *Lactobacillus* isolates to adhere to ectocervical epithelial cells (CaSki) was measured for the ten *Lactobacillus* isolates that were selected for in-depth analysis. All ten isolates adhered to CaSki cells with varying efficiency (***Figure 3.8***). *L. rhamnosus* strains tended to adhere better to CaSki cells (median 1.00%, IQR 0.63–1.34; n=6) compared to *L. acidophilus* (median 0.57%, IQR 0.31 – 0.72; n=3) and *L. reuteri* isolates (0.59%; n=1).

Lactobacillus strains isolated from European and South African probiotic products showed similar adherence profiles. However, the *L. casei rhamnosus* Lcr35 strain from Gynophilus® (Lot #0200; 0.9%) tended to be slightly more adherent to Ca Ski cells that the same strain from Provacare™ (Lot #0154, 0.7%, **Figure 3.8**).

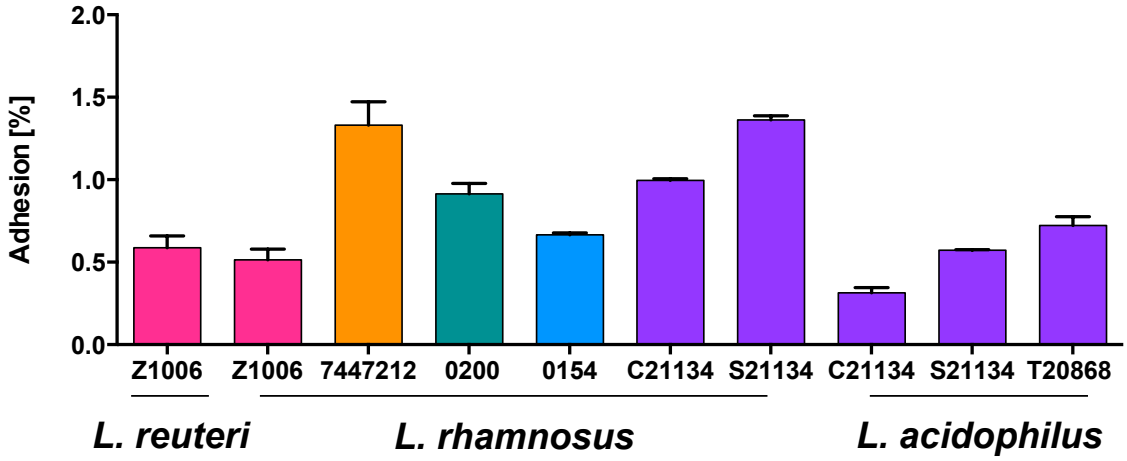


Figure 3.8. Adherence of probiotic *Lactobacillus* isolates to cervical epithelial (Ca Ski) cells. $4.18x10^6$ CFU of *Lactobacillus* cultures were added to Ca Ski cells and incubated for 3 h at 37 °C and 5% CO_2. and serial dilutions were plates onto MRS agar plates to determine the percentage of adhered bacteria of *L. reuteri* (Reuterina™ Femme® Z1006), *L. rhamnosus* (Reuterina™ Femme® Z1006, Provacare™, Gynophilus®, Muvagun®, Vagiforte® 21134 oral capsule and vaginal spray), and *L. acidophilus* (Vagiforte® 21134 oral capsule and vaginal spray, Vagiforte® 20868 vaginal tablet). The bars were color-coded based on the product the species and strains was isolated from (pink = Reuterina™ Femme®, purple = Vagiforte® PLUS, blue = Provacare™, green = Gynophilus®, orange = Muvagun®).

3.3.3.7 Production of lactate by probiotic Lactobacillus isolates

Levels of L- and D- lactate were measured in culture supernatants from only eight of the ten *Lactobacillus* isolates due to budgetary constraints for this part of the study. As such, Vagiforte® PLUS *L. rhamnosus* and *L. acidophilus* C21134 excluded as they adhered the poorest to Ca Ski cells. All eight *Lactobacillus* strains produced some L-lactate, which varied by isolates (**Figure 3.9**A). Concentrations of L-lactate produced by these eight probiotic *Lactobacillus* isolates were generally higher than the concentration of D-lactate (**Figure 3.9**B) produced (median of 7.65 [IQR 7.23-32.20] vs. 1.18 [IQR 0.54-22.71] ng/µL; p=0.0391). Only the *L. rhamnosus* isolate from Muvagun® did not produce any D-lactate. The *L. rhamnosus* isolate from Provacare™ (Lot #0154) and Gynophilus® (Lot #0200) produced almost identical amounts of L-lactate and very little D-lactate. Comparing the different *Lactobacillus* spp., *L. acidophilus* (n=2; median 41.32 [IQR 29.45-53.19] and 43.78 [IQR 29.43-58.14] ng/µL, respectively) isolates tended to produce more L- and D-lactate than the *L. rhamnosus* (n=5; median 7.49 [IQR 7.18-20.46] and 0.69 [IQR 0.24-1.18] ng/µL, respectively) and *L. reuteri* (n=1; 7.36 and 2.57 ng/µL, respectively) isolates (**Figure 3.9**).

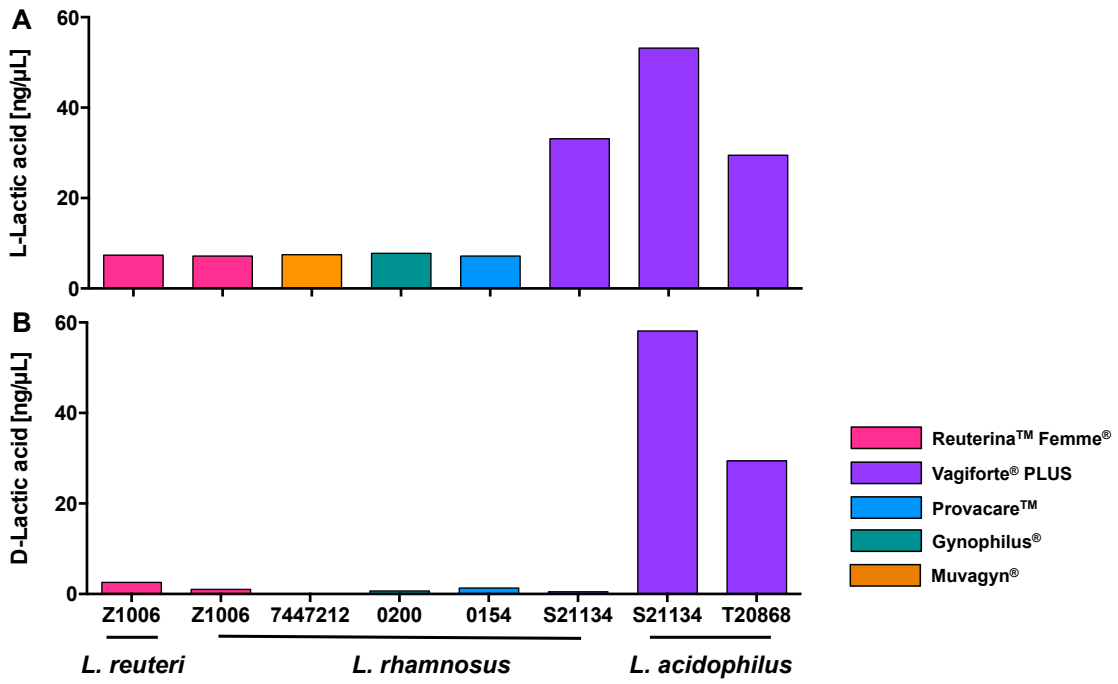


Figure 3.9. Production of L- (A) and D-lactate (B) by probiotic *Lactobacillus* isolates. *L. reuteri* (Reuterina™ Femme® Z1006), *L. rhamnosus* (Reuterina™ Femme® Z1006, ProvacareTM, Gynophilus®, Muvagun®, Vagiforte® 21134 vaginal spray), and *L. acidophilus* (Vagiforte® 21134 vaginal spray and 20868 vaginal tablet) were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS and incubated for 24 hours at 37°C under anaerobic condtions. The supernatant was harvested and filtered. The concentration of L – (A) and D- (B) lactic was measured using colorimetric assays (Sigma-Aldrich®, USA) The bars were color-coded based on the product the species and strains was isolated from (pink = Reuterina™ Femme®, purple = Vagiforte® PLUS, blue = Provacare™, green = Gynophilus®, orange = Muvagun®).

3.3.3.8 Production of H_2O_2 by probiotic Lactobacillus isolates

Under anaerobic conditions, all of the probiotic *Lactobacillus* isolates produced some H_2O_2, although only at low concentrations, ranging from 1.90 µM (*L. rhamnosus* Reuterina™ Femme® Lot #Z1006) to 5.80 µM (L. *rhamnosus* Vagiforte® Lot #C21134; **Figure 3.10**). *L. acidophilus* tended to produce more H_2O_2 than either *L. rhamnosus* or *L. reuteri*, although not significantly. The *L. rhamnosus* Lcr35 isolates from Provacare™ Lot #0154 and Gynophilus® Lot #0200 produced exactly the same concentration of H_2O_2 (2.7 µM, **Figure 3.10**). However, these amounts of H_2O_2, measured under anaerobic conditions, are ~10^6-fold lower than levels that are typically considered microbicidal (≥1000 mM). Because the lower FGT is a largely anaerobic environment, this brings into question the biological relevance of H_2O_2 production in the context of probiotics for vaginal health. Just like the other biological characteristics, the production of antimicrobial compounds did not depend on the manufacturing country.

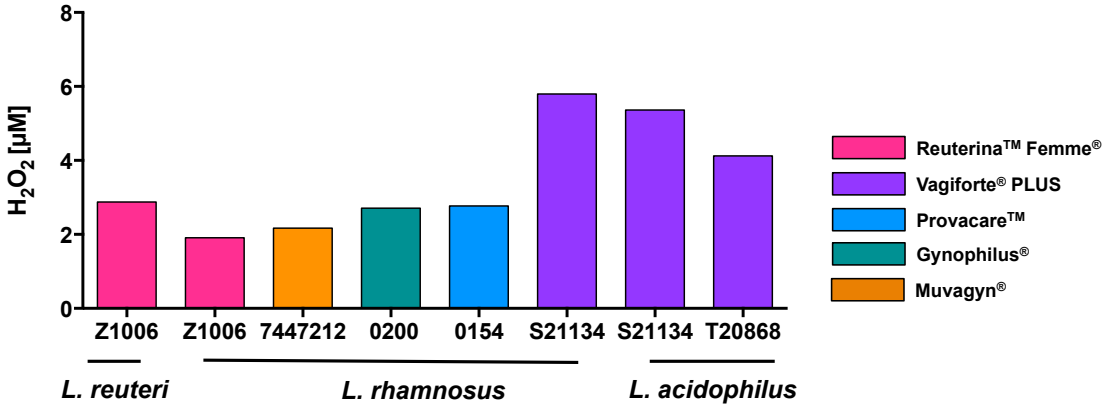


Figure 3.10. Production of H_2O_2 under anaerobic conditions by probiotic *Lactobacillus* isolates. *L. reuteri* (Reuterina™ Femme® Z1006), *L. rhamnosus* (Reuterina™ Femme® Z1006, ProvacareTM, Gynophilus®, Muvagun®, Vagiforte® 21134 vaginal spray), and *L. acidophilus* (Vagiforte® 21134 vaginal spray and 20868 vaginal tablet) were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS and incubated for 24 h at 37 °C under anaerobic condtions. The supernatant was harvested and filtered. H_2O_2 concentration was measured using a fluorometric assay (Sigma-Aldrich, USA). The bars were color-coded based on the product the species and strains was isolated from (pink = Reuterina™ Femme®, purple = Vagiforte® PLUS, blue = Provacare™, green = Gynophilus®, orange = Muvagun®). Experiments were performed in dubplicates.

3.3.3.9 Ability of cultured media from probiotic Lactobacillus isolates to inhibit growth of G. vaginalis, P. bivia and GBS

P. bivia and *G. vaginalis* are commonly found in women with BV, although they can also be found in the FGT in the absence of BV (Ravel *et al*. 2011; Gajer *et al*. 2012; Lennard *et al*. 2017), and GBS is commonly associated with adverse pregnancy outcomes (Shabayek & Spellerberg 2018; Martinez *et al*. 2000). Thus, the ability of antimicrobial compounds and metabolites present in *Lactobacillus*-conditioned abiotic media from the ten probiotic strains to inhibit growth of six anaerobically grown *G. vaginalis* (ATCC® 14018™ and five vaginal strains; kindly provided by Dr Remy Froissart, U. Montpellier, France) and *P. bivia* strains (ATCC® 29303™, one from semen, and four vaginal strains, kindly provided by Dr Remy Froissart) and six aerobically grown GBS strains (all vaginal, kindly provided by Dr Shameem Jaumdally) was evaluated (**Figure 3.11**).

Conditioned media from all *Lactobacillus* isolates inhibited the panel of *P. bivia*, *G. vaginalis* and GBS strains to varying extents, although they tended to inhibit *P. bivia* and GBS strains better than *G. vaginalis* strains (**Figure 3.11**). The *L. reuteri* strain Z1006 (isolated from Reuterina™ Femme®) and all *L. rhamnosus* strains inhibited the growth of all *P. bivia* strains by more than 80%, while *L. acidophilus* S21134

(isolated from Vagiforte® vaginal spray) inhibited *P. bivia* strains less effectively than either *L. reuteri* Z1006 (adj. p=0.0076) or *L. rhamnosus* (Z1006, adj. p=0.0060; 7447212, adj. p=0.0039; 0200, adj. p=0.0247; 0154, adj. p=0.0308; S21134, adj. p=0.1721; **Figure 3.11**A). As only one *L. acidophilus* strain was included, it is unclear whether this limited inhibitory effect is specific to this strain, or for all *L. acidophilus* strains. A highly comparable inhibition pattern was seen for GBS strains, with *L. reuteri* and *L. rhamnosus* strains inhibiting the growth of the majority of GBS stains by >90%, while *L. acidophilus* S21134 inhibited GBS growth by <80% (**Figure 3.11**C).

Lactobacillus-conditioned media generally inhibited *G. vaginalis* strains to a much lesser extent than *P. bivia* and GBS strains, with *L. reuteri* Z1006 inhibiting *G. vaginalis* more effectively than any of the *L. rhamnosus* strains (**Figure 3.11**B). *L. reuteri* Z1006 (isolated from Reuterina™ Femme®) inhibited all of the *G. vaginalis* strains to some extent, while *L. rhamnosus* 7447212 (from Muvagyn®) inhibited 5/6 *G. vaginallis* strains tested, *L. rhamnosus* Z1006, 0200 and 0154 (from Reuterina™ Femme®, Gynophilus® and Provacare™, respectively) inhibited 4/6 *G. vaginalis* strains, and the Vagiforte® isolates *L. rhamnosus* C21134 and *L. acidophilus* S21134 only inhibited only three and one *G. vaginalis* strains, respectively. The inhibitory effect appeared to be specific for each of the *G. vaginalis* strains. The ATCC® *G. vaginallis* strain was only inhibited by conditioned medium from *L. reuteri* Z1006, while one of the vaginal *G. vaginallis* isolates was inhibited by conditioned media from all of the *Lactobacillus* strains tested, and the other vaginal *G. vaginallis* strains were inhibited to different extents, depending on which *Lactobacillus* strain was used to condition the media. Conditioned media from *L. acidophilus* S21134 did not inhibit growth of *G. vaginalis* at all (**Figure 3.11**).

L. rhamnosus Lcr35 isolated from Provacare™ (Lot #0154) and Gynophilus® (Lot #0200) inhibited the growth of the *P. bivia* (median inhibition 96.11% [IQR 92.32 – 98.68] vs. 96.60% [94.91–98.22]) and GBS strains (98.74% [IQR 95.85-99.58] vs. 97.31% [IQR 94.49-99.43]) to almost identical extents. Furthermore, conditioned media from both of these strains inhibited the same four *G. vaginalis* strains (**Figure 3.11**) with very comparable levels of inhibition, again suggesting that probiotic characteristics are highly strain specific.

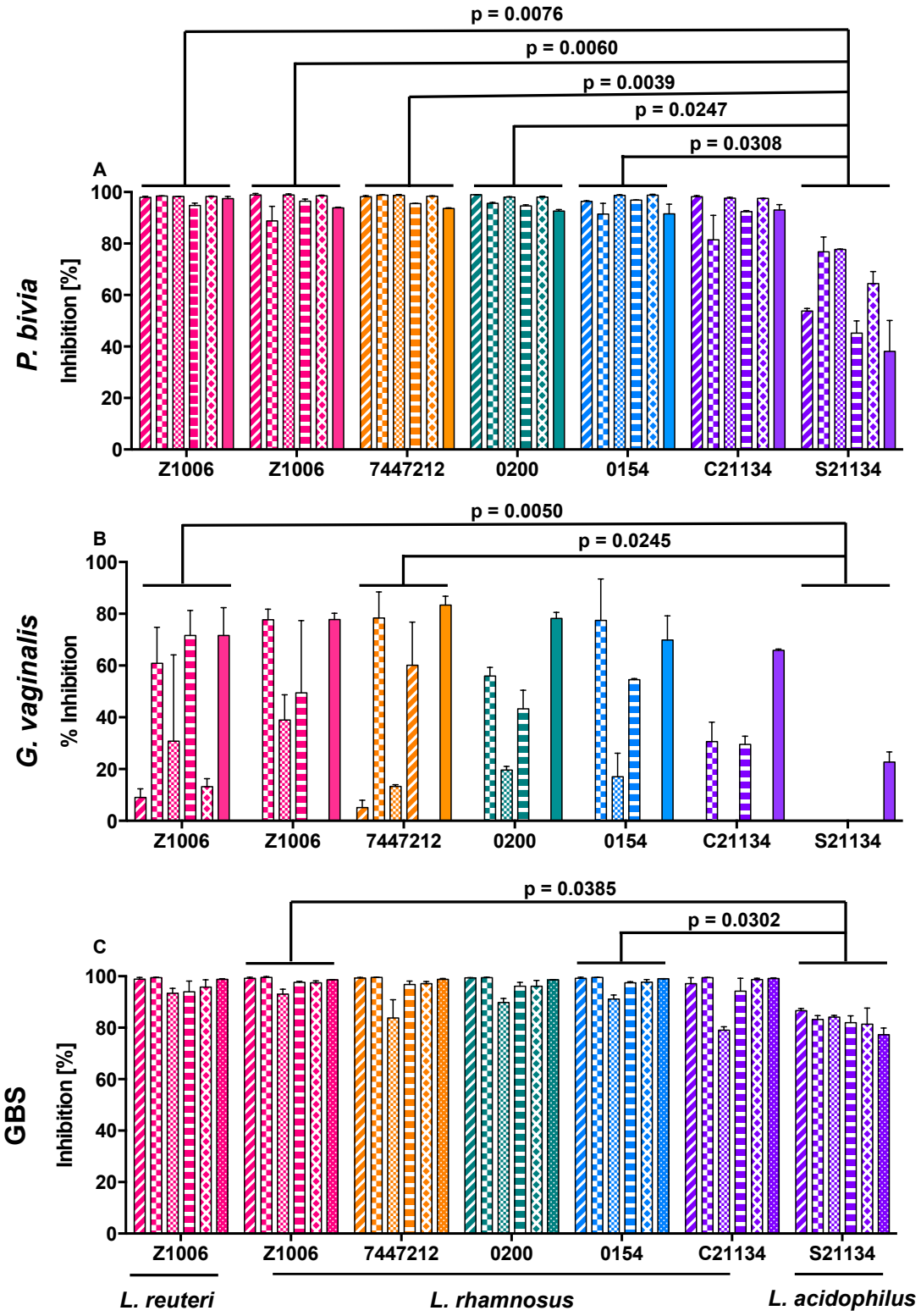


Figure 3.11. Inhibitory effect of *Lactobacillus*-conditioned media on the growth of FGT pathogens. *L. reuteri* (Reuterina™ Femme® Z1006), *L. rhamnosus* (Reuterina™ Femme® Z1006, ProvacareTM, Gynophilus®, Muvagun®, Vagiforte® 21134 oral capsule), and *L. acidophilus* (Vagiforte® 21134 vaginal spray) were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS and cultured for 24 hours at 37°C under anerobic conditions. The supernatants were sterile-filtered and added in a 1:1 ratio to overnight cultures of *P. bivia* (A), *G.vaginalis* (B) and GBS (C) strains. The absorbance of FGT pathogen cultures in *Lactobacillus*-conditioned and –unconditioned media (control) was measured at 3, 6, 12, 24, 30 and 48 h, and the the area under the curve (AUC) was calculated for each strain. The percentage of inhibition was determined by comparing the growht of the FGT pathogen strains in the presence of non-conditioned media (control) to the growth in the presence of *Lactobacillus*-conditions media. *P. bivia* and *G. vaginalis* strains used were: ATCC® (diagonal-striped, first bars), and isolated from semem (*P. bivia* only, squared pattern) and vaginal samples (remaining patterns). All GBS strains were vaginal (different pattern for each strain). The Kruskal-Wallis test was used to determine differences between isolates, and adjusted p-values are shown.

3.4 Discussion

It was important to determine whether locally sourced probiotics meet quality standards, including bacterial contents and dosing, before conducting a small clinical trial using a locally available probiotic product as adjunct to antibiotics in the treatment of BV (Chapter 4). This Chapter therefore evaluated the content, dose and biological characteristics of different batch numbers of *Lactobacillus* isolates from various off-the-shelf probiotics for vaginal health distributed in South Africa, and compared these to two European probiotics for vaginal health. Secondarily, probiotic characteristics that are thought to be important for vaginal health were determined *in vitro* as an estimate of each of the individual probiotic isolates' potential to improve vaginal health, which was also included in the selection of the product for the clinical trial (Chapter 4). Evidence presented in this Chapter suggested that the probiotics for vaginal health available OTC in South Africa [Provacare™ Probiotic Vaginal care (manufactured by Provacare, Ontario, Canada), Vagiforte® PLUS and Vagiforte® PLUS Combo Pack (manufactured by Bioflora CC, Pretoria, South Africa), Reuterina™ Femme® (manufactured by Ikacia, Isando, South Africa)] generally contained the bacterial content and dose as claimed by the manufacturers', similarly to the European products, like Muvagyn® (manufactured by Hälsa Pharma GmbH, Germany; distributed in Spain) and Gynophilus® (manufactured by Probinov, Paris; distributed in France)]. There was some variability noted in the biological characteristics of the *Lactobacillus* isolates from different product lot numbers. However, the evaluations conducted in this Chapter of some of the characteristics considered important for FGT health suggested that they might have good potential to improve vaginal health, as indicated by the manufacturers.

Most of the *Lactobacillus* spp. advertised by the manufacturers' were successfully isolated from the commercial probiotic products. The only *Lactobacillus* spp. that was not successfully isolated, despite numerous attempts, was *L. gasseri*, which was present only in Muvagyn®. Since Muvagyn® also contained *L. rhamnosus,* it is possible that competition made recovery of *L. gasseri* difficult. More likely, however, this particular strain may not have been viable in the product that was purchased for this study, as only few colonies grew when culturing the content of a whole capsule.

In contrast to *Lactobacillus* strains, fewer of the *Bifidobacterium* strains were successfully isolated. The fact that *B. bifidum* and *B. longum* were only isolated from some batch numbers of Vagiforte® products but not others may reflect that *Bifidobacterium* spp. have more fastidious growth requirements than *Lactobacillus* spp. (Zhou & Li 2015), rather than them being absent from Vagiforte® products. Supplemented BHI agar was used in this Chapter, and is commonly used to culture *Bifidobacterium* spp. as it is non-selective (Silvi *et al*. 1996; Pachenari *et al*. 2001). However, modified *Bifidobacterium* agar (BD™ Diagnostic Systems, Heidelberg, Germany) is now available, which may improve *Bifidobacterium* recovery in future studies.

Bacterial concentrations for some of the probiotics for vaginal health available in South Africa (Reuterina™ Femme® and Provacare™) were generally the same or higher than those listed on the packaging, and there was no significant variation between lot numbers. Further, bacterial concentrations in Vagiforte® vaginal spray per dose were higher than the manufacturer's claims, although the dose contained in each oral capsule and/or vaginal tablet was slightly lower than the manufacturer's claim. Three lot numbers of Vagiforte® were compared to determine batch variation within this product. The oral capsules of one of the batch numbers of Vagiforte® (Lot #20531) failed to grow, despite being tested within the expiry date and stored after purchase as stipulated by the manufacturer (at room temperature, <25ºC). Limited growth of bacteria was also observed for Muvagyn®, despite testing numerous times and being within the products expiry date. Reid *et al.* (2001) recommended a minimum oral daily dose of $1x10^8$ probiotic bacteria to restore a *Lactobacillus*-dominated vaginal microbiota in women with BV. Based on this recommendation, most of the products tested (including Reuterina™ Femme®, Provacare™, Gynophilus®, and Vagiforte® PLUS Combo Pack vaginal spray and oral capsules) contained concentrations >$1x10^8$ CFU/dose, indicating that the administration of these products might confer a beneficial effect on the vaginal health of the consumer.

It is important to note that discrepancies between manufacturer claims and contents (such as the failure to culture *L. gasseri* or limited success with isolating *Bifidobacterium* spp.) in this evaluation were by no means definitive proof of their absence. Anaerobic bacteria are generally fastidious organisms (Zhou & Li 2015), the

evaluations performed in this Chapter were not blinded or independently confirmed by an outside laboratory, and no or limited information was available on the delivery chain or storage conditions once leaving the manufacturers premises to the stores from which they were purchased. However, it was encouraging to find that the laboratory analysis of probiotics for vaginal health available in South Africa and Europe generally showed that they contained the bacterial species and doses claimed by the manufacturer.

Provacare™ and Gynophilus® contained the identical *L. casei rhamnosus* Lcr35 strain, and the isolates from both products behaved very similar for all of the biological characteristics tested. This serves as internal quality control for the accuracy and reproducibility of the performed assays, and suggests that the biological characteristics that were evaluated were indeed strain specific, and were useful to discriminate strains of the same bacterial species biologically.

It was hypothesized that the probiotic strains should be able to tolerate a wide range of pHs, since the FGT reaches pHs of ≤ 4.5 due to lactic acid production. Strains that grew well may also produce higher concentrations of metabolic byproducts, like lactic acid or H_2O_2, which confer a beneficial effect to the host. In this Chapter, probiotic-derived *Lactobacillus* spp. generally were more permissive to a range of pHs than *Bifidobacterium* spp., in addition to having a greater impact on pH of the culture medium. Both *Lactobacillus* and *Bifidobacterium* strains generally grew better at pH 6.0 than at lower pHs. This is consistent with observations that *Lactobacillus* spp. grow best at a culture pH of 6.5 (Hossein Nezhad *et al*. 2010; Corcoran *et al*. 2005; Hong *et al*. 1999; Huang & Adams 2004; Silva *et al*. 2005). While there was little variation between *Lactobacillus* isolates of different lot numbers growing at pH 6.0, some variation was evident between different lot numbers at lower culture pHs. This indicates that some strains might be more tolerant to acidic conditions than others, as previously suggested (Succi *et al*. 2005; Hassanzadazar *et al*. 2012; Jin *et al*. 1998). Since the FGT is typically acidic during health, those *Lactobacillus* strains growing well at low pHs might be more suitable to be included in probiotic formulations for vaginal health. The survival of *Lactobacillus* spp. is increased in the presence of sugars and amino acids that they can metabolize (Corcoran *et al*. 2005; Zhang *et al*. 2012), suggesting that a co-formulation of acid-tolerant *Lactobacillus* strains with

suitable prebiotics might enhance the growth of the administered probiotic strains in the FGT.

The ability of probiotic *Lactobacillus* isolates to lower pH *in vitro* was comparable within species and between isolates of different lot numbers of the same product. All of the probiotic *Lactobacillus* isolates lowered the culture pH, with a median pH of 4.4 (± 0.2) being recorded. However, previous studies have shown that some vaginal *Lactobacillus* strains isolated from healthy women can lower the culture pH to <4.0 (Boskey *et al*. 1999; María Silvina Juárez Tomás *et al*. 2003; Tomás *et al*. 2005), suggesting that vaginal *Lactobacillus* strains might have a greater capacity to lower pH than those included in the probiotics currently available in South Africa.

Antibiotic resistance is a major threat to global health (reviewed by Tagliabue & Rappuoli 2018) and antibiotic resistance profiles of *Lactobacillus* spp., which represent a significant component of the microbiota at various body sites, are important to consider. All probiotic *Lactobacillus* isolates tested in this Chapter were resistant to metronidazole, which has been described previously for clinical isolates (Danielsen & Wind 2003; Delgado *et al*. 2005; Ocaña *et al*. 2006). The *L. rhamnosus* Lcr35 strains isolated from Provacare™ and Gynophilus® were also resistant to clindamycin, while all other *Lactobacillus* strains were susceptible. Others have shown that susceptibility to clindamycin is not species but highly strain specific, and that clindamycin resistance is rare among clinical *Lactobacillus* isolates (Danielsen & Wind 2003; Delgado *et al*. 2005; Ocaña *et al*. 2006). This finding has important implications for the administration of probiotics as adjunct therapy for BV treatment, where metronidazole and clindamycin are used for the SOC treatment of BV in South Africa and internationally. The majority of probiotic trials that have assessed the effect of adjunctive probiotic treatment on BV cure rates have administered the probiotic after the course of antibiotics has been completed (Appendix I). Clear thought therefore needs to be given to BV trials that intend to co-administer probiotics concurrently with antibiotics.

In this Chapter, probiotic isolates were variable in their sensitivity to penicillin and amoxicillin, but all were highly sensitive to rifampicin. Considering the frequent use of penicillin and amoxicillin in South Africa for other infections (such as respiratory

tract infections and pharyngitis), the question arises whether these antibiotics could pose a threat to the usefulness and impact the efficacy of probiotics for vaginal health. More concerning is the fact that rifampicin is administered for at least six months for individuals with TB (Nahid *et al*. 2016), and reaches brain tissue and serum concentrations of 0.29 μg/mL and 10.90 μg/mL, respectively, (Mindermann *et al*. 1998), which are higher than the MICs determined for the probiotic isolates evaluated in this study. The only tissue concentrations available for rifampicin were for the brain and serum, so it would be interesting to determine the concentrations of this antibiotic reaching the mucosa of the lower FGT. However, the long duration of rifampicin treatment and its high tissue penetrations suggests that rifampicin may reach concentrations in vaginal tissue that would inhibit the growth of vaginal *Lactobacillus* spp., which might not only affect the efficacy of probiotics for vaginal health but also vaginal *Lactobacillus* colonization itself for prolonged periods of time. On the other hand, including bacterial strains resistant to commonly used antibiotics into probiotic formulations in the era of increasing antibiotic resistance is not desirable. Together, these data suggest that good practice, considering these concerns, would be to consider sequential rather than co-administration of probiotics with antibiotics.

Several authors have argued that it is necessary for probiotic strains to adhere to the vaginal mucosa and epithelial cells for them to inhibit FGT pathogens, by exclusion and competition for adherence (Atassi *et al*. 2006; Coudeyras *et al*. 2008; Zarate & Nader-Macías 2006). In this study, all the probiotic isolates that were tested adhered to ectocervical Ca Ski cells *in vitro*, with *L. rhamnosus* strains adhering better than *L. acidophilus* and *L. reuteri* isolates. Previously, Coudeyras *et al.* (2008) showed that adhesion of *Lactobacillus* spp. to vaginal, ecto- or endocervical cells did not differ significantly. Furthermore, they found that the level of adhesion of *Lactobacillus* strains to these different cells were similar after one and three hours of incubation, suggesting that bacterial cells adhere rapidly after making contact with the epithelial cells, indicating that the *in vitro* system used in this study might give a good estimate regarding the adhesion of these *Lactobacillus* strain to different FGT cells. However, it needs to be acknowledged that these *in vitro* results not necessarily correlate with *in vivo* adhesion in the FGT, as bacterial glycoproteins and carbohydrates on the bacterial cell surface, glycolipid receptors on vaginal cells, which presumably are

competitively engaged by *Lactobacillus* strains to exclude pathogenic microbes, and vaginal mucus properties influences bacterial adhesion to FGT cells (Boris *et al*. 1998; Otero & Nader-Macías 2007).

Concentrations of L- and D-lactate produced by the probiotic *Lactobacillus* strains tested here appear to be slightly lower than those previously reported for vaginal *Lactobacillus* strains (Atassi *et al*. 2006; Witkin *et al*. 2013), but the ratio of L- to D-lactate was comparable (Witkin *et al*. 2013). However, *L. rhamnosus* and *L. reuteri* strains consistently produced more L- than D-lactate, which is important because L-lactic acid inhibits HIV more effectively than D-lactic acid (Aldunate *et al*. 2013), although D-lactic acid is more important for inhibiting bacterial pathogens (Valore *et al*. 2002; Graver & Wade 2011; Nardini *et al*. 2016) and has been shown to be important for HIV trapping in mucus (Nunn *et al.* 2015).

Earlier research on the role of vaginal *Lactobacillus* spp. in protecting the lower FGT argued that H_2O_2 was the major mechanism promoting lowering the FGT pH as well as being microbicidal (Hawes *et al*. 1996; Hillier *et al*. 1993; Cherpes *et al*. 2008). However, much of this earlier research was conducted under aerobic culture conditions where *Lactobacillus* spp. produce higher concentrations of this metabolite, unlike the anaerobic conditions typically found in the FGT. As such, H_2O_2 concentrations were measured under anaerobic conditions in this study, where concentrations of <6 μM were found. This goes along with previous findings that vaginal *Lactobacillus* strains do not produce H_2O_2 under hypoxic conditions (O'Hanlon *et al*. 2010; O'Hanlon *et al*. 2011; María Silvin Juárez Tomás *et al*. 2003; Shalev *et al*. 1996). Even under aerobic conditions *in vivo* H_2O_2 concentration reached by *Lactobacillus* spp. in vaginal fluid were <23 μM (O'Hanlon *et al*. 2010), similarly to what the probiotic isolates reached in this study *in vitro*. Such low H_2O_2 levels are not able to inactivate FGT pathogens *in vitro* (O'Hanlon *et al*. 2010; O'Hanlon *et al*. 2011), confirming the implausible *in vivo* role of H_2O_2 as an antimicrobial factor produced by vaginal microbiota (Tachedjian *et al.* 2018). However, Tomás *et al*. (2005) showed that H_2O_2 and lactic acid production correlate well, suggesting that H_2O_2 might be a good indicator for the metabolic capacity of probiotics.

Conditioned media from the probiotic *Lactobacillus* isolates strongly inhibited the growth of all of the *P. bivia* and GBS isolates tested, which is promising as *P. bivia* is one of the organisms that it commonly associated with BV (Jacques Ravel *et al*. 2011; Lennard *et al*. 2017), and GBS causes a variety of adverse pregnancy outcomes (Shabayek & Spellerberg 2018; Martinez *et al*. 2000). In contrast, the inhibitory effect of *Lactobacillus* conditioned media on the growth of *G. vaginalis* strains was more variable and not as strong as against the *P. bivia* and GBS isolates. This might possibly be due to the differing abilities of these pathogens to tolerate acidity. Like *Lactobacillus* spp., both *G. vaginalis* and *P. bivia* are also able to acidify culture medium but to a much more moderate extent (pH 4.7-5.9*;* Boskey *et al*. 1999). Thus, the ability of *Lactobacillus* spp. to inhibit *G. vaginalis* and *P. bivia* might be due to the pH <4.5 achieved in the *Lactobacillus*-conditioned media. Previous studies have shown that *G. vaginalis* grows best at neutral pH (~pH 7), and *G. vaginalis* growth is severely compromised at pHs lower than 5.5 (Gottschick *et al*. 2016). Similarly, lowering the culture pH leads to a decreased adhesion of GBS strains to human vaginal and cervical cells *ex vivo* (Park *et al*. 2012) and increased biofilm formation (D'Urzo *et al*. 2014), suggesting that GBS does not tolerate low pH well. Additionally, the bacteriocidal and virucidal activity of lactic acid is thought to be dependent on pH, needing low pHs to be highly active (Aldunate *et al*. 2013; Gong *et al*. 2014). Together, this suggests that *Lactobacillus* strains with a better capacity to acidify their environment also have a better capacity to exert antimicrobial effects. Mechanism of inhibition are discussed in more detail in Chapter 5, but the precise metabolites or properties of *Lactobacillus*-conditioned media that conferred pathogen inhibition still need to be identified. Culture pH, lactate and H_2O_2 production were measured in this study, although it is also likely that other secreted substances, including bacteriocins (Holo *et al*. 1991; Flynn *et al*. 2002; Turovsky *et al*. 2009) and biosurfactants (Velraeds *et al*. 1998; Heinemann *et al*. 2000; Gudina *et al*. 2010), may also contribute to the ability of *Lactobacillus* spp. to inhibit FGT pathogens like *G. vaginalis, P. bivia* and GBS.

This study had some limitations worth noting. The tolerance of probiotic strains to low pH was determined by evaluating the growth in MRS adjusted to different pHs using HCl. However, it needs to be acknowledged that the tolerance observed is not to true environmental conditions in which low pH is driven by lactic acid not HCl. Only

one batch of each of the European probiotics was purchased, and due to discontinuation of Provacare™ no additional lots of this particular South African product could be purchased for this study. No genotypic analyses were performed on the various probiotic isolates, and therefore it was not possible to confirm that the different lot numbers of each probiotic product contained genetically identical strains. Reasons given by the South African distributor of Provacare™ for withdrawing this product from South Africa were poor sales and poor product uptake (personnel communication). Given the disproportionate HIV risk in young women in sub-Saharan Africa and the extremely high burden of recurrent BV, there is an urgent need for improved treatment of BV, including evaluating the effect of probiotics on BV cure and recurrence. One of the factors that may have contributed to Provacare™ selling poorly in South Africa was the cost of one treatment course for an individual, although this is a generalized factor since Provacare™ was not priced differently from Vagiforte® or Reuterina™ Femme®. South Africa is one of the countries in the world with the highest discrepancy between the rich and poor, with 27.2% of the population unemployed (53.7% of the youth), with an average monthly income of ~ZAR4000 (USD275) per month for low skilled workers compared to the average national wage being ~ZAR20000 (USD1400) per month[13]. As was reported in Chapter 2, the vaginal probiotics available OTC in South Africa equal approximately 1.6-4.9% of monthly income. This further emphasizes the need for affordable low cost probiotics, which are more easily accessible for the youth in the broader South African population.

Taking the information regarding probiotic ingredients (content), route of administration, recommended duration of treatment, and *in vitro* laboratory evaluations into consideration, Vagiforte® PLUS Combo Pack (and the *Lactobacillus* strains contained therein) appear to be the best locally licenced choice available for vaginal health. Some of the benefits of Vagiforte® PLUS included that it was manufactured in South Africa, making it easier to start exploring and testing the regulatory processes in South Africa, and it can easily be purchased by South African women if a clinical trial shows a promising outcome. Further, it remains the only product available in South Africa that is intended for both oral and vaginal administration, and for a prolonged period (treatment duration of 15 days). It was

[13] Census 2011, http://www.statssa.gov.za [02 January 2018]

hypothesized that targeting both topical and oral routes of administration and prolonged treatment duration would increase the chances of successful vaginal colonization, and therefore treatment effectiveness. Vagiforte® PLUS Combo Pack contained a bacterial load of $2x10^9$ CFU per dose, which is 20-fold higher than the recommended dose ($1x10^8$ CFU) of oral probiotic bacteria to successfully restore a *Lactobacillus*-dominant vaginal flora (Reid *et al*. 2001). Vagiforte® PLUS Combo Pack additionally contains two different *Lactobacillus* spp. (*L. rhamnosus* GG and *L. acidophilus*) as well as *B. bifidum* and *B. longum*, increasing the odds of conferring health benefits, as these different species might act jointly in the production of metabolites that lower the vaginal pH and have other beneficial antimicrobial activities. Vagiforte® PLUS *L. rhamnosus* and *L. acidophilus* strains grew well at various pHs, lowered the pH to <4.5, adhered to CaSki cells, produced high concentrations of L-lactate and moderate concentrations of D-lactate and H_2O_2, and inhibited a wide panel of clinical *G. vaginalis*, *P. bivia* and GBS isolates, thus showing the potential to improve vaginal health.

Although Vagiforte® PLUS Combo Pack was selected for further clinical testing for BV treatment in South African women, there were some important concerns about selecting this product that need to be acknowledged. Of the three lot numbers of Vagiforte® tested in this Chapter, no bacteria from one of the lots (#20531) of the oral capsules could be cultured *in vitro*. Furthermore, the concentration of probiotics contained in oral capsules of lot number 21134 were >40-fold lower than claimed by the manufacturer, and the vaginal spray from the same lot number yielded concentrations up to 16-fold higher than the manufacturer's claimed dose, casting some doubt on the quality control of this particular product. Prior to initiating a human trial with any of these probiotic products, it is therefore important to include batch testing. If the Vagiforte® PLUS Combo Pack is found to contain the bacterial load as stated, it could be a promising product as adjunct to conventional BV treatment based on its preferred product profile.

Conclusion

Products marketed in South Africa mainly contained the bacterial species and dose as claimed by the manufacturer and the bacterial isolates had promising probiotic characteristics, but there was some variability in the biological characteristics of

isolates from different lot numbers of the same products. However, there are few probiotics for vaginal health on the South African market that contain bacterial species that are considered healthy commensals in the human FGT. Thus, the development of a product containing FGT commensals should be considered. Further, it is important to assess the efficacy of currently available OTC probiotic products in treating BV and improving vaginal health of South African women. Due to the local availability, route of administration and treatment length, and performance of probiotic *Lactobacillus* isolates in *in vitro* testing of important probiotic characteristics, Vagiforte® PLUS Combo Pack was selected for clinical trial testing in BV positive South African women.

Chapter 4

Pilot clinical trial to evaluate the efficacy of a commercial probiotic for the treatment of BV in South African women

4. Preliminary results on the effect of adjunctive probiotics on BV cure in South African women enrolled in a small pilot trial

4.1 Introduction

BV treatment with antibiotics like metronidazole and clindamycin is effective in the short-term, although BV recurrence rates within six to twelve months are high (Barrons & Tassone 2008; Bradshaw *et al*. 2006). This may be related to the fact that BV is a polymicrobial dysbiosis, caused by many different pathobionts that may be differentially susceptible to antimicrobial therapy. The persistence of BV-associated biofilms, vaginal re-colonization by non-*Lactobacillus* organisms that have reservoirs in the GI tract, or re-colonization from sexual partners likely contribute to the inability of women to restore healthy *Lactobacillus*-dominated vaginal microbiomes and the high rates of recurrence (Beigi *et al*. 2004; Nyirjesy *et al*. 2006; Cribby *et al*. 2008). There is also some evidence that organisms associated with BV, like *G. vaginalis*, commonly develop resistance to metronidazole and/or clindamycin (Austin *et al*. 2005; Simoes *et al*. 2001; Anahtar *et al*. 2015; Zaga-Clavellina *et al*. 2012). There is thus an urgent need to rethink our options to treat BV.

There is clinical equipoise as to whether adjunctive probiotics (in combination with antibiotics) improve BV cure and recurrence rates. The latest Cochrane review states that adjunctive probiotics may improve BV cure rates, although there is currently not enough high-quality clinical evidence to support this as superior to antibiotic treatment alone[13]. RCTs have been performed in several African countries (including Nigeria and Tanzania), as well as European, Asian, and South America, and the majority have shown some beneficial effects (summarized in Appendix Table I). However, it is interesting to note that the effect size of probiotics on BV cure is considerably smaller when the effect of probiotics administered as adjunct to metronidazole is compared to placebo plus metronidazole (OR 0.09, 95% CI 0.03-0.26), than when comparing probiotics to placebo without antibiotic treatment (OR 1.21, 95% CI 0.67-2.19)[2]. In South Africa, no randomized trial assessing the effects of adjunctive probiotics on BV cure and recurrence has yet been performed. As the composition of the FGT is likely to be dependent on several factors, including geographic location, race and/or host genetics (Anahtar *et al*. 2015; Ravel *et al*. 2011;

[13] https://www.cochranelibrary.com/cdsr/doi/10.1002/14651858.CD006289.pub2/media/CDSR/CD006289/CD006289.pdf [13 Aug 2018]

Gajer *et al*. 2012; Lennard *et al*. 2017), it is important for a number of reasons to conduct a trial including South African women to evaluate adjunctive probiotics in BV treatment under local conditions in local populations.

Currently, registration of medicines, probiotics and other health supplements and their use in clinical trials in South Africa is regulated by SAHPRA. Before initiating this trial, neither a probiotic had been registered with SAHPRA, nor had SAHPRA approved or acknowledged a trial administering probiotics to South Africans. To test the regulatory environment in South Africa around conducting a clinical trial with probiotics for vaginal health and lay the path for future clinical studies on next generation vaginal probiotics being developed locally and internationally, the aim of this Chapter was to conduct a pilot clinical trial. This trial intended to enrol 50 BV positive (Nugent 7-10) but STI negative (including discharge-causing STIs, such as *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis* and *M. genitalium;* and HIV) South African women in a single-blinded, randomized trial comparing vaginal metronidazole treatment alone to a combination of metronidazole and a locally manufactured and distributed probiotic, Vagiforte® PLUS Combo Pack (characterized in Chapters 2 and 3). With the overall aim of testing the South African regulatory environment, it was desirable to use an already marketed product that was available OTC for this pilot probiotic trial. Of the four probiotic products for vaginal health evaluated in this study, Vagiforte® PLUS Combo Pack was selected based on its local production and availability, route of administration and treatment length, and performance of probiotic *Lactobacillus* isolates in *in vitro* testing of important probiotic characteristics (Chapter 3). Vagiforte® PLUS contains *L. rhamnosus*, *L. acidophilus*, *B. bifidum* and *B. longum,* bacterial species that are not commonly found in the lower FGT of South African women (Lennard *et al*. 2017), although none of the other products evaluated did either. The primary endpoint of this trial was BV cure (Nugent 0-3) at four weeks after the last administration of the probiotic. Secondary endpoints included: (1) the durability of any effect, focusing on microbiological cure (Nugent score) and vaginal symptoms three months after treatment; (2) lowering of vaginal inflammatory cytokines (using IL-1α as marker); (3) increase in *Lactobacillus* spp. and decrease in BV-associated bacteria abundances in the lower FGT; and evidence of the presence of the administered probiotic bacteria in the FGT. As of November 2018, screening and enrolment for the trial are still on-going (with 24

women having reached the primary end-point of the study), thus this Chapter presents an interim analysis of these first participants. Self-assessed improvement of vaginal symptoms, vaginal pH, changes in the composition of vaginal bacterial (*Lactobacillus* and BV-associated spp.), *Candida* communities, and genital inflammation assessed by vaginal IL-1α concentrations are reported in this Chapter.

4.2 Methods

4.2.1 Ethics statement and regulatory application approval process

Prior to starting the trial, ethics approval was obtained from UCT Human Research Ethics Committee (HREC Ref #706/2016), the National Health Research Ethics Council (NHREC Ref #4579), the City of Cape Town (relating to recruitment from the provincial Spencer Road clinic; Ref #7841) and from the UCT Department of Student Affairs (to recruit UCT students; DSA #100). In addition, SAHPRA was notified (SAHRPA Ref #20161201), and the trial registered with the South African National Clinical Trial Register of the Department of Health (DOH-27-1117-5579). All participants were given information through posters and leaflets to inform those interested of the details of this trial. After being given detailed information by the study coordinator and/or nurse, all women gave written informed consent at screening. The trial was conducted in accordance with the Declaration of Helsinki and current standards of good clinical practice (GCP), with regular auditing and bi-annual progress reports, in accordance with SAHPRA requirements

4.2.2 Details of the cohort and clinical trial design

Participants were recruited from two different wellness centres in Cape Town, South Africa: (1) the public sector "Spencer Road Clinic" in Salt River, which is a STI centre embedded within a general provincial wellness clinic; and (2) the UCT Student Wellness Centre, which is accessible for UCT students only and offers general care as well as syndromic STI management.

4.2.2.1 Eligibility criteria

To be eligible for enrolment, women had to be between the ages of 18-45 years, seeking care for an abnormal vaginal discharge although being in good general health otherwise. Exclusion criteria were being pregnant, breastfeeding, having pelvic inflammatory disease, a current STI (including *C. trachomatis, N. gonorrhoea, T. vaginalis, M. genitalium*) or HIV infection, serious medical conditions, known allergy to metronidazole, concomitant use of other antibiotics, and the use of natural remedies in the urogenital area. In addition, to be eligible, they should not be menstruating at the time of sampling or had unprotected sex or douched in the 48 hours prior to sampling. Only those who were BV positive (BV+, Nugent 7-10) but STI negative

(STI-) at the screening visit were enrolled. Those considered eligible for enrolment were asked to attend four study visits over a period of seven months (baseline, one, three and five months after treatment completion). Participants who acquired an STI or had recurrent BV over the course of the trial were provided with a referral letter but not excluded from the study. Any concomitant medication (including antibiotics) taken during the trial was recorded.

4.2.2.2 Testing for BV, STIs, HIV and pregnancy at screening

Participants providing genital samples were advised for 48 hours prior to sampling not to use anything with spermicide, insert anything into the vagina, which includes but is not limited to abstaining from protected or unprotected vaginal sex and douche. All participants were tested for HIV and pregnancy at the screening visit, in addition to being tested for BV (by Nugent Scoring) and discharge-causing STIs (*C. trachomatis*, *N. gonorrhoea*, *T. vaginalis*, *M. genitalium*, by Multiplex-PCR). HIV status was confirmed using the rapid Anti-HIV (1&2) test (InTec products, Inc., China). Pregnancy testing was done using the hCG Pregnancy test (Homemed^{TM}, South Africa), using midstream urine. A trained research nurse further collected a posterior fornix and lateral vaginal wall swab (Dryswabs^{TM}, Medical Wire and Equipment, UK) to diagnose BV. The swab was smeared onto a microscopic glass slide, which was air dried at the clinic and transported to the laboratory at room temperature where it was heat-fixed. It was shipped to the National Health Laboratory Service (NHLS) laboratory in Durban, South Africa, for Gram staining and Nugent scoring (performed by Nireshni Mitchev, Department of Medical Microbiology, Durban, South Africa). Slides were assessed microscopically and assigned a score between 0 and 10, with a score of 0-3 considered BV negative (BV-), 4-6 intermediate microbiota, and 7-10 BV+. The same swab was rolled onto a colour-fixed indicator strip to measure vaginal pH (pH range: 3.6-8.2; Macherey-Nagel, Germany). The pH was recorded on the participant data sheet at the time of sampling at the clinic. Further, another dacron swab (Dryswabs^{TM}, Medical Wire and Equipment, UK) was used to swab the vulva, the vaginal introitus and the lower vaginal wall to test for common discharge-causing STIs (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* and *Mycoplasma genitalium*) using an in house multiplex PCR, validated by the NHLS, using a Thermofisher platform and probes (performed by Dr Ravesh Singh, Department of Medical Microbiology,

Durban, South Africa). After sampling the vulvo-vaginal area, the swab was immediately placed back into its container and transported to the lab, where it was stored at -80 °C until being shipped to the NICD weekly. STI panels were obtained from Thermo Fisher Scientific existing Woman's Health panel, and the qPCR was performed as per manufacturers recommendation. As positive controls, genomic DNA extracts prepared from the following ATCC® strains were included: *N. gonorrhoeae* (ATCC® 700825), *C. trachomatis* (ATCC® VR-885), *T. vaginalis* (ATCC® 30001) and *M. genitalium* (ATCC® 33530).

4.2.2.3 Randomization and dosing regime

As part of this SAHPRA acknowledged clinical trial, randomisation, enrolment and follow-up took place at the UCT Clinical Research Centre (CRC; http://www.crc.uct.ac.za), a state of the art facility developed by UCT for clinical research, which provided both administrative, regulatory, and monitoring support, in addition to providing the clinical facilities to conduct this trial. The pharmacy team at the CRC dispensed all investigational products, and a trained study nurse collected FGT samples and administered questionnaires. Eligible participants were randomised by pharmacists at the UCT CRC (using a randomisation generator) to either receive topical metronidazole only (0.75% gel, 5 g vaginally, once a day for 5 days; MetroGel™ V, iNova Pharmaceuticals, South Africa, package insert Appendix III; considered the standard of care or control group; n=20) or topical metronidazole and probiotic in combination (Vagiforte® PLUS Combo Pack; intervention group; n=30) at baseline (enrolment visit). The randomisation list was kept confidential at the CRC pharmacy. Participants in the intervention arm received MetroGel™ V for five days followed by a 15-day treatment course of Vagiforte® PLUS Combo Pack (Bioflora, South Africa, Lot # 21918; expiry date 03/2019); including five days of oral probiotic capsules followed by ten days of oral capsules together with twice daily vaginal spray. Vagiforte® PLUS Combo Pack is scheduled as a nutritional supplement (Act 54/1972) in South Africa, and is indicated for bacterial vaginosis and vaginal thrush induced by antibiotic treatment, or the excessive use of strong soaps, disinfectants and deodorants (according to the package insert, Appendix IV). Each Combo Pack contains 15 oral capsules and a vaginal spray with 30-metered dosages per spray bottle. As described in Chapters 2 and 3, the Vagiforte® PLUS oral capsules were reported by the manufacturer to contain 25.8 mg of lyophilized *L. acidophilus*, *L.*

rhamnosus GG, *B. bifidum* and *B. longum*, yielding ≥ $2x10^9$ CFU per dose unit. Each metered dose of the probiotic spray releases a volume of 250 µL, containing the same bacterial species and concentration per dose unit as the capsule. The contents and dose of Vagiforte® PLUS Combo Pack Lot #21918 was confirmed prior to initiation of the trial (section 4.2.3), and all women in the intervention arm received the same Lot number.

4.2.2.4 Collection of socio-bevioural questionnaire and study medical diary data

At enrolment, all participants completed a questionnaire on demographics (age, ethnicity and marital status), their reproductive health (menstrual history, vaginal hygiene practices, pregnancy history, contraceptive use and vaginal discharge), and sexual behaviour (recent sexual intercourse, condom usage, number of lifetime partners and risk factors). A sample of the questionnaire is included in Appendix V. At follow up visits, participants completed a questionnaire assessing their experience of using the administered products, and any self-reported changes in symptomatic vaginal discharge during the course of the study (sample questionnaire provided in Appendix VI). At the last visit, a final questionnaire assessed the use and acceptability of the products (Appendix VII). One, three and five months after treatment, participants returned their study medication diary and reported any AEs that occurred, and completed a questionnaire (Appendices V-VII). Telephone follow-up visits were conducted between study visits to review any AEs, and to remind participants about their next study visit (**Table 4.1**).

4.2.2.5 Collection of samples from the lower FGT

At enrolment and follow up visits, the nurse collected the following genital tract samples from each participant in the following order, under speculum examination: (1) a vulvo-vaginal swab for STI testing (section 4.2.2.2); (2) a posterior fornix and lateral wall swab to screen for BV (Nugent Scoring) and to measure vaginal pH (section 4.2.2.2), (3) a lateral wall swab for measurement of genital tract cytokines (section 4.2.4); (4) a lateral wall swab for STI and *Candida* screen (section 4.2.5); and (5) one lateral wall and one ectocervical swab for future studies (**Table 4.1**).

Table 4.1. Details of study visits and samples taken

Visit	Screen	Enrol	Follow-up [Months after treatment]					
			Tel*	1	Tel*	3	Tel*	5
Administrative procedures								
Informed consent	✔							
Inclusion/Exclusion criteria	✔							
Participant identification card	✔							
Medical history	✔							
Treatment allocation		✔						
Review study medication diary		✔		✔		✔		✔
Clinical Safety Evaluations								
Weight		✔						
Height		✔						
Vital Signs		✔						
Pregnancy test	✔							
HIV test	✔							
Review adverse events		✔	✔	✔	✔	✔	✔	✔
Patient reported outcomes								
Questionnaire		✔		✔		✔		✔
Female genital tract sampling								
Vulvo-vaginal swab (for STI testing)	✔	✔		✔		✔		✔
Lateral vaginal wall swab (for cytokine measures)		✔		✔		✔		✔
Lateral vaginal wall swab (for future studies)		✔		✔		✔		✔
Ectocervical swab (for future studies)		✔		✔		✔		✔
Lateral vaginal wall swab (for qPCR)		✔		✔		✔		✔
Lateral vaginal wall/posterior fornix swab (for Nugent Score and pH testing)	✔	✔		✔		✔		✔

*Telephone visit

4.2.3 Laboratory analysis of Vagiforte® PLUS Combo Pack Lot #21918

To ensure that the Vagiforte® PLUS Combo Pack Lot number used for the trial contained the bacterial species and doses as claimed by the manufacturer, three individual boxes of Lot #21918 were tested prior to the study starting, using the same approaches as described in Chapter 3 (section 3.2.2 and 3.2.3). All contained *L. acidophilus*, *L. rhamnosus*, *B. bifidum* and *B. longum* (determined by MALDI-TOF, Chapter 3 section 3.2.3), at a similar dose as stated on the package leaflet (**Figure 4.1**), confirming lot and dose conformity for this lot of Vagiforte® PLUS Combo Pack.

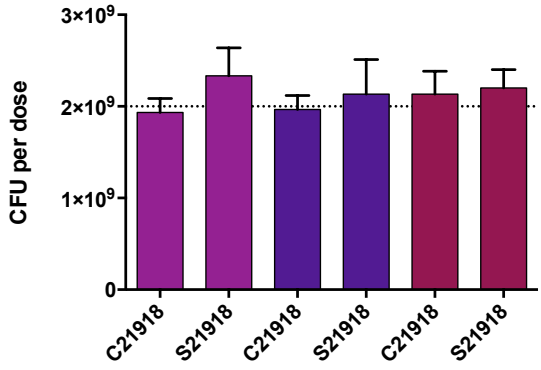


Figure 4.1. Bacterial concentration per dose unit of Vagiforte® PLUS Combo Pack. The CFU per dose of three products (pink, purple and red) of Vagiforte® Plus Combo Pack lot #21918 containing oral calsules (C) and vaginal spray (S) was determined using serial dilutions and compared to the manufacturers claim of $2x10^9$ CFU per dose (dotted line).

4.2.4 Measurement of genital IL-1α concentrations

A dacron swab (Dryswabs^{TM}, Medical Wire and Equipment, UK) was used to swab the lateral vaginal wall under speculum examination, placed into 1 mL of PBS in a 2 mL cryotube (Sigma-Aldrich®, USA), transported to the laboratory at 4 °C, and stored at -80 °C. An IL-1α ELISA kit was used to measure cytokine concentrations in genital specimens (E-EL-H008, Elabscience®, USA; detection range 125.0-1.25 pg/mL). All samples were thawed on ice and filtered by centrifuging at 1950g (Microcentrifuge, Labnet Inc., USA) for 10 minutes at 4°C in SPIN-X® 0.2μM cellulose acetate filters (Sigma-Aldrich®, USA) to exclude mucus and debris prior to performing the ELISA. Genital samples were randomly allocated on the plate, and the ELISA was conducted according to the manufacturer's instructions. Briefly, 100 μL of sample was added per well and incubated for 90 min at 37°C. The liquid was removed and 100 μL of biotinylated detection antibody was added. The plate was incubated for one hour at 37°C, the solution was aspirated and washed three times with washing buffer (provided with the kit). The horseradish peroxidase (HRP) conjugate (100 μL) was added, and the plate was incubated for 30 min at 37°C. The solution was aspirated and the plate was washed five times using washing buffer (provided with the kit). The substrate reagent (90 μL) was added, and the plate was incubated for 15 min at 37°C, before addition of 50 μL of stop solution (provided with the kit). The absorbance (OD_{450nm}) was read immediately and concentrations were calculated based on the standard curve, using standards provided with the kit. The lower detection limit for this kit was 1.25 pg/mL, and all values below the detection limit were recorded as half of the lowest concentration measured.

4.2.5 Quantitative PCR to determine vaginal bacterial and fungal concentrations

A FLOQSwab™ was used to swab the lateral vaginal wall under speculum examination, and placed into 1 mL of liquid Amies medium (both are part of the ESwab™ Liquid Amies Collection and Transport System, Copan Diagnostics Inc., USA). Upon receipt in the laboratory, the tube was vortexed, and 300 μL were transferred to an Eppendorf tube that was stored at – 80 °C. DNA was extracted and the Thermo Fisher qPCR platform (described in section 4.2.2.2) was used to quantify common vaginal *Lactobacillus* spp., including *L. crispatus*, *L. jensenii*, *L. iners*, *L. gasseri*, *L. vaginalis, L. mucosae,* and bacterial species contained in Vagiforte®,

including *L. acidophilus, L. rhamnosus*, *B. bifidum* and *B. longum.* In addition, the BV-associated spp. *G. vaginalis*, *P. bivia*, BVAB2, *Megasphaera* 1 and *A. vaginae*, as well as the *Candida* spp. *C. albicans, C. dubliniensis, C. glabrata, C. krusei, C. lusitaniae, C. parapsilosis,* and *C. tropicalis* were quantified using Applied Biosystems™ TaqMan® assays. A Quant Studio 5 Real-Time PCR Detection System (Thermo Fisher Scientific, USA) was used to quantify each of the targets. The quantification was performed using amplicons generated from an ATCC® control strain for each of the targets (TaqMan™ Vaginal Microbiota Extraction Control; cat no. A32039). A serial dilution from each control strain was used to determine the accurate quantification from the patient samples for all targets. Serial dilutions ranged from $1x10^9$ to $10x10^9$ molecules per mL of sample.

4.2.6 Statistical Analysis

GraphPad Prism6® (GraphPad Software, USA) and STATA version 11.0 (StataCorp, USA) were used for statistical analyses, and R version 3.5.0 was used to generate a heatmap. The non-parametric Mann-Whitney U test was used to compare groups of continuous variables, and for three or more groups, the Kruskal-Wallis one-way analysis of variance was used. Non-parametric Wilcoxon matched-pairs signed rank tests were used to compare paired data (pre- and post-treatmet) within treatment groups. 95% confidence intervals and p values <0.05 were used to assess statistical significance.

4.3 Results

4.3.1 Navigating the regulatory environment in South Africa around probiotic trials

Before conducting this trial, SAHPRA had not registered a probiotic product. Thus, discussions with SAHPRA authorities were initiated in March 2016, and key areas that needed addressing were identified. These included a decision by SAHPRA on whether a full application was necessary or a notification sufficient. Typically, SAHPRA would need to consider a full application if the product or medicine was not registered in South Africa already, if it was not used for its registered indication/dose/formulation, or if it was not sourced in South Africa[14]. Generally, SAHPRA will consider notification studies when the intention is to undertake a phase IV clinical study of an approved medication within its approved dosage, formulation and indication[14]. In October 2016, SAHPRA decided that an application for a notification (containing the following documents: CVs and declarations of investigators, protocol, informed consent forms, UCT HREC approval letter, NHREC registration, participant materials and questionnaires, participant insurance certificate, auditing monitor's declaration, and package inserts of administered products) for an UCT-sponsored, investigator-driven trial would be sufficient, with the understanding that the probiotic product was not a medicine but a health supplement, and currently already available OTC in South Africa. As such, SAHPRA required bi-annual progress and monitoring report submissions. After this decision from SAHPRA, the notification application was submitted on 21 November 2016 and granted on 8 December 2016 to conduct this single-blind trial, after which the first participant was screened.

4.3.2 Screening and enrolment considerations

A total of 226 women showed interest in this pilot trial (**Figure 4.2**). The majority (215/226; 95.1%) were from the UCT Wellness Centre. After explaining more about the study and pre-screening potential participants, 149/226 (65.9%) women were either no longer interested in participating, did not have a vaginal discharge (n=29), had a known STI or were HIV positive (n=2), were less than 18 years of age (n=1),

[14] https://www.sahpra.org.za/documents/ff4ecbe86.24_Notification_template_Phase_IV_Apr17_v1.pdf [03 November 2018]

had recently been treated for BV (n=3); or could not commit to the length of the study (n=6). The remaining 77/226 women were further screened for BV (by Nugent scoring) and discharge-causing STIs (including *C. trachomatis*, *N. gonorrhoea*, *T. vaginalis*, *M. genitalium* and HIV). The majority of those screened for eligibility were UCT students, with a median age of 22 years (IQR 20 – 26 years). The majority self-identified as black (68.8%; 53/77), 13/77 as mixed race (16.9%) and 11/77 as white (14.3%). Of these 77, one woman tested positive for HIV and thus, screening was aborted.

Despite seeking care for vaginal discharge, only about half (37/76, 48.7%) were BV+ (Nugent 7-10). However, the prevalence of STIs at the screening visit was high: 21.1% had an STI (16/76), with *T. vaginalis* (9/76, 11.8 %) and *C. trachomatis* (7/76, 9.2 %) being the most common. This left a total of 27 women with vaginal discharge who were BV+ but STI- eligible to enrol. A further three eligible women were not enrolled as one opted to take medication to treat her BV before attending the enrolment visit, one moved away from Cape Town, and one was not available anymore (could not be contacted). This resulted in 24 women being enrolled and randomised (**Figure 4.2**). Of the remaining non-eligible women, 10/76 (13.2%) were BV+/STI+, 6/76 were BV-/STI+ (7.9%), and 33/76 (43.4%) BV-/STI-.

These results confirm that symptomatic vaginal discharge is a poor predictor for either BV and/or STIs, with positive predictive values (PPV) of 41.5% for BV+/STI-, 15.2% for STI+/BV+ and 5.8% for BV-/STI+ women. The PPV of symptomatic vaginal discharge for BV was 64.9%, if one did not take into account their STI statuses, suggesting that only 65% of these young women that were seeking health care for vaginal discharge had BV. Furthermore, the high rate of screen-failures in the pilot trial raises concerns about the feasibility of excluding those that had concurrent STIs.

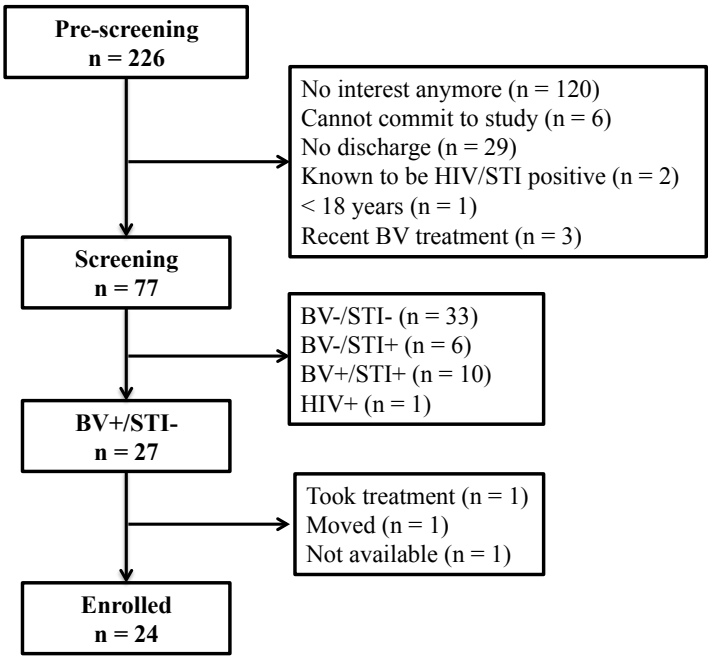


Figure 4.2. Overview of cohort. Participants were pre-screened (criteria: 18-45 years old, abnormal vaginal discharge, not being pregnant or breastfeeding, absence of pelvic inflammatory disease, not known to have an STI and HIV, absence of other serious medical conditions, no allergy to metronidazole, not using natural medication in the urogenital area) and the trial was explained in detail before the screening visit (HIV and preganancy test, medical history, BV and STI testing). BV+/STI- participants were enrolled.

In the final randomized group (n=24), ten women were randomised to the SOC and 14 women to the intervention arm. The majority of young women were single and self-identified as black South-Africans (16/24; **Table 4.2**). They had a median age of 22 years (IQR 20-28), with a median age of menarche of 14 (IQR 12-15). Almost all participants reported having had sex in the past six months, and the majority was not in monogamous relationships. Some participants used some sort of hormonal contraceptive (7/24; n=3 combined oral contraceptive, n=2 progesterone-only injectable [Net-EN], n=1 implant), while 20/24 reported using condoms and/or the withdrawal method of contraception. The majority of participants reported regular condom use during the past six months, and about 60% reporting use of a condom at their last sex act. Despite good condom use, one-third (8/24) of these young women had been pregnant before (**Table 4.2**).

Participants reported a median age at sexual debut of 17 years (IQR 16-18), and a median of five sexual partners in their lifetimes (IQR 4-8). Reporting of oral sex was common (15/24), while reporting of anal sex was less common (1/24). Very few participants reported having been previously diagnosed or treated for STIs (**Table 4.2).** About half reported that they were currently smoking, a factor that has been previously associated with the risk of BV (Hellberg *et al*. 2000; Brotman *et al*. 2014; Cherpes *et al*. 2008). No statistical differences of these baseline socio-behavioural

characteristics of enrolled participants were noted between treatment groups, besides age of menarche, which was higher in the intervention group (**Table 4.2**).

Table 4.2. Socio-behavioural characteristics of enrolled participants

Characteristic	All [n=24]	SOC [n=10]	Intervention [n=14]	p-value*
Age participant [years, median (IQR)]	22 (20-28)	24 (22-37)	21 (20-27)	0.1804
Race [% (n/N)]				
Black	66.7 (16/24)	60.0 (6/10)	71.4 (10/14)	0.6734
Coloured	16.7 (4/24)	20.0 (2/10)	14.3 (2/14)	>0.9999
White	16.7 (4/24)	20.0 (2/10)	14.3 (2/14)	>0.9999
Age menarche [years, median (IQR)]	14 (12-15)	12 (11-14)	15 (13-16)	**0.0121**
Marital Status [% (n/N)]				
Single	83.3 (20/24)	80.0 (8/10)	85.7 (12/14)	>0.9999
Married	4.2 (1/24)	10.0 (1/10)	0 (0/14)	0.4167
Separated/Divorced	12.5 (3/24)	10.0 (1/10)	14.3 (2/14)	>0.9999
Widowed	0 (0/24)	0 (0/10)	0 (0/14)	>0.9999
Pregnancies and contraception [% (n/N)]				
Ever been pregnant	33.3 (8/24)	30.0 (3/10)	35.7 (5/14)	>0.9999
Currently using hormonal contraception	29.2 (7/24)	20.0 (2/10)	35.7 (5/14)	0.6529
Reported current smoking [% (n/N)]	45.8 (11/24)	50.0 (5/10)	42.9 (6/14)	>0.9999
Sexual risk behaviour				
Age of sexual debut [median (IQR)]	17 (16-18)	17 (17-20)	17 (16-19)	0.2964
Number of lifetime partners [median (IQR)]	5 (4-8)	6 (3-10)	5 (4-8)	0.7178
Number sex partners during last 6 months [median (IQR)]	1 (1-2)	1 (1-2)	1 (1-2)	>0.9999
Vaginal sex during last 6 months [% (n/N)]	91.7 (22/24)	90.0 (9/10)	92.9 (13/14)	>0.9999
Anal sex during last 6 months [% (n/N)]	4.2 (1/24)	0 (0/10)	7.1 (1/14)	>0.9999
Oral sex during last 6 months [% (n/N)]	62.5 (15/24)	50.0 (5/10)	71.4 (10/14)	0.4028
Monogamous relationships [% (n/N)]	16.7 (4/24)	20.0 (2/10)	14.3 (2/14)	>0.9999
Reported condom use [% (n/N)]				
Always	29.1 (7/24)	30.0 (3/10)	28.6 (4/14)	>0.9999
Most of the time	37.5 (9/24)	40.0 (4/10)	35.7 (5/14)	>0.9999
Occasionally	4.2 (1/24)	0 (0/10)	7.1 (1/14)	>0.9999
Never	16.7 (4/24)	20.0 (2/10)	14.3 (2/14)	>0.9999
Regular condom use during last 6 months	62.5 (15/24)	70.0 (7/10)	57.1 (8/14)	0.6785
Condom use at last sexual act	54.2 (13/24)	70.0 (7/10)	42.9 (6/14)	0.2397
STI history [% (n/N)]				
Ever been diagnosed or treated for a STI	12.5 (3/24)	10.0 (1/10)	14.3 (2/14)	>0.9999
Partner or participant STI in last 6 month	8.3 (2/24)	0 (0/10)	14.3 (2/14)	0.4928

*Mann-Whitney test comparing SOC and intervention groups

For the majority of young women who were enrolled, vaginal discharge was reported to be a long-term problem, self-reported to be creamy in consistency, and with a strong smell. Participants were questioned about their knowledge of different vaginal practices, whether they thought that any of these practices caused vaginal irritation or symptoms, and which of these they actually practiced (**Table 4.3**). Of all the vaginal practices included in the questionnaire, the young women were most familiar with vaginal cleansing with water and soap, wiping with paper, insertion of vaginal creams

and douching. About half of the participants use their fingers and water to clean their vagina, while other vaginal practices are less common (such as cleansing with cloth or using traditional herbs). About a third of participants thought that using vaginal products was beneficial for their vaginal health. Similarly, about a third expressed concern that these vaginal products were causing their vaginal discharge and smell, although only a few reported experiencing problems (such as vaginal itching and discomfort). More than half reported previously using medicine applied vaginally that was prescribed by a doctor or nurse, to reduce vaginal discharge and smell, confirming that vaginal discharge and/or infections may have been a long-term problem for these young women. In addition, more than half used tampons when they were menstruating, suggesting that they were likely to be comfortable with the vaginal application of the probiotic study product (**Table 4.3**). Again, no differences were noticed between participants randomised to the two treatment arms (**Table 4.3**).

Table 4.3. Description of vaginal practices and symptoms

Characteristic	All [n=24]	SOC [n=10]	Intervention [n=14]	p-value*
Knowledge about vaginal practices [% (n/N)]				
Cleansing with water	100 (24/24)	100 (10/10)	100 (14/14)	>0.9999
Cleansing with soap	95.8 (23/24)	90.0 (9/10)	100 (14/14)	0.4167
Wiping with cloth or paper	91.7 (22/24)	90.0 (9/10)	92.9 (13/14)	>0.9999
Insertion of creams	75.0 (18/24)	80.0 (8/10)	71.4 (10/14)	>0.9999
Douching	58.3 (14/24)	50.0 (5/10)	64.3 (9/14)	0.6785
Use of traditional herbs/medicine	29.2 (7/24)	20.0 (2/10)	35.7 (5/14)	0.6529
Cleansing with salt solutions	20.8 (5/24)	10.0 (1/10)	28.6 (4/14)	0.3577
Cleansing with lemon juice	16.7 (4/24)	10.0 (1/10)	21.4 (3/14)	0.6146
Cleansing with detergents (other than soap)	12.5 (3/24)	30.0 (3/10)	0 (0/14)	0.0593
Thoughts about using products in vagina [% (n/N)]				
Good for me	33.3 (8/24)	40.0 (4/10)	28.6 (4/14)	0.6734
Cause discharge and/or smell	37.5 (9/24)	30.0 (3/10)	42.9 (6/14)	0.6785
Increases chances of getting STIs	8.3 (2/24)	0 (0/10)	14.3 (2/14)	0.4928
Partner want me to use them	4.2 (1/24)	10.0 (1/10)	0 (0/14)	0.4167
Increases chances of getting HIV	0 (0/24)	0 (0/10)	0 (0/14)	>0.9999
Vaginal practices actually reported [% (n/N)]				
Use of medicine from doctor/nurse	58.3 (14/24)	70.0 (7/10)	50.0 (7/14)	0.4212
Use of tampon	54.2 (13/24)	50.0 (5/10)	57.1 (8/14)	>0.9999
Cleansing with fingers	45.8 (11/24)	40.0 (4/10)	50.0 (7/14)	0.6968
Sex during period	37.5 (9/24)	30.0 (3/10)	42.9 (6/14)	0.6785
Cleansing with water	33.3 (8/24)	30.0 (3/10)	35.7 (5/14)	>0.9999
Douching to clean vagina	12.5 (3/24)	0 (0/10)	21.4 (3/14)	0.2391
Cleansing with soap	8.3 (2/24)	10.0 (1/10)	7.1 (1/14)	>0.9999
Cleansing with cloth/sponge	4.2 (1/24)	0 (0/10)	7.1 (1/14)	>0.9999
Use of traditional herbs/medicine	4.2 (1/24)	0 (0/10)	7.1 (1/14)	>0.9999
Use of drying/tightening agents for sex	4.2 (1/24)	0 (0/10)	7.1 (1/14)	>0.9999
Insertion of cloth/paper	0 (0/24)	0 (0/10)	0 (0/14)	>0.9999
Experiences problems after vaginal product use [% (n/N)]				
Yes	16.7 (4/24)	10.0 (1/10)	21.4 (3/14)	0.6146

Itching/irritation	100 (4/4)	100.0 (1/1)	100.0 (3/3)	-
Discomfort	50.0 (2/4)	100.0 (1/1)	33.3 (1/3)	-
Vaginal Infections	25.0 (1/4)	100.0 (1/1)	0 (0/3)	-
Description of symptomatic discharge [% (n/N)]				
Long-term symptoms	91.7 (22/24)	80.0 (8/10)	100 (14/14)	0.1630
New symptoms	8.3 (2/24)	20.0 (2/10)	0 (0/14)	0.1630

*Mann-Whitney test comparing SOC and intervention groups

4.3.3 Spontaneous resolution of BV between screening and enrolment

Because this pilot trial relied on screening for BV by Nugent scoring and laboratory-diagnosis of STIs that were conducted at a centralized, South African National Accreditation System (SANAS) accredited laboratory, the average time between screening and enrolment was 14 days (IQR 9-15). Of the 24 women that were considered eligible to enrol after their screening visit, based on BV Nugent scores 7-10 and absence of tested STIs, 14/24 (6/10 SOC and 8/14 intervention participants, p>0.9999) appeared to have spontaneously resolved BV by their enrolment visit, of which 4/14 had Nugent scores 4-6 and 10/14 had Nugent scores 0-3. Because this microbiological information was not available when the women were enrolled and randomized, all 24 participants started on treatment. In addition, 5/24 participants acquired an STI between screening and enrolment (2/24 had *C. trachomatis*, 2/4 *T. vaginalis,* and 1/24 *N. gonorrhoea*). Because of these new infections, these participants subsequently took a second higher dose of metronidazole (2 g orally in a single dose, or 500 mg orally twice a day for seven days) before returning for their follow-up visits. For these 5/24 individuals, it was not possible to determine if their BV had resolved with the metronidazole treatment or the combination with Vagiforte PLUS®, or if the second dose of metronidazole contributed to BV cure. This underscores the major limitations to having centralized screening infrastructure for STIs/BV that depend on swift care. Despite this high rate of spontaneous BV clearance and acquisition of STIs between screening and enrolment, the primary analysis of treatment efficacy was the intention-to-treat analysis (ITT), which included all 24 participants (section 4.3.5). A second analysis that included only participants who followed the protocol (Per-Protocol analysis, PP) could not be conducted because of very small numbers.

This finding, although not anticipated, shows that spontaneous BV clearance rates were extremely high in this cohort of young sexually active women, and highlights a potentially important confounder that may exist in all such clinical trials for treating

BV. Most of the other clinical studies on the efficacy of probiotics have not re-screened for BV at enrolment and randomization (Appendix I), therefore it is likely that BV prevalence at enrolment would be over-estimated.

4.3.4 Use of study products

All 24 enrolled participants reported that they administered MetroGel™ according to the manufacturer's instructions, and completed the entire course. However, almost half (11/24) reported to have experienced vaginal problems while or shortly after using the product, including: vaginal itching/irritation (8/24), vaginal discomfort (3/24) or bleeding (spotting; 1/24), which are recognized side effects of MetroGel™ use (Appendix III). Vaginal itching/irritation are considered typical symptoms of candidiasis, and this was the most common complaint after MetroGel™ application.

All participants randomised to the intervention group (14/24) reported that they administered Vagiforte® PLUS, and completed the entire course of oral capsules. The majority (12/14) also reported that they completed the administration of the vaginal spray for the full ten days, suggesting that adherence and acceptance of the probiotic product was generally high. A few of the women randomized to the intervention group reported to have experienced some sort of problem while using Vagiforte® PLUS (4/14), including vaginal itching/irritation (2/14), discomfort (1/14), increased nipple sensitivity (1/14), and nausea (1/14). All participants returned empty packages of both study products at the next visit, one month after treatment completion (month 1).

4.3.5 ITT analysis of BV cure at month 1

The overall BV cure rate (defined as achieving a Nugent score 0-3) was 41.7% (10/24) one month after treatment completion, with cure rates tending to be higher in the MetroGel™ alone SOC arm (60%; 6/10) than the probiotic intervention arm (5/14, 35.7%), although not significantly (p=0.4081; **Figure 4.3**). Furthermore, 1/10 woman in the SOC arm and 3/14 women in the intervention arm had intermediate Nugent scores (Nugent score 4-6). Overall, BV Nugent Scores decreased significantly in both arms (possibly reflecting MetroGel™ efficacy), with the Nugent scores decreasing from a median of 7.5 (IQR 7-8) at screening to 2 (IQR 0-6) and month 1 in

the SOC arm (MetroGel™ alone; p=0.0091), and from a median Nugent score of 8 (IQR 7.75-8) to 6 (IQR 0-8) in the probiotic intervention arm (p=0.0016; **Figure 4.3**).

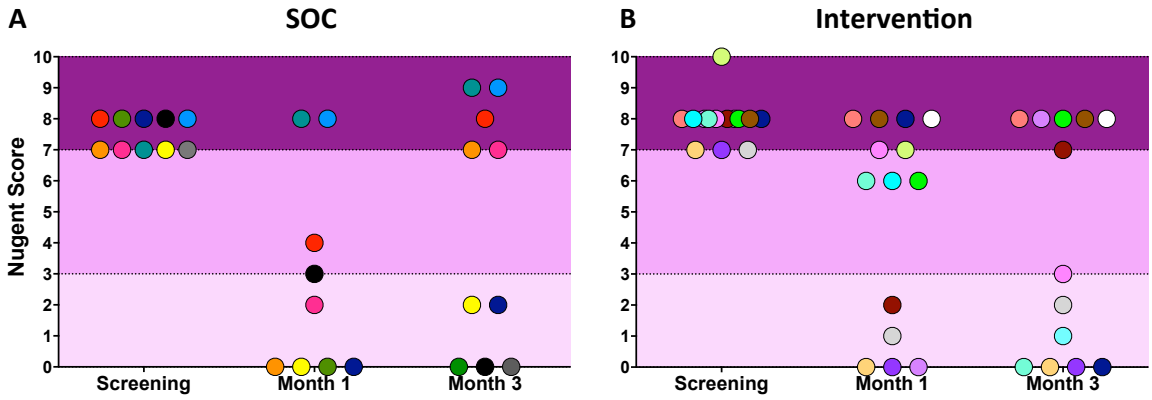


Figure 4.3. Nugent scoring of participants in the SOC (A) and intervention (B) groups. Nugent Scores were determined at screeninng, one month (month 1) and three months (month 3) after treatment completion. Each participant is represented by a colour-coded dot. Women with a Nugent Score ≤3 were considered BV negative (light purple), with a Nugent 4-6 had an intermediate microbiota (pink), and women with a Nugent ≥7 were considered BV positive (dark purple).

4.3.6 Changes in vaginal symptoms at month 1

One month after treatment completion, about 80% of women reported an improvement of their vaginal symptoms (**Table 4.4**), which was similar in the SOC and probiotic arm. Of those that reported an improvement in symptoms, >80% noted a decreased amount of discharge, and more than half reported a change in smell and/or colour. Again, this was independent of the treatment arm (**Table 4.4**). However, there was a significant difference between women who were BV- (11/12 said discharge improved, 91.7 %, p=0.1089) and BV intermediate (4/4, 100 %, p=0.2081) compared to BV+ (4/8, 50.0 %; p=0.0485) one month after treatment completion noticing an improvement of symptoms, indicating that BV cure in these women might have gone along with symptom relief.

Table 4.4. Vaginal symptoms of participants after treatment

Month 1	Overall	SOC	Intervention	p-value*
Improvement of discharge [% (n/N)]	79.2 (19/24)	80.0 (8/10)	78.6 (11/14)	>0.9999
Less discharge	89.5 (17/19)	100 (8/8)	81.8 (9/11)	0.4854
Change in colour	52.6 (10/19)	50.0 (4/8)	54.5 (6/11)	>0.9999
Change in smell	63.2 (12/19)	62.5 (5/8)	63.6 (7/11)	>0.9999
Month 3				
Improvement of discharge [% (n/N)]	78.3 (18/23)	80.0 (8/10)	76.9 (10/13)	>0.9999
Less discharge	83.3 (15/18)	87.5 (7/8)	80.0 (8/10)	>0.9999
Change in colour	55.6 (10/18)	75.0 (6/8)	40.0 (4/10)	0.0601
Change in smell	66.7 (12/18)	75.0 (6/8)	60.0 (6/10)	0.3077
No change in symptoms	21.7 (5/23)	20.0 (2/10)	15.4 (2/13)	>0.9999
Symptoms worsened	4.3 (1/23)	0 (0/9)	7.7 (1/13)	>0.9999

*Mann-Whitney test comparing SOC and intervention groups

4.3.7 Recurrence of BV at month 3

In the ITT analysis, the BV cure rate was 52.1% overall three months after treatment completion, being similar in the SOC and the intervention group (p>0.9999; **Figure 4.3**). Overall, the recurrence rate was 26.1% within the two months between the first and second follow-up visit, with 30% of women in the SOC and 23.1 % in the intervention group (p>0.9999) having recurrent BV (**Figure 4.3**).

4.3.8 Recurrence of symptomatic vaginal discharge at month 3

Three months after treatment completion, about 80% (18/23) of the participants still reported that their vaginal symptoms had improved, which was comparable to the previous follow-up visit. Independent of treatment arm, the majority noticed a decreased in the amount of discharge, a change in smell, and/or colour (**Table 4.4**). However, one woman in the intervention arm noticed an increase in discharge.

4.3.9 Achieving a protective vaginal pH

A vaginal pH<4.5 is thought to be beneficial for vaginal health (Boskey *et al*. 1999; Aroutcheva *et al*. 2001), forming one of the clinical criteria used in Amsel's criteria to diagnose BV (Amsel *et al*. 1983). Thus a decrease of pH <4.5 after BV treatment would be considered favourable. In the SOC arm, 6/10 (60.0 %) participants had a vaginal pH <4.5 one month after treatment completion, compared to 9/14 (64.3 %) in the intervention group (p>0.9999; **Figure 4.4**). Significantly fewer women who were BV+ (25.5%, n=8) had a vaginal pH <4.5 than those who were BV- (81.3%; n=16;

p=0.0215), indicating that clearance of BV coincided with a decrease in vaginal pH. In agreement, Nugent scores and vaginal pH correlated significantly (Spearman rho = 0.72; p=0.0001).

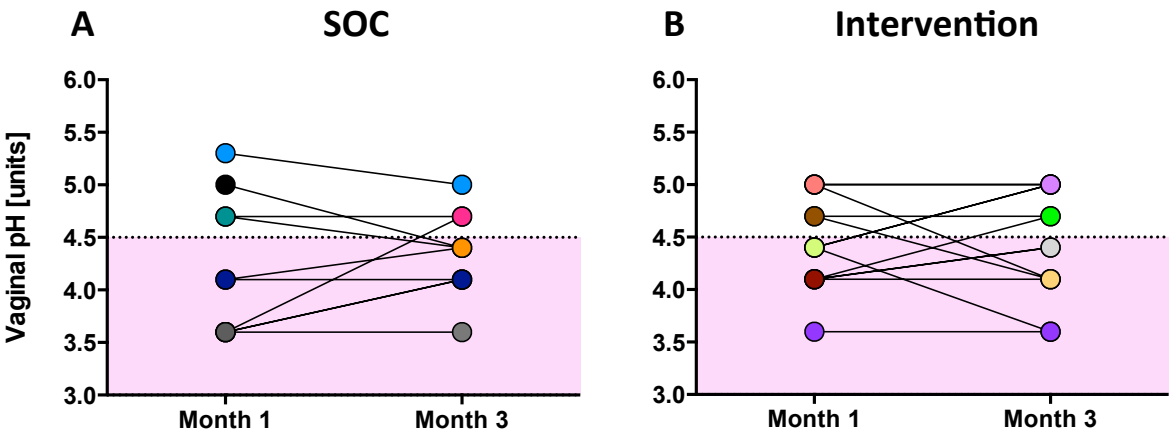


Figure 4.4. Vaginal pH in participants of the SOC (A) and intervention (B) group. Vaginal pH was measured one month (month 1) and three months (month 3) after treatment completion. Each participant is represented by a colour-coded dot. A vaginal pH <4.5 (light purple) is considered beneficial for vaginal health.

Three months after treatment completion, 7/10 participants in the SOC and 7/13 in the intervention group had a protective pH <4.5 (p=0.6693; **Figure 4.4**). Again, less women who were BV positive had a vaginal pH <4.5 (3/13, 23.07%) compared to those with a Nugent score <7 (11/12, 91.67%; p=0.0010).

4.3.10 Reducing levels of genital inflammatory cytokines

Previously, BV has been associated with elevated levels of genital inflammatory cytokines, which may increase the risk of HIV infection (Liebenberg *et al*. 2017; Masson *et al*. 2015; Mlisana *et al*. 2012). To investigate whether treatment influenced inflammatory markers in the FGT, the levels of IL-1α, a marker for genital tract inflammation (Lennard *et al*. 2017), was measured pre- (at enrolment) and one month post-BV treatment (**Figure 4.5**). IL-1α levels were neither associated with vaginal pH (Spearman rho=0.17, p=0.2912) nor the presence of other STIs (ANOVA BV-/STI- vs. BV-/STI+ vs. BV+/STI- vs. BV+/STI+ p=0.3994).

In the SOC arm, the median IL-1α levels measured in the SOC pre-treatment were 28.07 pg/mL (IQR 8.59-48.26) compared to 18.63 pg/mL (IQR 2.11-64.8) post-

treatment (p=0.8368). In the intervention arm, median IL-1α levels of 14.17 pg/mL (IQR 0.63 – 30.79) were measured pre-treatment compared to 9.55 pg/mL (IQR 1.10 – 23.27; p=0.5017) post-treatment. The median decrease of IL-1α from pre-to post-treatment was slightly stronger in the intervention (-3.17 pg/mL [IQR -20.71 - +13.69]) than the control group (-0.8 pg/mL [IQR -11.81 – +16.65]; p=0.8556; **Figure 4.5**).

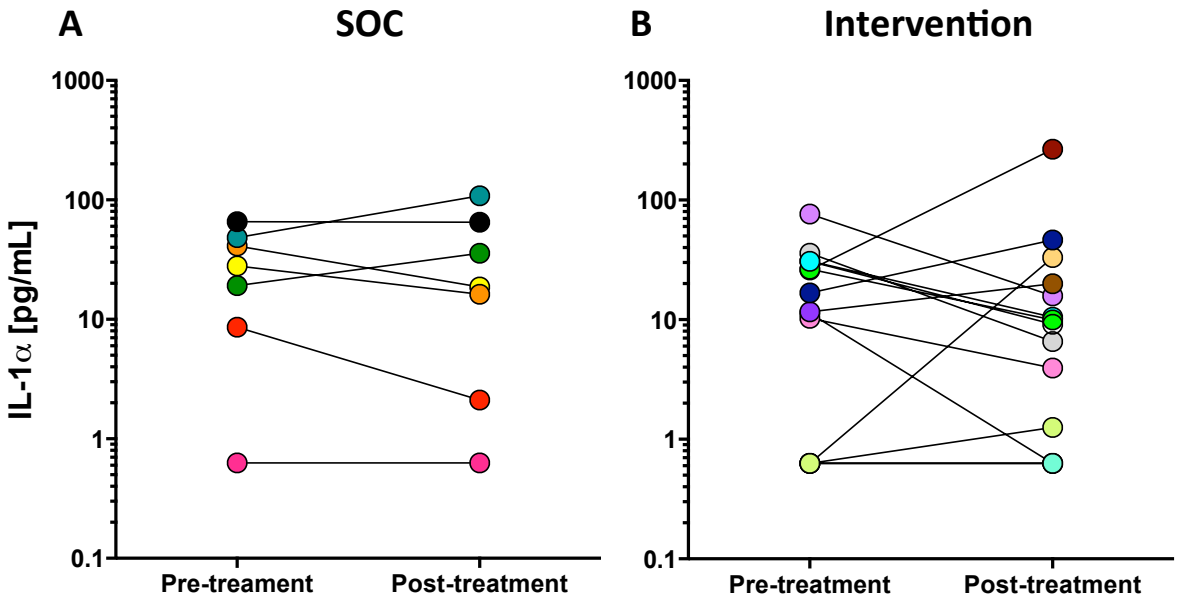


Figure 4.5. Levels of IL-1α pre- and post-treatment in participants of the SOC (A) and intervention (B) group. IL-1α levels were measured in genital secretions by ELISA at enrolment (pre-treatment) and one month after treatment completion (post-treatment). Median and IQR are shown. Each participant is represented by a colour-coded dot. The lower detection limit was 1.25 pg/mL, and all values below the detection limit were recorded as half of the lowest concentration measured.

4.3.11 Changes in vaginal bacterial communities following treatment

4.3.11.1 Changes in Lactobacillus and BV-associated species

While *L. crispatus, L. gasseri, L. vaginalis*, and *L. jensenii* are considered to be healthy vaginal commensals, the role of *L. iners* in vaginal health is controversial. In this trial, *L. gasseri* and *L. crispatus* were detected in 30% (7/21) of the participants, *L. jensenii* and *L. vaginalis* in 50%, and *L. iners* (19/21) and *L. mucosae* (21/21) was present in the majority of women (**Figure 4.6**) Overall, the concentrations of *L. crispatus, L. gasseri, L. jensenii*, and *L. vaginalis* did not change from pre- to post-treatment, while *L. mucosae* and *L. iners* decreased, although not statistically significantly. Further, there were no clear differences between the SOC and

intervention group regarding the changes of *L. crispatus, L. gasseri, L. vaginalis*, *L. jensenii, L. mucosae*, and *L. iners* after treatment (**Figure 4.6**).

Along with an increase of *Lactobacillus* spp., a decrease of microbes associated with BV, including *G. vaginalis*, *A. vaginae*, *P. bivia*, BVAB2 and *Megasphaera* 1 would be favourable, as these bacterial species have been associated with genital inflammation and other adverse outcomes (Lennard *et al*. 2017; Libby *et al*. 2008; Zaga-Clavellina *et al*. 2012). *Megasphaera* 1 was present in about half, and the remaining species were detected in the majority (>80%) of the participants, at a much higher level than *Lactobacillus* spp., both before and after treatment (**Figure 4.7**).

Overall, the concentrations of *Megasphaera* 1 did not change after BV treatment, while *G. vaginalis*, *P. bivia*, *A.vaginae* and BVAB2 decreased. This trend was similar between the SOC and intervention group, however *G. vaginalis* and *P. bivia* tended to decrease more in the intervention than SOC group, while the opposite was observed for *A. vaginae*, BVAB and *Megasphaera* 1 (**Figure 4.7**), but neither was statistically significant.

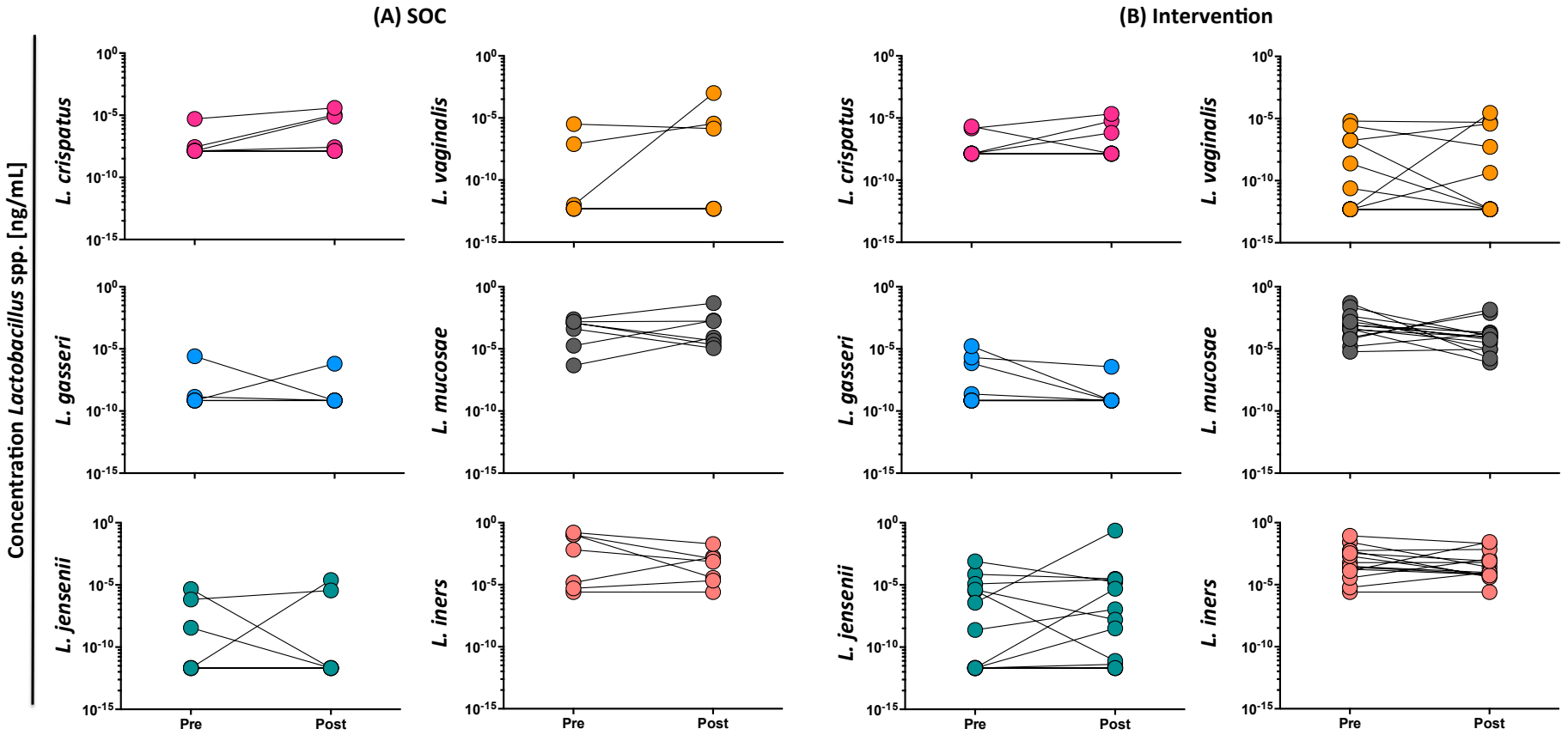


Figure 4.6. *Lactobacillus* spp. concentration in the FGT of women in the SOC (A) and intervention (B) group. The concentrations of *Lactobacillus* spp., including *L. crispatus* (pink), *L. gasseri* (blue), *L. jensenii* (green), *L. vaginalis* (orange), *L. mucosae* (grey) and *L. iners* (salmon) in FGT secretions were measured of each participant in the SOC (n=7) and intervention (n=14) group pre- (at enrolment) and post- (1 month after treatment completion) treatment using qPCR, and normalised to 16S bacterial concentration.

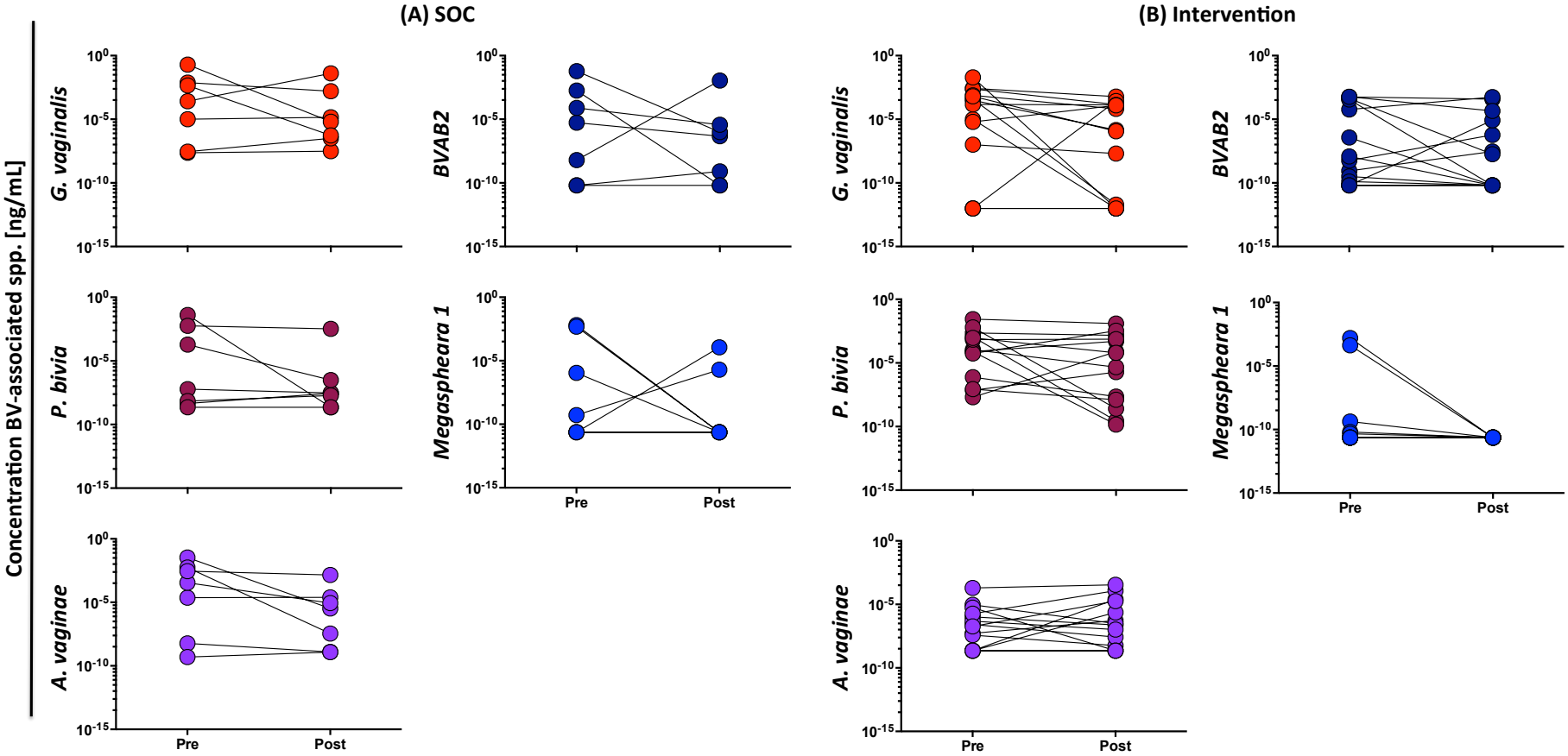


Figure 4.7. BV-associated spp. concentration in the FGT of women in the SOC (A) and intervention (B) group. The concentrations of BV-associated spp., including *G. vaginalis* (red), *P. bivia* (plum), *A. vaginae* (purple), *BVAB2* (dark blue) and *Megaspheara 1* (light blue) in FGT secretions were measured of each participant in the SOC (n=7) and intervention (n=14) group pre- (at enrolment) and post- (1 month after treatment completion) treatment using qPCR, and normalised to 16S bacterial concentration.

4.3.11.2 Changes in bacterial species contained in Vagiforte®

To estimate whether the bacterial species contained in Vagiforte® PLUS Combo Pack (*L. rhamnosus, L. acidophilus, B. bifidum* and *B. longum*) were detected in the participants, the quantities of these species was determined pre-treatment (at enrolment), after which Vagiforte® was administered, and the first follow up visit one month after treatment completion (**Figure 4.8**).

L. acidophilus was present in the majority (20/21) of participants. *L. rhamnosus* was only detectable in the FGT of each one woman from the SOC and one woman from the intervention group. *B. bifidum* was only measurable in three and two woman of the two groups, respectively, while *B. longum* was detectable in seven woman of the intervention but in only three women of the SOC group.

L. rhamnosus was also below detection limit before and after treatment in the majority of women, independently to which treatment group they belonged to (**Figure 4.8**), while *L. acidophilus* only increased marginally from pre- to post-treatment in the intervention arm, which was similar to the SOC group. However, *B. bifidum* and *B. longum* tended to increase in the intervention arm while decreasing in the SOC arm, although this was not statistically significant (**Figure 4.8**). Overall, this data does not confirm a successful colonization and proliferation of the *Lactobacillus* spp. contained in Vagiforte® PLUS in the FGTs of women randomised to the intervention arm, while *Bifidobacterium* spp. might have undergone a successful colonization.

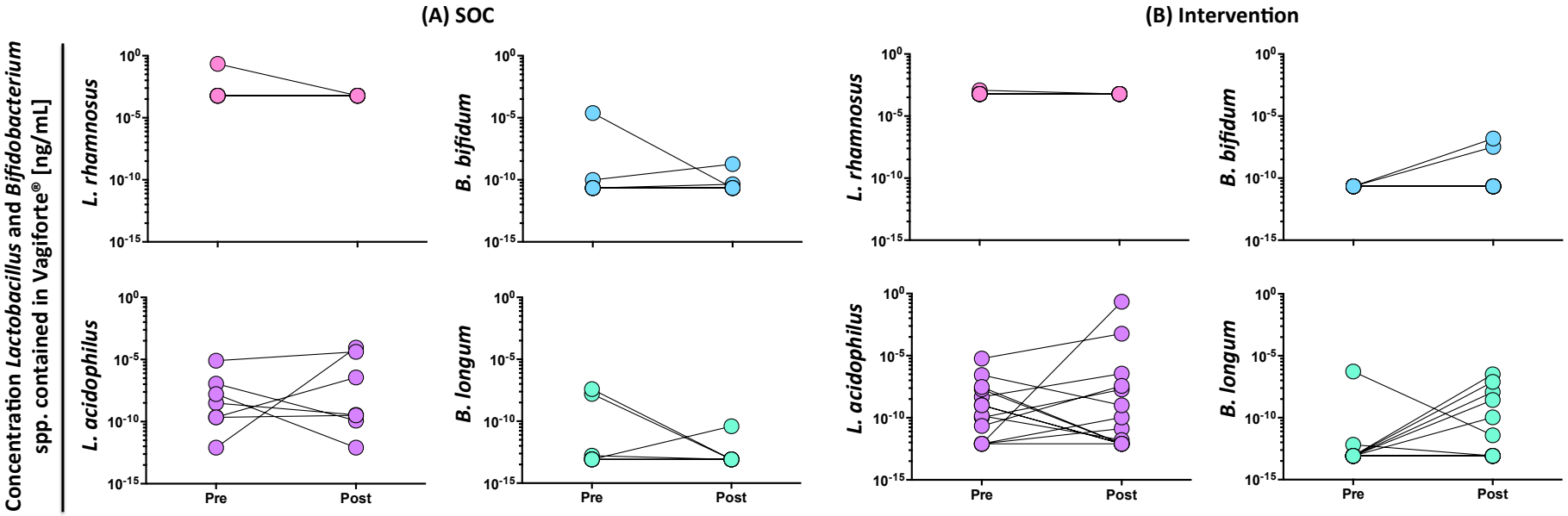


Figure 4.8. Vagiforte® bacterial concentration in the FGT of women in the SOC (A) and Intervention (B) group. The concentrations of bacterial species contained in Vagiforte®, including *L. rhamnosus* (rose), *L. acidophilus* (lilac), *B. bifidum* (ice-blue) and *B. longum* (bright green) in FGT secretions were measured of each participant in the SOC (n=7) and intervention (n=14) group pre- (at enrolment) and post- (1 month after treatment completion) treatment using qPCR, and normalised to 16S bacterial concentration.

4.3.12 Comparison of microbial and inflammatory changes between trial groups

To determine whether treatment influenced the relationship between vaginal microbial composition and inflammatory cytokines, unsupervised clustering of IL-1α and microbial concentration changes from pre- (enrolment) to post (month 1)-BV treatment was performed. Women did not cluster separately by intervention arm, but those who cleared BV by (assessed by Nugent score) and who had lower vaginal pHs, appeared to also have decreased BV-associated bacteria (**Figure 4.9**). Direct correlations between Nugent Score, vaginal pH, IL-1α and abundances of vaginal bacterial and *Candida* spp. are shown in section 4.3.14.

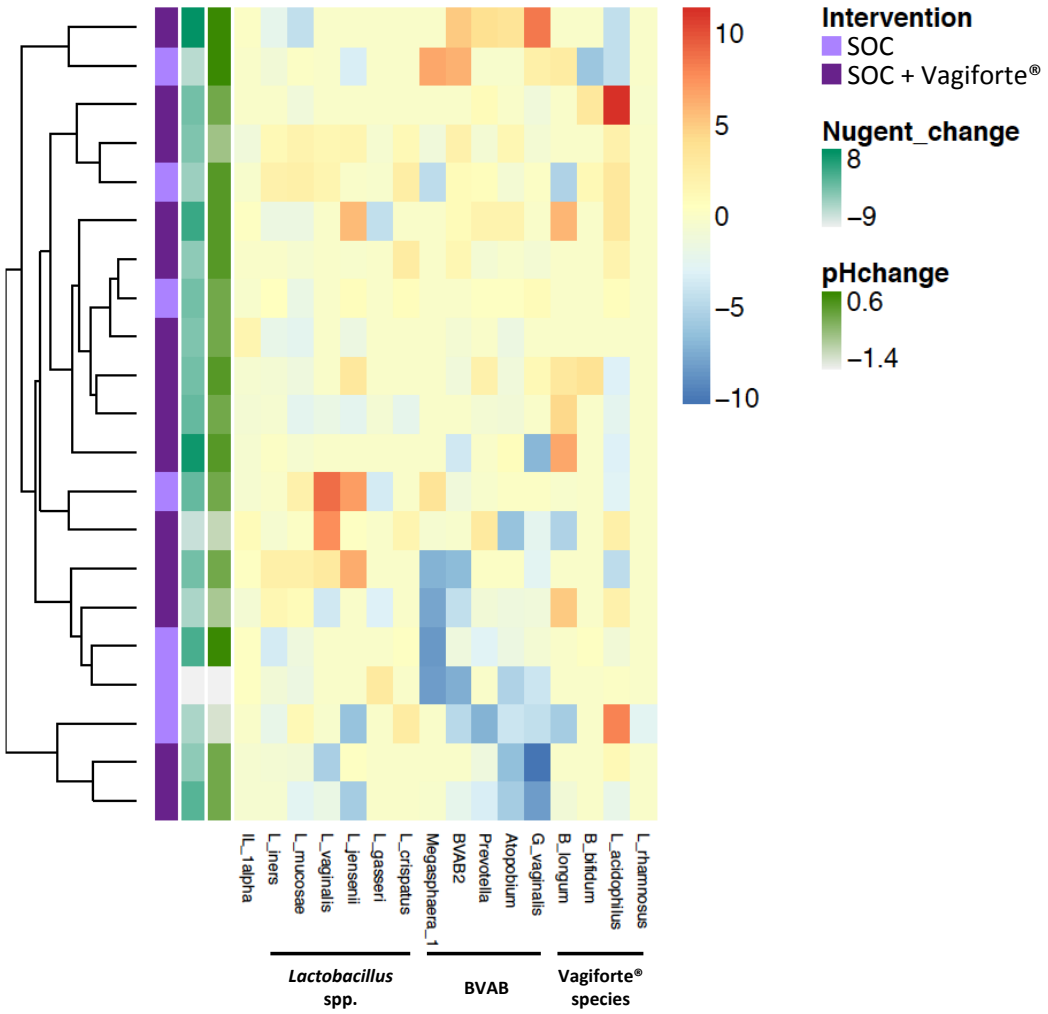


Figure 4.9. Unsupervised hierachical clustering analysis of IL-1α concentrations and microbial community abundence by study arm. The heatmap shows an unsupervised hierarchical clustering of log-differences in IL-1α and bacterial species by participant between enrolment (pre-treatment) and month 1 (post-treatment). Highest log-differences between visits are shown in red and lowest log-differences shown in blue. The heatmap was generated using the Euclidean distance algorithm abd the clustering algorithm was complete. All zero values were approximated to 0.0001. Allocation to SOC is shown in light purple, and to the intervention arm in dark pruple. Changes in Nugent Score are shown in dark green, and vaginal pH changes in light green.

4.3.13 Changes in Candida communities following treatment

More than half of the participants (13/24) reported symptoms of vaginal *Candida* infections one month after treatment completion, which is a recognised side effect of MetroGel™ use (as described on the package leaflet, Appendix III). To investigate this, changes in concentrations of several *Candida* spp. were assessed, including *C. albicans, C. glabrata, C. parapsilosis, C. dubliniensis, C. krusei, C. lusitaniae* and *C. tropicalis* (**Figure 4.10**). Almost all women had at least one *Candida* spp. detected (20/21). The most common *Candida* spp. that was detected was *C. albicans* (12/21), with 7/21 having *C. albicans* measurable before MetroGel™ use and 7/21 after finishing MetroGel™. The next most common fungal species detected was *C. tropicalis* (9/21; 5/21 before, 4/21 after MetroGel™), *C. dubliniensis* (8/21; 5/21 before, 4/21 after MetroGel™), *C. krusei* (7/21; 4/21 before, 3/21 after MetroGel™), *C. lusitaniae* (5/21; 4/21 before, 1/21 after MetroGel™) and *C. glabrata* (4/21; 2/21 before, 2/21 after MetroGel™) and *C. parapsilosis* (4/21; 0/21 before, 4/21 after MetroGel™). Despite most woman reporting symptoms of *Candida* infections after MetroGel™ use, there was no change in the concentration of any of the *Candida* spp. overall. Abundance and diversity of *Candida* spp/ detected neither differed by study arm (**Figure 4.10**), although Vagiforte® PLUS is indicated for vaginal thrush induced by antibiotic treatment (according to the package leaflet), nor between women reporting symptomatic candidiasis and those not experiencing any symptoms (data not shown).

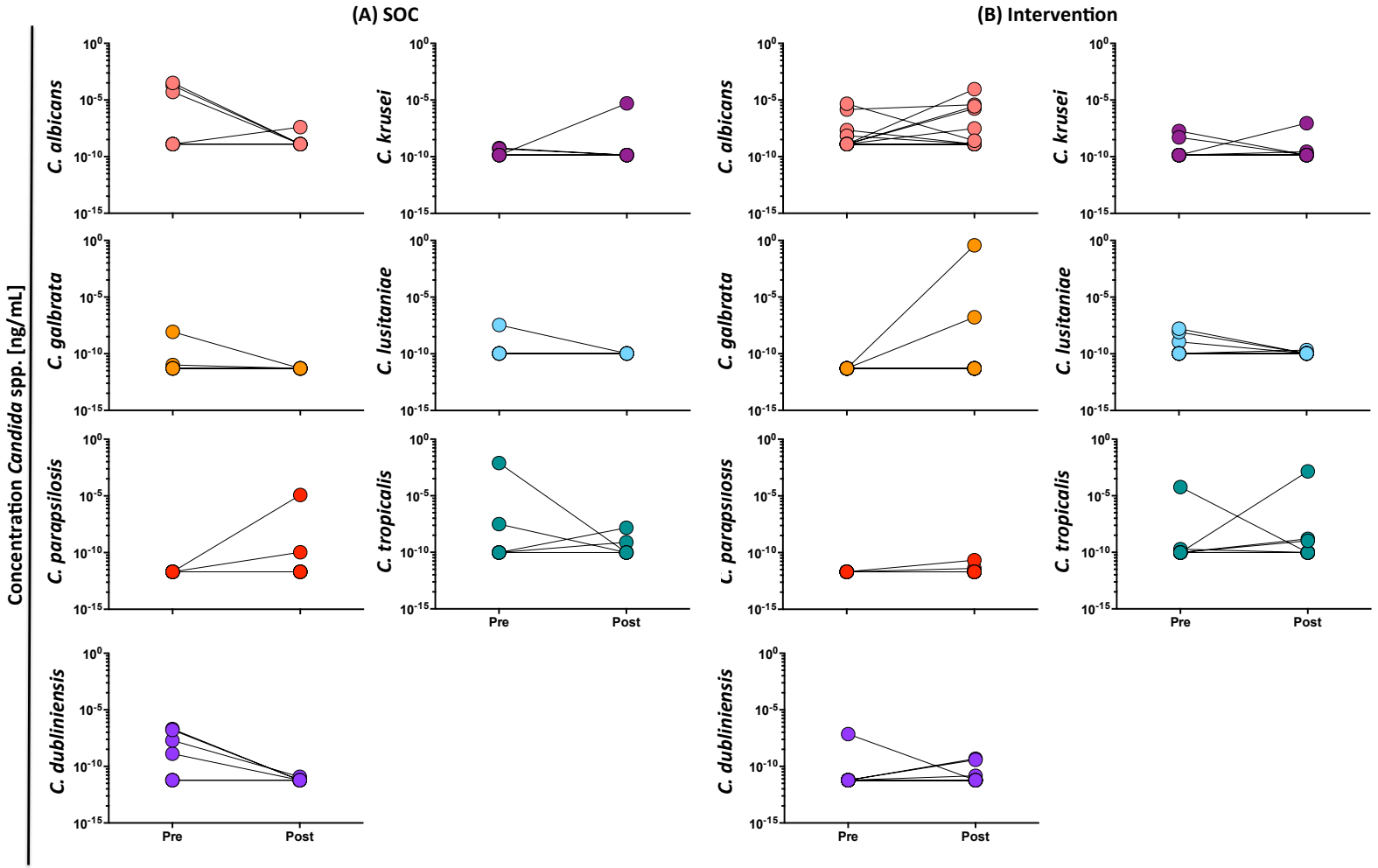


Figure 4.10. Concentration of *Candida* spp. in the FGT of women in the SOC (A) and intervention (B) group. The concentrations of *C. albicans* (salmon), *C. galbrata* (orange), *C. parapsilosis* (red), *C. dubliniensis* (purple), *C. krusei* (purple), *C.lusitaniae* (ice-blue) and *C. tropicalis* (green) in FGT secretions were measured of each participant in the SOC (n=7) and intervention (n=14) group pre- (at enrolment) and post- (1 month after treatment completion) treatment using qPCR.

4.3.14 Interplay between BV Nugent scores, pH, inflammation and microbial communities in the FGT

In the vaginal niche, the interaction between host immunity with abundance and/or diversity within and between bacterial species is likely to be complex. These may further be influenced by or have an influence on vaginal pH, genital inflammation and BV status of women. Thus, the relationship between vaginal pH, Nugent scores, vaginal IL-1α concentrations and microbial concentrations (bacterial and *Candida* spp.) were evaluated (**Figure 4.2**).

As described previously, Nugent scores correlated significantly with vaginal pH (Spearman rho=0.6717, unadj. p=0.0009, adjust. p=0.0104). In addition, Nugent scores correlated positively with the concentration of vaginal *Atopobium* spp. detected (rho=0.5972, unadj. p=0.0043, adjust. p=0.0494). Vaginal pH correlated positively with the concentration of BVAB (rho=0.6, unadjust. p=0.004, adjust. p=0.046) and *Atopobium* spp. measured (rho=0.5635, unadjust. p=0.0078, adjust. p=0.0897).

The concentration of IL-1α (as a marker of inflammation) correlated weakly inversely with the concentration of *L. iners* measured, but only before adjusting for multiple comparisons (rho=-0.4430, unadjust. p=0.0443, adjust. p=0.5095). Interestingly, vaginal concentrations of *L. crispatus* correlated negatively with concentrations of the *B. longum* (rho=-0.6850, unadjust. p=0.0006, adjust. p=0.0069), suggesting that *L. crispatus* was less likely to be detected in women who were colonized with *B. longum*. Similarly, concentrations of *L. rhamnosus* correlated positively with *Prevotella* spp. (rho=0.6687, unadjust. p=0.0009, adjust. p=0.0104), and negatively with *L. acidophilus* (rho=-0.538, unadjust. p=0.0199, adjust. p=0.2286), although the later was not significant after adjusting for multiple comparisons. Although concentrations of several *Candida* spp. correlated positively with quantities of *B. bifidum* [including *C. albicans* (rho=0.4609, unadjust. p=0.0355, adjust. p=0.4083) and *C. dubliniensis* (rho=0.4775, unadjust. p=0.0286, adjust. p=0.3289), these were not significant after adjusting for multiple comparisons. Further, *C. albicans* concentrations correlated significantly with *C. dubliniensis* (rho=0.7714, unadjust. p<0.0001, adjust. p=0.0012). Overall, this suggests that colonization with either *L. rhamnosus* or *B. bifidum* tended to favour an environment with high concentrations of

BV-associated bacteria (like *Prevotella* spp.) rather than *L. crispatus*, and vaginal colonization with *Candida* spp.

Although the concentrations of several of the *Lactobacillus* spp. considered being healthy (including *L. crispatus, L. mucosae, L. vaginalis* and *L. jensenii*) tended to correlate, none of these associations were significant after adjusting for multiple comparisons. Similarly, BV-associated species tended to correlate with one another (including *Megashphera* 1 with BVAB2 (rho=0.6333, unadjust. p=0.0021, adjust. p=0.0242), *G. vaginalis* with *Atopobium* (rho=0.6298, unadjust. p=0.0029, adjust. p=0.0334; **Figure 4.2**). *L. iners* also tended to correlate positively with *L. mucosae* concentrations (rho=0.4492, unadjust. p=0.0026, adjust. p=0.0299) but negatively with *C. albicans* abundance (r=0.5912, unadjust. p=0.0048, adjust. p=0.0552).

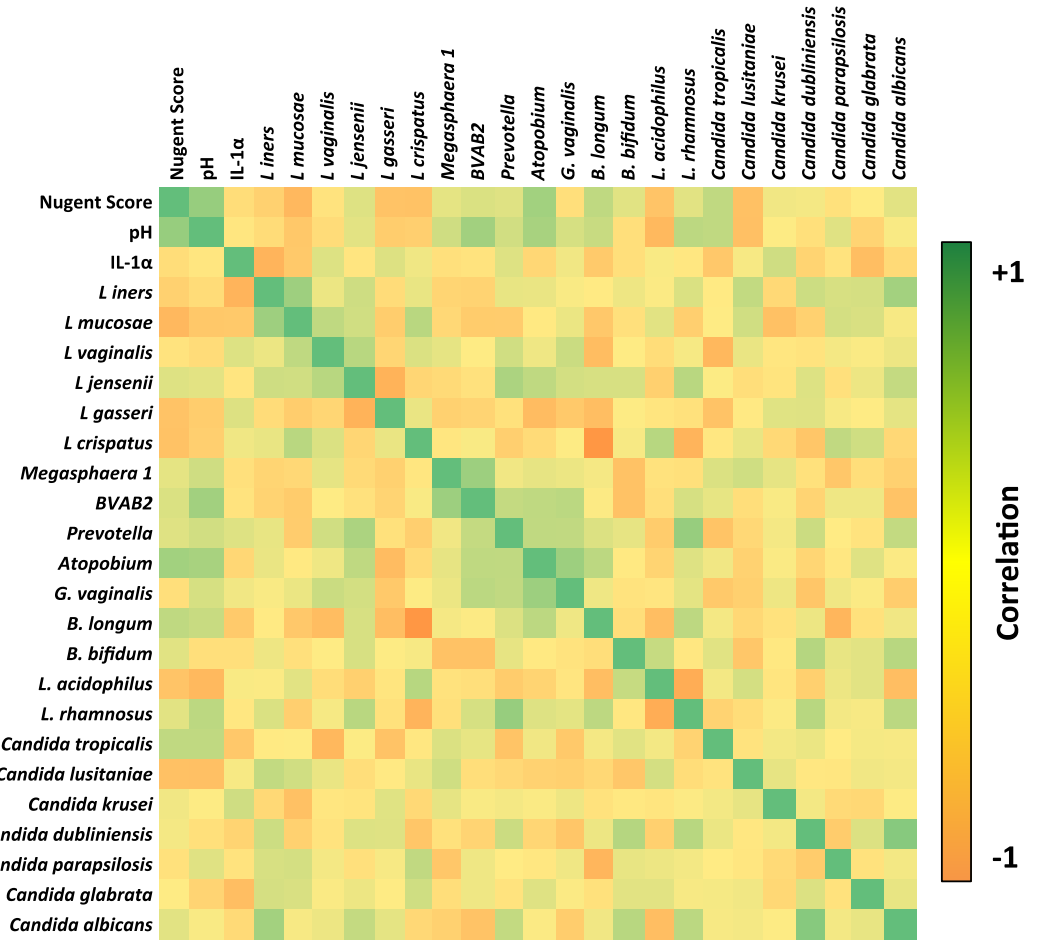


Figure 4.1. Correlation matrix of changes in Nugent Scores, vaginal pH, genital inflammation marker, and vaginal microbes. Correlations between changes (pre- and post-treatment) in Nugent score and vaginal pH units, log-fold changes of concentrations of a maker of genital inflammation [IL-1α], and log-changes of the concentrations of vaginal *Lactobacillus* spp. [*L. iners, L. mucosae, L. vaginalism L. jensenii, L. gasseri, L. crispatus*], BV-associated bacteria [*Megashaera 1, BVAB2, Prevotella, Atopobium, G. vaginalis*] and *Candida* spp. [*C. tropicalis, C. lusitaniae, C. kruesi, C. dubliniensis, C. parapsilosis, C. glabrata, C. albicans*] were calculated and colour-coded (green=postive correlation, orange=negative correlation).

4.3.15 Reported adverse events

A total of 76 AEs occurred during the trial up to date. Of these, only one AE (forced sexual intercourse) was rated as severe by a physician but not related to product use. The majority of AEs (72.4%; 55/76) were noted by participants in the intervention arm, but few (nausea [1/55], vaginal itching/irritation [2/55], discomfort [1/55], sensitive nipples [1/55]) were considered related to the administration of Vagiforte® PLUS Combo Pack. In contrast, vaginal *Candida* infections (13/55), vaginal discomfort (4/55) and constipation (1/55) were considered to be related to MetroGel™ use, and all have previously been reported as side effects of this product (as indicated on the package leaflet, Appendix III). The most common unrelated AEs were headache (14/55) and flu symptoms (9/55).

AEs related to vaginal health were also noted, including recurrent BV (7/55), and infections with *T. vaginalis* (6/55), *C. trachomatis* (3/55), *M. genitalium* (1/55) and *N. gonorrhoea* (1/55). Other AEs included stomach cramps, pre- and post-menstrual cramps, lower abdominal pain (a common symptom of ascending *C. trachomatis* infection; noticed by a participant diagnosed with *C. trachomatis*), and diarrhoea. Overall, these results indicate that the administration of MetroGel™ commonly causes *Candida* infections, but that the oral and vaginal administration of Vagiforte® PLUS Combo Pack is safe.

4.4 Discussion

Since this pilot trial was the first probiotic trial in South Africa recognized by SAHPRA, which assessed the effect of adjunctive probiotics on BV cure, the exercise of obtaining regulatory approval and conducting this randomised trial gave valuable insight into potential problems that could be encountered, as well as experience within UCT to conduct these trials. This was the first time conversations with SAPHRA were initiated around the regulation of probiotics in South Africa when making medicinal claims, thus were important in shaping the regulatory landscape of probiotics in South Africa to lay the path for future probiotic trials with better vaginal products.

Vagiforte® PLUS Combo Pack was selected based on its local production and availability, route of administration and treatment length, and performance of probiotic *Lactobacillus* isolates in *in vitro* testing of important probiotic characteristics, although it did not contain FGT commensals, as it was desirable to use an already marketed product that was available OTC to test the regulatory environment to conduct probiotic trials in South Africa.

There were several major findings in this pilot trial: (i) the screen failure rate was high, as lots of women with vaginal discharge needed to be screened to identify those with BV but no STIs; (ii) the majority of eligible participants who were BV+ at screening had self-resolved BV by enrolment; and (iii) participants who were colonized with *B. longum* and *B. bifidum* and/or *L. rhamnosus*, which were co-formulated in the administered probiotic, tended to be colonized with higher abundances of some BV-associated bacteria and yeast, while *L. crispatus* was less abundant in these women.

Symptomatic vaginal discharge was confirmed to be a poor predictor of the presence of BV in this cohort. Only about 35% of screened women were found to be BV+ and STI-, and thus eligible for the trial, while the major proportion of women with vaginal discharge was BV-. To reduce this high screen-failure rate, pre-screening participants for BV using a rapid test with high sensitivity and high specificity would be useful in this setting before performing STI testing. There are rapid tests available for BV

diagnosis, such as the BVBlue® test that detects sialidase activity, however, a pilot study performed in our laboratory showed poor sensitivity (data not shown), in line with previous findings (Madhivanan *et al*. 2014). Alternatively, a trained person could perform Nugent scoring in real-time at the clinic to immediately determine the BV status of screened participants, although staining and evaluating slides on site is time consuming and requires some specialized laboratory infrastructure and experienced staff (Chawla *et al*. 2013). This would allow only BV+ women (with a confirmed Nugent 7-10 on site) to be tested for discharge-causing STIs. Alternatively, vaginal pH could be measured to estimate BV status, as Nugent scores and pH correlated significantly in this trial and Amsel's criteria include a pH>4.5 as one of the diagnostic criteria for BV (Amsel *et al*. 1983), but vaginal pH alone has been shown to have a poor specificity (61%) in predicting BV (Masson *et al*. 2018; Hoffman *et al*. 2017), thus preference should be given to in-house Nugent Scoring. Point-of-care testing for other STIs (such as GeneXpert®) should optimally also be performed to exclude BV+/STI+ women, since women with other STIs may require additional antibiotics that would influence the outcome of a trial administering probiotics as adjunct to antibiotics.

Although the majority of previous trials have not retested women for BV after screening (Appendix I), more than half of the participants in this pilot trial had self-resolved their BV (by Nugent criteria <7) by enrolment. Rapid changes of the vaginal microbiota over one month have been described before, especially with onset of menstruation (Noyes *et al*. 2018; Vodstrcil *et al*. 2017; Srinivasan *et al*. 2010; Gajer *et al*. 2012). Although spontaneous clearance of BV at the rates seen in this study were not expected, it is likely that the two week interval been screening and enrolment was too long. It is thus critical to reduce the window between screening and enrolment significantly in future trials; and participants should optimally be screened and enrolled within one day using point-of-care diagnostics. To allow easy, quick and accurate STI testing (< 90 min), GenXpert® assays could be done for *C. trachomatis*, *N. gonnorhoea* and *T. vaginalis*. Unfortunately, no GenXpert® test is available for *M. genitalium* yet, but this STI was not prevalent in this cohort and other cohorts from South Africa (Barnabas *et al*. 2017). Optimally, genital sampling of participants should further take place at the same time point in their menstrual cycle at each study

visit, to avoid biasing Nugent Scoring and microbiome results, although this might be hard to implement.

Another unexpected complication in the pilot trial was that ~20% of participants became infected with a STI within the first month, for which they took additional antibiotics. This finding is consistent with a recent observational study in young women from Cape Town reported that the burden of STIs was unexpectedly high compared to South African national averages (Barnabas *et al*. 2017). In addition, participants enrolling in this pilot trial were largely University students that are generally considered high risk sexually, less likely to be in stable relationships (reviewed by Brown *et al*. 2016) and already accessing health care for vaginal conditions. Despite difficulty in screening for STIs and excluding based on a positive result, it was an important criteria for exclusion since trial participants would possibly receive additional antibiotics, which would confound the outcome. As such, a loss of ~20% of participants (as observed in this cohort) due to them acquiring an STI should be accounted for in calculating power for a larger study.

In the ITT analysis, the BV cure rate was 41.7% overall at the first follow up visit, which took place one month after treatment completion, with the cure rate being slightly higher in the MetroGel™ only group compared to the probiotic intervention group, although this was not significant. By three months after treatment completion, the BV cure was similar in both arms, suggesting that the probiotics tested in this pilot trial did not improve BV cure rates compared to MetroGel™ alone. This no benefit result, while disappointing, may reflect the fact that Vagiforte® PLUS did not contain commensal vaginal *Lactobacillus* spp. associated with reproductive health, although the trial was also underpowered to definitively determine impact of probiotics.

Most studies tested for BV cure immediately after completion of the treatment course (Grewal *et al*. 2018; Laue *et al*. 2018; Arooj *et al*. 2017; Schwiertz *et al*. 2015; Tomusiak *et al*. 2015; Kriplano *et al*. 2014; Vicariotto *et al*. 2014; Ling *et al*. 2013; Sudha *et al*. 2012; Hantoushzadeh *et al*. 2012; and others), thus any future clinical studies that are designed should test for BV cure shortly after treatment completion to ensure that women did not have recurrent BV when being tested for cure. Further, reporting cure before the first menstruation after treatment completion might results in

higher cure rates, as menstruation is commonly associated with vaginal microbial shifts (Gajer *et al*. 2012; Srinivasan *et al*. 2010).

It was interesting to note that the abundances of the *Lactobacillus* spp. contained in Vagiforte® were similar in women in the intervention arm compared the MetroGel™ alone arm, although specific probiotic strain typing and DNA fingerprinting (Hemmerling *et al*. 2010) was not performed to confirm colonisation efficacy of the specific administered strains in this trial. It therefore cannot ultimately be concluded that the *Lactobacillus* contained in Vagiforte® strains did not colonise the FGT, but it is questionable whether the colonized, and subsequently proliferated, especially as the species contained in Vagiforte® are not commonly highly abundant in the FGT of South African women (Lennard *et al*. 2017). Of concern, women in the intervention group tended had an increased colonization with *B. bifidum* and *B. longum* after administration of Vagiforte®, which tended to go along with higher abundances of some BV-associated bacteria and *Candida* spp., and lower levels of *L. crispatus*, which is contrary to the desired effect of administering probiotics for vaginal health. It has been previously described that *Bifidobacterium* spp. are not commensals in the FGTs of healthy South African women (Lennard *et al*. 2017), and that they are associated with BV in pregnant women (Rosenstein *et al*. 1996). Thus, while the inclusion of *Bifidobacterium* spp. in probiotics for gut health may be beneficial (reviewed by Siezen & Wilson 2010; Yuan *et al*. 2017), it is questionable to co-formulate *Bifidobacterium* spp. in products intended to improve vaginal health, and if done so, *Bifidobacterium* strains need to be carefully selected and evaluated to ensure only strains with beneficial properties are included. Taking these findings into considerations, future trials should make use of a product that contains FGT commensals, such as vaginal *Lactobacillus* strains that were isolated from healthy women, to increase chances of successful FGT colonisation and proliferation, as well as to increase the likelihood to improve vaginal health and contribute to BV cure.

While Vagiforte® PLUS Combo Pack did not appear to confer better BV cure rates than MetroGel™ alone, the product was found to be generally safe. In contrast, more than half of the participants reported symptomatic *Candida* infections after MetroGel™ use, although the quantitative analysis (*Candida* spp. qPCR) did not confirm this. However, *C. albicans* was the most common species as described

previously in sub-Saharan women (Mnge *et al*. 2017), and symptomatic infections might have been caused by an overgrowth of other *Candida* spp. than those tested here. Further, the sampling time point chosen in this pilot trial was one month after treatment completion and not immediately after MetroGel^{TM} use, and thus *Candida* communities might have had normalised already by then.

There are several clinical trials that have evaluated the efficacy of probiotics as adjunctive treatment (with metronidazole) for BV, with mixed results compared to the SOC arm. Anukam *et al.* (2006) observed an 88% BV cure rate in the intervention group compared to 40% in the SOC arm in a trial in 125 premenopausal Nigerian BV+ women using oral metronidazole (500 mg) twice daily from days one to seven, and oral capsules containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (1×10^9 CFU/strain/dose) twice daily from days one to 30. In contrast, many other studies with similar study designs had lower differences in BV cure rates between intervention and SOC groups (averaging 88% cure in the probiotic arm versus 66% in the SOC arm; Marcone *et al*. 2008 [n=84]; Martinez *et al*. 2009 [n=64]; Ehrstrom *et al*. 2010 [n=95]; Recine *et al*. 2016 [n=250]; Arooj *et al*. 2017 [n=60]). This pilot trial was clearly underpowered to determine a beneficial effect of probiotics on BV cure in South African women over metronidazole treatment alone, although it provided important regulatory and clinical criteria that need to be considered in the design of future studies. Based on cure rates of the above-cited studies, a sample size of 106 women (enrolled in a ratio 1:1 for the SOC: intervention arm) should detect a difference in BV cure of 0.88 versus 0.66, with 80% power, with the alpha level set at 0.05. Taking into consideration that ~20% of women will be excluded over the course of the trial due STI infections, at least 130 women (n=65 per treatment group) should be enrolled in a future trial assessing the effect of adjunctive probiotics on BV cure in South African women. Considering the high screen failure rate observed in this trial (only 35% were eligible), more than 370 would have to be screened to ensure that 130 BV-/STI- participants would be enrolled, unless the screening algorithms were improved.

Some of the strengths of this study were that all FGT sampling and pH reading were done by the same trained research nurse, all samples were processed by one researcher, and laboratory assays were also consistently performed by the same

researchers. Detailed information on vaginal practices, behavioural and sexual risk factors, study product use, side effects, and concomitant medications were collected, as well as whether the vaginal symptoms are a new or long-term problem, being an estimate for initial of recurrent BV. So far, no participant was lost to follow up. Some of the limitations of this pilot study were the low number of participants fulfilling the protocol in terms of eligibility and interventions, no information being available about any new sexual partners, and administration of the study drugs and the medical history (including previous BV) being self-reported.

In summary, the present pilot trial showed the safe use of oral and vaginal probiotics in South African BV+ women, and a high acceptance of the study product. Vaginal colonization with *B. bifidum* and *B.longum* was negatively associated with vaginal health, as it went along with higher abundances of *Candida* spp. and lower abundances of *L. crispatus*. Future double-blind, randomized, placebo-controlled trials with adequate screening and enrolment algorithms and sample sizes, using a product containing vaginal *Lactobacillus* spp., that are acknowledged by the regulatory authorities in South Africa, are needed to determine the efficacy of adjunctive probiotics on BV cure and recurrence in South African women.

Chapter 5

The potential of vaginal *Lactobacillus* strains from healthy South African women to improve vaginal health

5. Evaluation of the probiotic *in vitro* characteristics of South African vaginal *Lactobacillus* isolates compared to commercially available probiotic strains

5.1 Introduction

Only a few probiotic products are explicitly marketed for vaginal health in South Africa, and few of these contain bacterial species commonly found in a healthy FGT (Chapter 2 and 3). Bacterial strains with probiotic potential should fulfil specific criteria if their intended purpose is to be developed into a product to improve FGT health – collectively referred to as the preferred product profile (PPP). For the purposes of this study, the following characteristics of vaginal bacterial isolates were considered important for a vaginal PPP: (1) the organism/s selected should originate from the FGT of healthy women (with no BV or STIs); (2) these should include vaginal strains of *Lactobacillus* spp. that adhere well to vaginal and cervical cells; (3) these should grow well at a range of pHs and be tolerant of low pH; (4) in addition to themselves lowering culture pH through production of lactic acid; (5) these should produce metabolites that have anti-microbial and virucidal potential (including lactic acid, H_2O_2, biosurfactants and bacteriocins); (6) these should be capable of inhibiting adhesion and growth of pathogens or pathobionts; (7) be resistant to certain antibiotics used to treat BV and other common infections; and (8) these should not cause genital inflammation or epithelial cell cytotoxicity.

Vaginal *Lactobacillus* spp. commonly found in the lower FGT are highly adapted for this specialized niche, with significantly smaller genome sizes than their probiotic *Lactobacillus* counterparts, which suggest also that they may be more dependent on their host and other bacteria in their community to survive (reviewed by Mendes-Soares *et al*. 2014). *L. crispatus, L. gasseri, L. jensenii, L. vaginalis,* and/or *L. mucosae* have been associated with vaginal health in white, Hispanic and African American, and sub-Saharan African women (Ravel *et al*. 2011; Gajer *et al*. 2012; Anahtar *et al*. 2015; Lennard *et al*. 2017). *L. iners* was not considered in this Chapter, as the role of this species in vaginal health is controversial, since it is found at similar frequencies in women without BV, in those with intermediate Nugent scores, and in BV+ women (Ravel *et al*. 2011; Anahtar *et al*. 2015; Lennard *et al*., 2017).

Lactobacillus strains being considered for probiotic development should adhere well to vaginal and cervical cells, as adherent isolates are possibly more likely to remain resident in this niche and confer their health benefit to the host. Adherent bacterial strains are thought to be better able to competitively prevent the adhesion of FGT pathogens or pathobionts that comprise BV (Tomás *et al*. 2005; Atassi *et al*. 2006; Coudeyras *et al*. 2008; Mastromarino *et al*. 2002). It is also desirable that the isolates should produce stable biofilms. Biofilm formation promotes the colonization and long-term persistence of *Lactobacillus* strains in the lower FGT, thereby forming a stable ecosystem, which discourages pathogens from invading an already colonized ecological niche (Jones and Versalovic 2009; Ventolini *et al*. 2015; Terraf *et al*. 2016).

Lactobacillus strains being considered for probiotic product development should be better able to tolerate low pHs than pathogens or pathobionts, which would give them a growth advantage and therefore increase their ability to confer a health benefit to the host (Hossein Nezhad *et al*. 2010; Huang and Adams 2004; Silva *et al*. 2005). In addition, these isolates should be able to decrease vaginal pH to <4.5, predictive of vaginal health, as acidification of the vaginal milieu has been shown to inhibit viral and bacterial pathogens (Boskey *et al*. 1999; Barrons and Tassone 2008; O'Hanlon *et al*. 2011; Nardini *et al*. 2016). In addition to achieving low pHs, the selected *Lactobacillus* strains should be able to produce anti-microbial metabolites, including L-and D-lactic acid, which are involved in inhibition of viral and bacterial pathogens (Aldunate *et al*. 2013; Valore *et al*. 2002; O'Hanlon *et al*. 2011). In addition, it would be beneficial if *Lactobacillus* strains produce biosurfactants and bacteriocins, which have been shown to inhibit the adhesion of pathogens and pathobionts found in the urogenital tract and during BV (Velraeds *et al*. 1996; Gudina *et al*. 2010; Turovsky *et al*. 2009). The selected *Lactobacillus* strains should also be able to inhibit the growth of BV-associated bacterial species (such as *G. vaginalis* and *P. bivia*) and other FGT pathogens associated with adverse reproductive outcomes (such as GBS and *C. trachmonatis*; Atassi *et al*. 2006; Castro *et al*. 2015; Teixeira *et al*. 2012; Andreeva *et al*. 2016; Coudeyras *et al*. 2008; Mastromarino *et al*. 2014).

It is desirable that *Lactobacillus* strains being considered for probiotic product development are resistant to antibiotics commonly used for the treatment of BV

(Stecher *et al*. 2012; Van Reenen & Dicks 2011; Gueimonde *et al*. 2013). However, antibiotic resistance has become a concern in the selection of probiotic strains, since beneficial bacteria can transfer resistance genes to potentially pathogenic bacteria via horizontal gene transfer (Barlow 2009), thus antibiotic resistance profiles should be determined. It is also desirable that the full genome of the potential probiotic strains be sequenced, as this would allow verification that antibiotic resistance and toxicity genes are absent, thus allowing an *in silico* estimation of safety and identification of genes responsible for carbohydrate transport, metabolism and stress responses (Douillard *et al*. 2018; Siezen and Wilson 2010; Fukao *et al*. 2013; Li *et al*. 2016).

The chosen *Lactobacillus* strains should not induce genital inflammation when administered vaginally. While genital inflammation is an important part of the mucosal immune response that controls infections, cytokines produced during this inflammatory response are able to recruit activated HIV target cells to the lower FGT, thereby increasing the risk of HIV acquisition (Tomalka *et al*. 2016; Masson *et al*. 2015; McKinnon *et al*., 2018). Given the high rates of both BV and HIV in South Africa, this is particularly important.

It is desirable that the selected *Lactobacillus* strains can be co-formulated with prebiotics that can be metabolized by these strains, as previous studies have shown that *Lactobacillus* survival is increased in the presence of sugars and amino acids that the strains can metabolize (Corcoran *et al*. 2005; Zhang *et al*. 2012). Furthermore, they should demonstrate stability during the manufacturing process, maintaining good viability and reach high concentrations in mass culture (biofermenters). Ideally, the *Lactobacillus* strains chosen for probiotic production should have shelf-life >3 years at room temperature (~30°C) once formulated, to ensure easier shipment, distribution and storage logistics, especially within Africa (Anukam 2007; Anal and Singh 2007). This would also contribute to lower product pricing. Currently available probiotics on the South African market are not affordable for those most in need, so keeping prices low would be critical (Happel *et al*. 2017).

While it would be desirable for the selected strains to be formulated for vaginal delivery, oral capsules might be cheaper and more feasible. Although the efficacy of oral delivery of probiotics for reproductive health is still being debated, previous

studies have suggested that probiotics do improve vaginal health when given orally, and orally administered *Lactobacillus* strains have been recovered vaginally, although at low concentrations (Reid *et al*. 2001; Reid *et al*. 2003; Morelli *et al*. 2004).

The aim of this Chapter was to (i) isolate and then evaluate several of these key PPP characteristics of vaginal *Lactobacillus* strains from young South African women, including growth under various pH conditions, ability to decrease culture pH, adherence to cervical cells, L- and D-lactic acid and H_2O_2 production, antibiotic susceptibility profiles, inhibitory effect on the growth of *G. vaginalis, P. bivia,* and GBS, and their effect on inflammatory markers and cervical cell viability *in vitro*, and (ii) to compare these to commercially available probiotic strains that are currently used in probiotic formulations for vaginal health (described in Chapter 3). To objectively rank the vaginal *Lactobacillus* isolates and compare them with commercially in South Africa available probiotic *Lactobacillus* strains, a PPP scoring system was developed to select the top vaginal *Lactobacillus* strains with the most critical probiotic characteristics.

5.2 Methods

5.2.1 Cohort description and genital samples collected

Between November 2013 and February 2015, the Women's Initiative in Sexual Health (WISH) study enrolled 149 female adolescents girls and young women (AGYW; 16-22 year old) from Masiphumulele, an area with a high HIV and STI incidence in Cape Town, South Africa, through the Desmond Tutu HIV Foundation (DTHF) Youth Centre, to investigate biomedical factors associated with increased susceptibility to HIV in young women (Barnabas *et al*. 2018). Adolescents who were ≥18 years provided informed written consent, while participants <18 years old provided written assent, in addition to parental consent being obtained from their parent(s)/legal guardian(s). Ethics approval was obtained from the UCT Human Research Ethics Committee (HREC Ref 267/2013). For the parent study, eligible AGYW were HIV-negative, sexually active, generally healthy and neither pregnant nor menstruating at the study visits. In addition, they were only eligible to enrol if they had not taken antibiotics in the previous two weeks, as one of the aims of the parent study included vaginal 16S sequencing (Lennard *et al*. 2017). In addition to the standard risk reduction counselling offered at the DTHF, AGYW were asked to abstain from sex, douching or using spermicides for 48 hours prior to their study visit. From each AGYW, the following genital tract samples were collected: Softcup® menstrual cup for collection of cervicovaginal mucus to measure genital cytokines and to isolate *Lactobacillus* strains, vulvovaginal swab to test for STIs, lateral vaginal wall swab to prepare a wet mount slide to test for BV, candidiasis and to measure vaginal pH (measured at the clinic by a trained nurse using a colour-fixed pH indicator strip; pH range: 3.6-8.2; Macherey-Nagel, Germany), lateral vaginal wall swab to measure the presence of prostate specific antigen (PSA) as a marker of recent sex, endocervical cytobrush to collect cervical mononuclear cells, and a lateral vaginal wall swab to sequence bacterial 16S rRNA (Lennard *et al*., 2017). The young women were tested for STIs (including *C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium* in the discharge panel; and HSV-1, HSV-2, *H. ducreyi, T. pallidum* and *Lymphogranuloma venerum* in the genital ulcer panel; using two validated in house Multiplex PCRs; Lewis *et al*. 2012; Barnabas *et al*. 2017), and BV by Nugent Scoring.

Softcup® menstrual cup samples from twelve participants were selected based on being negative for BV (Nugent 0-3) for this sub-study, which was approved by UCT as part of this PhD thesis (HREC Ref 319/2016), to isolate vaginal *Lactobacillus* strains. For comparison, vaginal *Lactobacillus* isolates were also isolated from four women with Nugent scores 4-6, and ten women with BV (Nugent 7-10).

5.2.2 Isolation of vaginal Lactobacillus strains from FGT samples

To prepare primary vaginal *Lactobacillus* isolate stocks, 50 µL of Softcup® secretions were diluted with 250 µL of PBS and 150 µL of 60% glycerol (20% v/v final), which were stored at -80°C. The isolation of pure bacterial strains has been described in detail in Chapter 3, section 3.2.3. Briefly, a sterile loop was used to streak the frozen cervicovaginal secretions onto MRS agar plates (to enrich for *Lactobacillus* spp.). These were incubated under anaerobic conditions at 37ºC for 48 hours, and single colonies with distinct morphologies were picked, re-streaked onto new MRS agar plates, and incubated under the same conditions until homogenous colonies were obtained. At least three rounds of purification were performed to ensure purity of colonies. Single, pure colonies were picked, inoculated into MRS broth, grown at 37ºC for 48 hours, and stocks were frozen down with 60 % glycerol (20% v/v final) and stored at –80ºC. The bacterial species of the primary stocks were identified by MALDI-TOF by Dr Rémy Froissart (at the University of Montpellier, Montpellier, France). In addition, the identification by MALDI-TOF was repeated at the end of the study once the characterisation of vaginal *Lactobacillus* strains was completed to confirm the initial species determination and no change in species determination over the study period.

5.2.3 Determining bacterial counts at a standardized optical density (OD)

The method used for determining bacterial counts (CFU/mL) was described in Chapter 3, section 3.2.5. Briefly, overnight cultures of primary *Lactobacillus* stocks were adjusted to OD_{600nm} 0.1 (±0.01), and serial dilutions (10^0, 10^{-2} and 10^{-4}) were plated onto MRS agar plates, incubated anaerobically at 37ºC for 48 hours, colonies were counted, and the CFU per mL at OD_{600nm} 0.1 (±0.01) was calculated for each isolate. Experiments were repeated a total of three times.

5.2.4 Acid tolerance of Lactobacillus growth

The method for determining the growth kinetics of vaginal *Lactobacillus* isolates at various pHs to evaluate the extent of acid tolerance was described in Chapter 3 section 3.2.6. In brief, overnight cultures were standardized to OD_{600nm} of 0.1 (±0.01) in MRS broth that was adjusted to pHs 2.0, 3.5, 4.0 and 4.5 or 6.0 using hydrochloric acid, and incubated in triplicate wells under anaerobic condition in a sealed 96-well plate at 37ºC for 48 hours. To track the growth kinetics over 48 hours, absorbance (OD_{600nm}) was measured at 0, 3, 6, 12, 24, 32 and 48 hours. Area under the curve (AUC) was calculated for each of the *Lactobacillus* isolates. Experiments were repeated a total of three times.

5.2.5 Influence of bacterial growth on culture pH

The method for determining the effect of vaginal *Lactobacillus* isolates on culture pH was previously described in Chapter 3 section 3.2.7. Briefly, cultures adjusted to OD_{600nm} 0.1 (±0.01) in MRS broth were incubated anaerobically at 37ºC for 3, 6, 12, 24 and 48 hours, and the pH of the culture supernatant was measured at each time point using a calibrated pH Meter (Jenway Bench pH Meter 2510, Bibby Scientific, UK). Experiments were performed in duplicate.

5.2.6 Measurement of lactate and H_2O_2 production in culture

The colorimetric method to measure concentrations of D- and L-lactate (MAK058 and MAK064, Sigma-Aldrich®, USA) and the fluorimetric method to measure H_2O_2 (MAK165, Sigma-Aldrich®, USA) in culture supernatants was described in Chapter 3, section 3.2.10. The concentrations of all three of these metabolites were determined from anaerobically grown *Lactobacillus* culture supernatants, harvested at 24 hours, in duplicate (section 5.2.5)

5.2.7 Adhesion of Lactobacillus isolates to human ecto-cervical Ca Ski cells

Adhesion of *Lactobacillus* strains to Ca Ski cells was measured using the same method as described in Chapter 3, section 3.2.9, by adding $4.2x10^6$ CFUs of *Lactobacillus* isolates to Ca Ski cells grown to 80-90% confluence, incubating them

for 3 hours at 37°C and 5% CO_2, washing unbound bacterial cells off using PBS, lysing the Ca Ski cell monolayer, harvesting the cell lysate to plate serial dilutions onto MRS agar plates that were then incubated anaerobically at 37ºC for 48 hours, counting colonies to determine the CFU/mL of adhered bacteria, and finally calculating the percentage of adhered bacteria. Experiments were repeated a total of three times.

5.2.8 Antimicrobial activity of vaginal Lactobacillus strains

The ability of culture supernatant from a panel of 25 vaginal *Lactobacillus* strains to inhibit a panel of six *G. vaginalis* (anaerobic culture), six *P. bivia* (anaerobic culture), and six Group B Streptococcus isolates (GBS; aerobic culture) has been described in Chapter 3, section 3.2.10. The *G. vaginalis* and *P. bivia* isolates were kindly provided by Dr Rémy Froissart (CNRS, Montpellier, France) while the GBS isolates were kindly provided by Dr Shameem Jaumdally (Division of Immunology, UCT, South Africa). Selected *Lactobacillus* strains included: *L. crispatus* 70.6 PA. 73.55 a, 96.9 PB, 100.16a and ATCC®; *L. gasseri* 114.1 PA, 117.73 PA, 107.10 PB, 94.98 PB, 114.30 PA and ATCC®; *L. jensenii* 88.33 PA, 95.1 PA, 92.1 PA, 92.27 PA, 95.31 PA and ATCC®; *L. vaginalis* 79.24 PA, 80.3 b, 80.23 b, 91.8 a, 100.13 PA and ATCC®; *L. mucosae* 80.3 a, 86.30 PA, 87.21 PA, 90.13 PA, 102.33 PA. This section focused on only 25/57 isolates because of how labour intensive each evaluation was; the 25 strains were selected based on the BV status of their donor and their performance in previous experiments. Experiments were repeated a total of three times.

5.2.9 Impact of Lactobacillus strains on cervical cell viability and cytokine responses

To evaluate whether *Lactobacillus* isolates influenced cervical epithelial cell viability or induced inflammatory cytokine responses *in vitro*, Ca Ski cells were seeded into 24-well tissue culture plates ($1x10^5$ cells/well) in antibiotic-free DMEM supplemented with 10% FCS and grown for 24 hours at 37°C and 5% CO_2 to 80-90 % confluence. For this part of the analysis, the screening panel was further reduced to 15 of the 57 vaginal *Lactobacillus* isolates, due to budget restrictions regarding the Luminex® assay, representing three strains per species: *L. crispatus* [100.16 a, 96.9

PB, 95.34 PA], *L. gasseri* [117.73 PA, 100.46 PA, 114.30 PA], *L. jensenii* [88.33 PA, 92.27 PA, 95.31 PA], *L. vaginalis* [80.3 b, 79.24 PA, 100.13 PA], and *L. mucosae* [102.33 PA, 86.30 PA, 80.23 a]. Bacterial overnight cultures were adjusted to $4.2x10^6$ CFUs in 500 µL of antibiotic-free DMEM supplemented with 10% FCS, added in duplicate to the cell monolayer and incubated for 24 hours at 37°C and 5% CO_2. After the incubation period, the supernatants were transferred to an Eppendorf tube, and the cells were lifted by adding 250 µL of 0.25% trypsin/EDTA to each well. The cells were transferred to the same Eppendorf tube, and viability was evaluated by staining Ca Ski cells with 1:1 ratio with trypan blue solution (0.4 %, Sigma-Aldrich, USA). The number of cells per mL and percentage of viable cells was determined using a TC20™ Automated Cell Counter (Bio-Rad®, USA).

Luminex® was used to measure the concentrations of seven cytokines (including MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1RA and IL-10) in cell culture supernatants from Ca Ski cells co-cultured with the 15 vaginal *Lactobacillus* strains. These were compared to six of the probiotic strains described in in Chapter 3, including *L. reuteri* and *L. rhamnosus* RFZ1006 from Reuterina™ Femme®, *L. rhamnosus* 7447212 from Muvagyn®, *L. rhamnosus* 0154 from Provacare™, *L. rhamnosus* 0200 from Gynophilus®, and *L. acidophilus* S21134 from Vagiforte®. Culture supernatants from Ca Ski cells that had not been co-cultured with *Lactobacillus* isolates were used as a negative control. Culture supernatants were filtered using Corning® Costar® Spin-X® Plastic Centrifuge Tube Filters (0.22 µm, Sigma-Aldrich®, USA) prior to performing the Luminex® assay to exclude cellular debris. The Bio-Plex Pro™ Human Cytokine Luminex kit (Lot 64064139, Bio-Rad®, USA) was used to measure the concentrations of cytokines. The chemokines MIP-1α, MIP-1β, IP-10, IL-8 and MCP-1 were selected because they have previously been linked to risk of HIV acquisition in South African women (Liebenberg *et al*. 2017; Masson *et al*. 2015). In addition, IL-1RA and IL-10 were selected because they are anti-inflammatory and therefore may impart a protective environment in the lower FGT. The assay was conducted according to the manufacturer's instructions, using 50 µL of sample filtrate, and each sample was measured in duplicate. The data was acquired using the Bio-Plex™ Suspension Array Reader (Bio-Rad Laboratories Inc®, USA), and a 5PL regression line was used to determine the cytokine concentrations from the standard curves using the according Bio-Plex™ manager software. All values below the

detection limit were recorded as half of the lowest measured concentration for each cytokine.

5.2.10 Antibiotic susceptibility profiles of Lactobacillus strains

Sensitivity of the full panel of 57 vaginal *Lactobacillus* isolates to various commonly used antibiotics was determined using either the disc diffusion method (for metronidazole, clindamycin, penicillin and amoxicillin, described in in Chapter 3 section 3.2.8), or MIC assays (for rifampicin and rifabutin; described in Chapter 3 section 3.2.8). For rifabutin (R3530, Sigma-Aldrich®, USA), concentrations ranging from 5 – 0.00488 µg/mL were tested. For rifampicin (R3501, Sigma-Aldrich®, USA), concentrations ranging from 25 – 0.024 µg/mL were tested. For 14/57 vaginal and six probiotic *Lactobacillus* strains (including the same strains that were included in the Luminex® assay, section 5.2.9), broader antibiotic susceptibility profiles were determined using Sensititre^{TM} GPALL1F plates (for ampicillin, cefoxitin, chloramphenicol, ciprofloxacin, clindamycin, daptomycin, erythromycin, gentamycin, levofloxacin, linezolid, moxifloxacin, nitrofurantoin, oxacillin with 2% NaCl, penicillin, quinupristin/dalfopristin, rifampin, streptomycin, tetracycline, tigecycline, trimethoprim/sulamethoxazole and vancomycin; Thermo Fisher Scientific Inc., USA).

For the Sensititre^{TM} plates, overnight cultures of bacterial isolates were standardised to $1x10^5$ CFU (as per manufacturer's instructions) and microplates containing lyophilized antimicrobials were inoculated with 100 µL of the CFU-adjusted broth. The plates were incubated anaerobically (sealed with plate sealers) at 37 °C for 48 hours. MICs were read by visual inspection, as recommended by the Clinical and Laboratory Standard Institute (Wayne, PA, USA). The lowest concentration of antibiotic that prevented bacterial growth (turbidity) was considered to be the MIC.

5.2.11 Development of a scoring system to rank Lactobacillus strains

To rank the vaginal *Lactobacillus* strains, taking the PPP criteria into consideration, a scoring system was developed that included: (1) growth kinetics at various pHs, (2) ability to lower pH, (3) adherence to cervical cells, (4) production of L-/D-lactate and H_2O_2, (5) inhibition of *G. vaginalis*, *P. bivia* and GBS. For each of these

characteristics, isolates were ranked relative to the whole group into quartiles; and assigned a score of 0 if a particular characteristic for that isolate was < 25th percentile of all the isolates; scored 1 if it was between the 25th and 50th percentile; 2 if it was between the 50th and 75th percentile, and 3 if it was above the 75th percentile for all the isolates tested. Because some of these characteristics may be related (eg. ability to lower pH and production of lactate), the scoring system was then weighted to account for relative co-linearity between characteristics using a Spearman rank correlation co-efficient (as described further in section 5.3.11 and **Figure 5.19**) using the following equation: Weighting=1-[sum of column Spearman Rho ÷ sum of column sums).

5.2.12 Statistical Analyses

GraphPad Prism6® (GraphPad Software, USA) and STATA version 11.0 (StataCorp, USA) was used for statistical analysis. The Mann-Whitney U test was used to compare two groups of non-parametric continuous variables, and Spearman Rank tests were used for non-parametric correlations. The Kruskal-Wallis one-way analysis of variance was used to compare variables with three or more groups. A false-discovery rate step-down procedure was used to adjust p-values for multiple comparisons, and adjusted p-values <0.05 were considered significant. A step-up strategy was used where the different variables that could potentially contribute to a measured characteristic were included one at a time, and R^2 values were compared as a measure of model fit. An R^2 value gives the proportion of the variance that can be accounted for by the regression model. The step-up model was checked for the assumption of linearity, homogeneity of variance of residuals, multi-collinearity, influential points were detected using Cooks residuals and Leverage, outliers were detected and dropped, and the model was re-run if suitable. Multivariate logistic regressions were carried out to assess the contribution of several factors measured on pathogen inhibition. In addition, 95% confidence intervals and p-values were used to assess statistical significance.

5.3 Results

5.3.1 Description of vaginal Lactobacillus isolates and their donors

In order to evaluate characteristics of vaginal *Lactobacillus* strains from South African young women important for the development of a local probiotic product for vaginal health, vaginal anaerobic microbes were enriched and isolated from twelve young women, aged between 16-22 years (median 18 years) from Masiphumulele in Cape Town, South Africa, who were BV- using Nugent criteria (Score 0-3). As a comparison group, four young women who had intermediate Nugent scores (Score 4-6) and ten women who were BV+ (Nugent score 7-10) were included. The majority of BV- young women were also STI- (9/12). The three BV- women with STIs (STI+) had infections with *C. trachomatis* and *N. gonorrhoea, C. trachomatis, N. gonorrhoea* and *T. vaginalis*, and *N. gonorrhoea* only, respectively. Similarly to BV- women, 7/10 BV+ and 3/4 women with intermediate scores were STI-. Of those with STIs, they either had *C. trachomatis* infections (n=2) or were DNA positive for HSV-2 (n=1). As anticipated, vaginal pH was lower in BV- women (median 4.7; IQR 4.1 – 5.0) than those with BV (median 5.2; IQR 4.9-5.4, p=0.0365), although the median vaginal pH for those without BV was still higher than considered healthy according to Amsel's criteria (pH $\leq$ 4.5).

A total of 57 vaginal *Lactobacillus* strains were isolated from these young women, including ten *L. crispatus* (from seven women), nine *L. gasseri* (from five women), 18 *L. jensenii* (isolates from ten women), eight *L. vaginalis* (from seven women), and twelve *L. mucosae* isolates (from eight women; **Table 5.1**). *L. crispatus* (9/10), and *L. jensenii* strains (13/18) tended to be isolated from women who were BV-, while *L. gasseri* (5/9) and *L. vaginalis* (5/8) were obtained from both BV- and BV+ women (**Table 5.1**). *L. mucosa*e strains tended to come from women who were BV+ (10/12 from BV+ women). Since sequencing has not been performed yet, *Lactobacillus* isolates of the same species that were derived from the same woman were only included if they showed different biological characteristics, to increase the likelihood that they were not identical on strain level. In particular, vaginal samples from 7/26 women (5/7 BV-) yielded several different *Lactobacillus* spp. For example, one *L. crispatus* and four *L. jensenii* isolates were obtained from PID 95, who was BV- and

STI- (**Table 5.1**). Single *Lactobacillus* spp. were obtained from the remaining 19/26 participants.

Table 5.1. Details of included *Lactobacillus* strains

Species	N Isolates	SC ID[±]	Age [years]	BV[#] Status	STI[@] status	Details*
L. crispatus	10	**70**	19	-	-	**70.1 PA, 70.6 PA**
		80	18	-	-	**80.3 a**
		94	18	-	-	**94.77 PA**
		95	17	-	-	**95.34 PA**
		96	17	-	-	**96.9 PA, 96.9 PB, 96.27 PA**
		100	18	-	-	**100.16 a**
		73	16	+	-	73.55 a
L. gasseri	9	**94**	18	-	-	**94.98 PB**
		100	18	-	-	**107.10 PB, 107.7 PA**
		107	19	-	+	**100.5 PA, 100.46 PA**
		114	17	Int.	+	114.1 PA, 114.30 PA, 114.12 PB
		117	17	+	-	117.73 PA
L. jensenii	18	**88**	20	-	-	**88.10 PA, 88.33 PA**
		94	18	-	-	**94.70 PA**
		95	17	-	-	**95.1 PA, 95.22 PA, 95.31 PA, 95.37 PA**
		96	17	-	-	**96.8 PA, 96.45 PA, 96.45 PB**
		72	16	-	+	**72.14 PA, 72.22 PA**
		93	17	-	+	**93.18 PA**
		84	18	Int.	-	73.2 PA,
		73	16	+	-	84.35 PA
		89	18	+	-	89.50 PA
		92	18	+	-	92.1 PA, 92.27 PA
L. vaginalis	8	**80**	18	-	-	**80.3 b, 80.23 b**
		88	20	-	-	**88.5 b**
		91	18	-	-	**91.8 a**
		100	18	-	-	**100.13 PA**
		73	16	+	-	73.27 PA
		81	18	+	-	81.17 a
		79	18	+	+	79.24 PA
L. mucosae	12	**80**	18	-	-	**80.23 a**
		99	20	-	-	**99.1 PA**
		85	16	Int.	-	85.1 PA, 85.30 PA
		102	17	Int.	-	102.33 PA
		87	19	+	-	87.5 PA, 87.21 PA
		98	21	+	-	98.46 PA, 98.52 PA
		86	22	+	+	86.4 PA, 86.30 PA
		90	18	+	+	90.13 PA
Total	**57**	**26**				

[±]Softcup® Identification Number (PID), **bold SC IDs indicate which were from BV-/STI- participants**

[#]BV status: Nugent 0-3 (-), 4-6 (Int.), 7-10 (+)

[@]STIs included *C. trachomatis, T. vaginalis, N. gonorrhoe, M. genitalium*, HSV-2 DNA; negative for all (-), positive for ≥1 (+), not determined (n/a)

*Labelling as the following: First two digits refer to participant, following numbers refer to isolate number; PA/PB refers to pure (P) isolate A (picked first) and B (picked afterwards from the same plate), species colour-coded (pink=*L. crispatus*, blue=*L. gasseri*, green=*L. jensenii*, orange=*L. vaginalis*, grey=*L. mucosae*), **Bold labels indicate those obtained from BV- women.**

The characteristics of these 57 vaginal *Lactobacillus* strains were compared to four *Lactobacillus* ATCC® reference strains (*L. crispatus* 33197™, *L. gasseri* 9857™, *L. jensenii* 25258™, and *L. vaginalis* 49540™). In addition, they were also compared to ten probiotic *Lactobacillus* strains (described in Chapter 3): *L. reuteri* and *L. rhamnosus* RFZ1006 (from Reuterina™ Femme®), *L. rhamnosus* 7447212 (from Muvagyn®), *L. rhamnosus* 0154 (from Provacare™), *L. rhamnosus* 0200 (from Gynophilus®), *L. rhamnosus* C21134 (C=oral capsule) and S21134 (S=vaginal spray) and *L. acidophilus* C21134, S21134 and T20868 (T=vaginal tablet, from Vagiforte®).

5.3.2 Standardized bacterial counts at OD_{600nm}

As described in Chapter 3, *Lactobacillus* strains sometimes differ considerably by size, which may influence their relative counts at a standardized absorbance (OD_{600nm}). Therefore, the actual bacterial concentration at a standardized OD_{600nm} of 0.1 (±0.01) was measured for all 57 clinical isolates. The bacterial concentrations varied by up to one log between *Lactobacillus* spp. (**Figure 5.1**), with *L. jensenii* being detected at the highest concentration at this standardized OD (median $6.3x10^7$ CFU/mL; IQR $9.0x10^6$– $8.3x10^7$; **Figure 5.1**D) and *L. gasseri* at the lowest relative concentration ($1.4x10^6$ CFU/mL; IQR $5.2x10^5$ – $5.6x10^6$; **Figure 5.1**B). The bacterial counts of isolates from BV- women did not differ to isolates from women with Nugent scores 4-10 (**Figure 5.1**). Vaginal *L. jensenii* species demonstrated the lowest intra-species variance in concentration (65.4% CV; n=18) while *L. gasseri* strains demonstrated the highest within species variation in concentration (216.1% CV; n=9). The standardised bacterial counts of the vaginal isolates within each *Lactobacillus* spp. were comparable to the ATCC® reference strains, with exception of *L. gasseri* ATCC®, which yielded higher concentrations ($1.1x10^8$ CFU/mL) than the vaginal *L. gasseri* isolates (**Figure 5.1**B). The median CFU/mL at this standardised OD did not differ between vaginal and probiotic *Lactobacillus* spp. (*L. reuteri* $1.9x10^7$ CFU/ml [IQR 1.6–$2.1x10^7$], *L. rhamnosus* $3.3x10^7$ CFU/ml [IQR 2.2–$6.0x10^7$]; *L. acidophilus* $6.0x10^6$ CFU/ml [IQR 4.8–$9.0x10^6$]; Chapter 3 section 3.3.3.1)

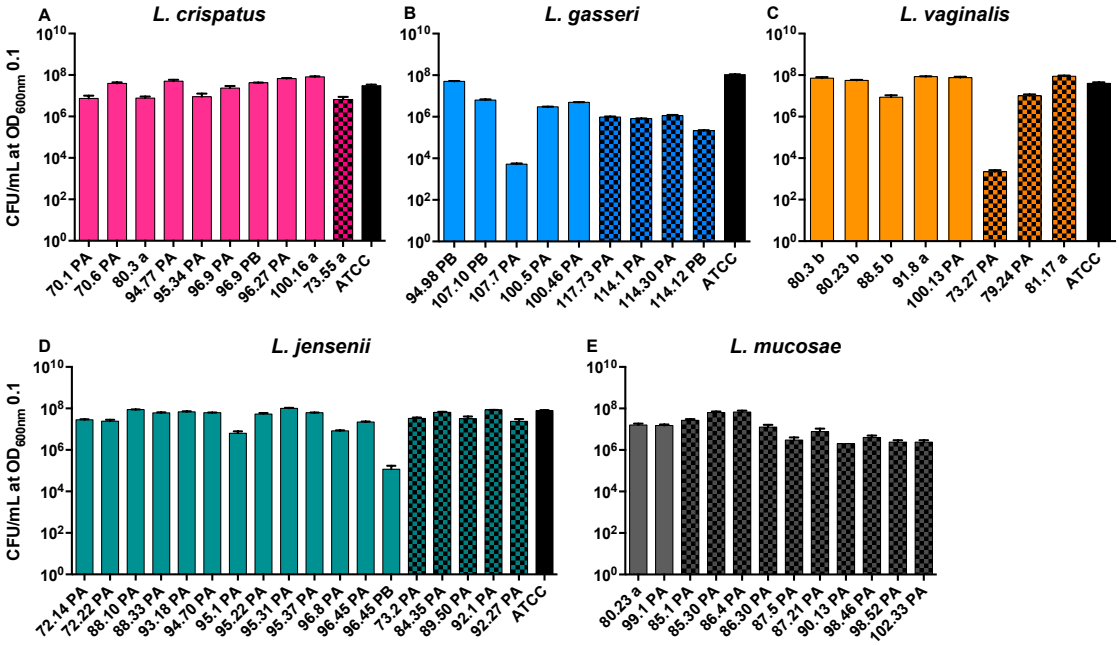


Figure 5.1. Comparison of vaginal *Lactobacillus* spp. concentrations achieved at a standardized OD_{600nm} 0.1 (±0.01). The CFU/mL at OD_{600nm} 0.1 (±0.01) of *L. crispatus* (A, pink bars), *L. gasseri* (B, blue bars), *L. vaginalis* (C, orange bars), *L. jensenii* (D, green bars), and *L. mucosae* (E, grey bars) isolates was determined by plating serial dilutions. The ATCC® strains are shown by black bars. Solid bars represent isolates that were obtained from BV- women (n=12), while patterned bars indicate isolates that were obtained from women with a Nugent score above 3 (BV intermediate, n=4 and BV+, n=10).

5.3.3 Adhesion of vaginal Lactobacillus spp. to ectocervical cells

All *L. crispatus* (9/9), *L. vaginalis* (5/5), the majority of *L. jensenii* (11/13) and about half of the *L. gasseri* (3/5) and *L. mucosae* (1/2) strains obtained from BV- women showed some degree of adhesion to ectocervical epithelial Ca Ski cells, which was similarly observed in strains isolated from women had a Nugent >3 (**Figure 5.2**). While adherence to this ectocervical cell line was a general feature of the vaginal isolates, some strains showed better adhesion than others (defined as >2% of added bacteria adhering to cells, including 2/9 *L. crispatus*, 1/5 *L. gasseri*, 3/13 *L. jensenii*, 1/5 *L. vaginalis* and 1/2 *L. mucosae* strains from BV- women. In contrast, only four isolates from women with Nugent scores 4-10 showed >2% adhesion (including *L. gasseri* 117.73 PA, *L. vaginalis* 73.27 PA and *L. mucosae* 85.1 PA and 98.52 PA). Overall, *L. crispatus* vaginal isolates from BV- women tended to adhere best [median of 1.6% (IQR 0.5–2.2%) of bacilli adhering to Ca Ski cells], followed by *L. mucosae* (1.3%, IQR 0–2.6%), *L. jensenii* (0.8%, IQR 0.2–2.1%), *L. vaginalis* (0.6%, IQR 0.2–1.7%) and *L. gasseri* (0.4%, IQR 0–2.0%; **Figure 5.2**). Although not significant, *L. mucosae* strains from BV- women tended to adhere more strongly than strains isolated from

women with Nugent scores 4-10 (0.4%, IQR 0.1–1.8%; **Figure 5.2**E). Of all the *Lactobacillus* spp. tested, adherence of *L. crispatus* was the least variable (CV 58.8%). In contrast, adhesion variability of *L. gasseri, L. jensenii*, *L. vaginalis* and *L. mucosae* isolates were largely similar (CVs of 111.5%, 99.9%, 115.0%, and 118.3 %, respectively). This was not influenced by the BV status of the donors. These data suggest that adhesion to Ca Ski cells is somewhat strain-specific, but *L. crispatus* isolates tended to generally adhere better than isolates from other *Lactobacillus* spp.

Several of the vaginal isolates (2/10 *L. crispatus*, 2/9 *L. gasseri*, 6/8 *L. vaginalis*, and 11/18 *L. jensenii*) also adhered better than their respective ATCC® reference strains. Compared to the vaginal isolates, probiotic *Lactobacillus* strains generally showed intermediate levels of adhesion, with 1.0% (IQR 0.6–1.3%) of *L. rhamnosus*, 0.57% (IQR 0.3–0.7%) of *L. acidophilus* and 0.6% of *L. reuteri* isolates adhering (Chapter 3 section 3.3.3.6).

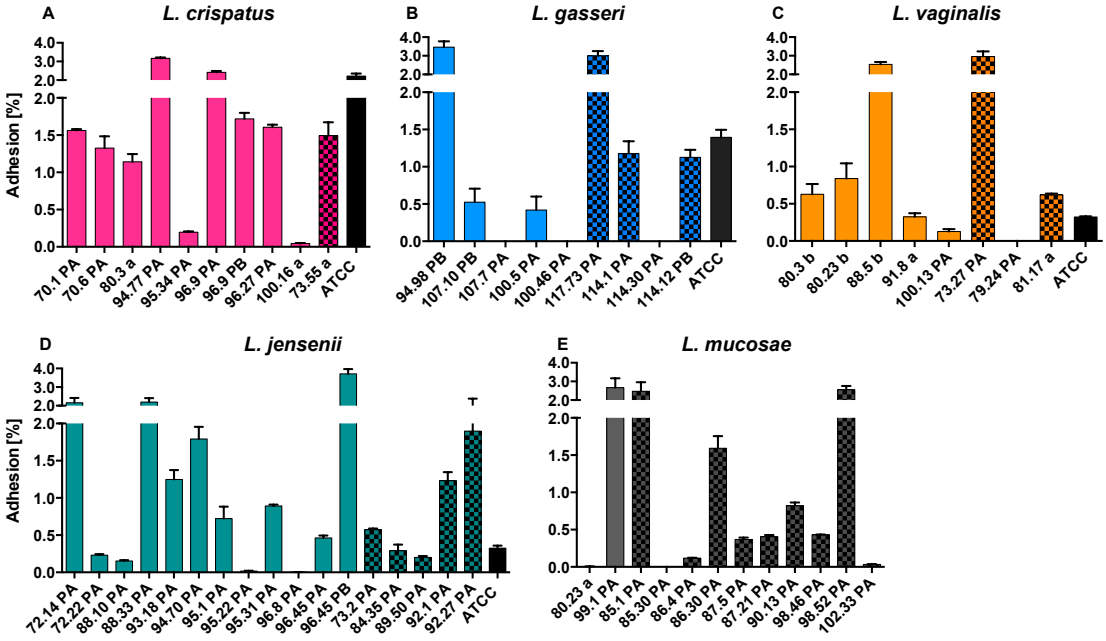


Figure 5.2. Adherence of vaginal *Lactobacillus* isolates to ectocervical Ca Ski cells. $4.18x10^6$ CFUs of *Lactobacillus* cultures were added to Ca Ski cells and incubated for 3 hours at 37°C and 5% CO_2. Unbound bacteria were washed off, cells were lysed, and serial bacterial dilutions were plates onto MRS agar plates to determine the percentage of adhered bacteria for *L. crispatus* (A, pink bars), *L. gasseri* (B, blue bars), *L. vaginalis* (C, orange bars), *L. jensenii* (D, green bars), and *L. mucosae* (E, grey bars). ATCC® strains are shown by black bars. Solid bars represent isolates that were obtained from BV- women (n=12), while patterned bars indicate isolates that were obtained from women with Nugent Scores 4-10 (BV intermediate n=4 and BV+ n=10).

5.3.4 Growth kinetics of vaginal Lactobacillus isolates at low pHs

The growth kinetics and maximum titre achieved for *Lactobacillus* strains at a culture pH 6.0 (previously determined to be optimal for *Lactobacillus* spp.; Hossein Nezhad *et al*. 2010) is critical to taking these isolates to scale in probiotic production. It was also important to investigate the ability of these primary vaginal isolates to survive lower pHs typically associated with vaginal health in the lower genital tract of women (pH <4.5; ranging from 3.5 to 4.5; Boris & Barbés 2000; Boskey *et al*. 2001). In addition, their ability to resist extreme pHs, such as found in the upper GIT (pH ~2), was measured as a surrogate for their ability to survive oral delivery.

At pH 6.0, the vaginal *Lactobacillus* isolates grew variably between and within species (**Figure 5.3**, first column). *L. crispatus* and *L. jensenii* strains from BV- women grew the best at pH 6.0 (median area under the curve [AUC] of 68.1 [IQR 66.7-68.7] and 60.3 [IQR 11.7-68.0], respectively), followed by *L. mucosae* (AUC 56.4, IQR 44.3-68.6), *L. vaginalis* (AUC 47.1, IQR 18.5-67.8) and *L. gasseri* (AUC 41.7, IQR 8.4-60.1) strains (**Figure 5.3**). The variability in growth at this pH followed the same order, with CVs ranging from 29.6% for *L. crispatus* to 76.4% for *L. gasseri*. *Lactobacillus* isolates from women with Nugent scores 4-10 tended to grow to lower optical densities than those from women who were BV-, albeit not significantly.

At pHs 4.5, 4.0, and 3.5, all nine of the *L. crispatus* strains tested from BV- women showed reduced growth compared to pH 6.0, although they appeared to tolerate the lower pH better than other *Lactobacillus* spp. (**Figure 5.3**, top panel). Only a single *L. crispatus* strain (73.55a from a BV+ woman) did not grow at any of the lower pHs tested. In contrast, *L. jensenii*, *L. vaginalis, L. gasseri,* and *L. mucosae* strains from BV– women appeared to be less acid tolerant, with the number of strains able to grow decreasing with decreasing pHs. None of the *L. gasseri* and *L. vaginalis* strains from women with Nugent scores 4-10 grew at lower pHs (pH 3.5-4.5; **Figure 5.3**). In contrast, the two *L. mucosae* isolates from BV- women did not grow at the lower pHs, while 5/10 *L. mucosae* isolates from BV+ women demonstrated some level of acid tolerance (**Figure 5.3**). All of the *Lactobacillus* strains tested did not tolerate pH 2.0 (**Figure 5.3**, last column).

The probiotic strains showed growth at pH 6.0 that was comparable to the vaginal *L. vaginalis* isolates, and 9/10 showed some acid tolerance at pHs 3.5 – 4.5, although none grew at pH 2.0 (**Figure 5.3**, bottom panel). The majority of the vaginal *L. crispatus*, *L. vaginalis* and *L. jensenii* strains grew better than their respective ATCC® strain at all pHs tested. *L. gasseri* 94.98 PB (from a BV- woman) showed comparable growth to the *L. gasseri* ATCC® reference strain.

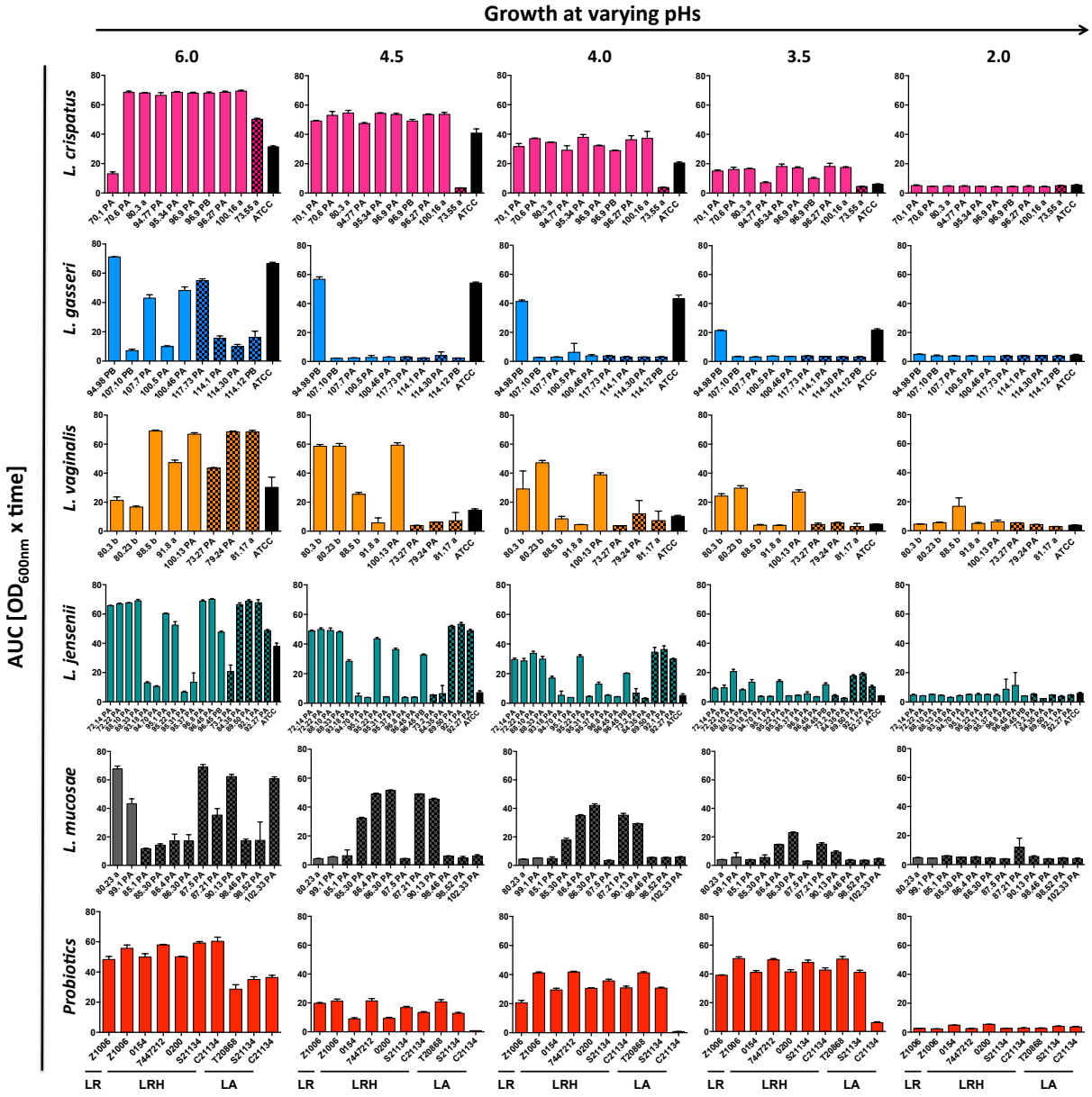


Figure 5.3. Growth of *Lactobacillus* strains at varying pHs. The growth of vaginal *L. cripsatus* (pink bars, n=10), *L. gasseri* (blue bars, n=9), *L. vaginalis* (orange bars, n=8), *L. jensenii* (green bars; n=18) and *L. mucosae* (grey bars, n=12) and probiotic strains (red bars, n=10, LR – *L. reuteri*, LRH = *L. rhamnosus*, LA = *L. acidophilus*) at pH 6.0, 4.5, 4.0, 3.5 or 2.0 was measured over 48 hours at 0, 3, 6, 12, 24, 32 and 48 hours. The AUC was calculated for each strain, bars shows mean and standard deviation. ATCC® reference strains are shown by black bars. Solid bars represent isolates that were obtained from BV- women (n=12), while patterned bars indicate isolates that were obtained from women with a Nugent Score 4-10. (BV intermediate n=4 and BV+ n=10).

Overall, vaginal *L. crispatus* strains from BV- women demonstrated significantly better growth than other vaginal *Lactobacillus* isolates tested, and slightly better growth than commercially available probiotic *Lactobacillus* isolates at both low and neutral pHs.

5.3.5 Influence of bacterial growth on culture pH

The ability of *Lactobacillus* isolates to lower vaginal pH is of importance, as lower vaginal pHs have been shown to inactivate both viral and bacterial sexually transmitted pathogens (O'Hanlon *et al*. 2011; Valore *et al*. 2002; Graver & Wade 2011; Nardini *et al*. 2016). All vaginal *Lactobacillus* strains tended to have similar kinetics in their ability to lower pH (**Figure 5.4**). All *L. crispatus* strains lowered the pH from 6.0 to a median pH of 3.7 by 48 hours. In contrast, the other vaginal *Lactobacillus* spp. lowered culture pH more variably, with only 4/5 *L. gasseri*, 9/13 *L. jensenii*, and 3/5 *L. vaginalis* strains from BV- women lowering pH to <4.0. Fewer strains from women with Nugent scores 4-10 appeared to lower the pH to <4.0. The probiotic strains achieved a median pH of 4.2 (IQR 4.1 – 4.4) at 48 hours (**Figure 5.4**). With the exception of *L. crispatus,* where the ATCC® strain showed a similar ability to lower the pH as the vaginal isolates, the majority of the other vaginal *Lactobacillus* strains lowered culture pH more dramatically than their corresponding ATCC® strains.

L. gasseri and *L. vaginalis* isolates from BV- women tended to lower the pH stronger than those from BV+ women, while BV status of the donors did not seem to influence pHs achieved for *L. crispatus, L. jensenii,* and *L. mucosae* strains. Overall, *L. crispatus* lowered the pH significantly more than *L. gasseri*, *L. mucosae*, *L. vaginalis* and the probiotic strains at 48 hours, after adjusting for multiple comparisons (**Figure 5.5**). It was interesting that growth kinetics of individual isolates at pH 6.0 (measured as AUC) did not significantly predict their ability to lower culture pH at 48 hours (Spearman Rho=-0.17, p=0.165), indicating that differences in growth rates between strains did not account for differences in their ability to lower culture pH.

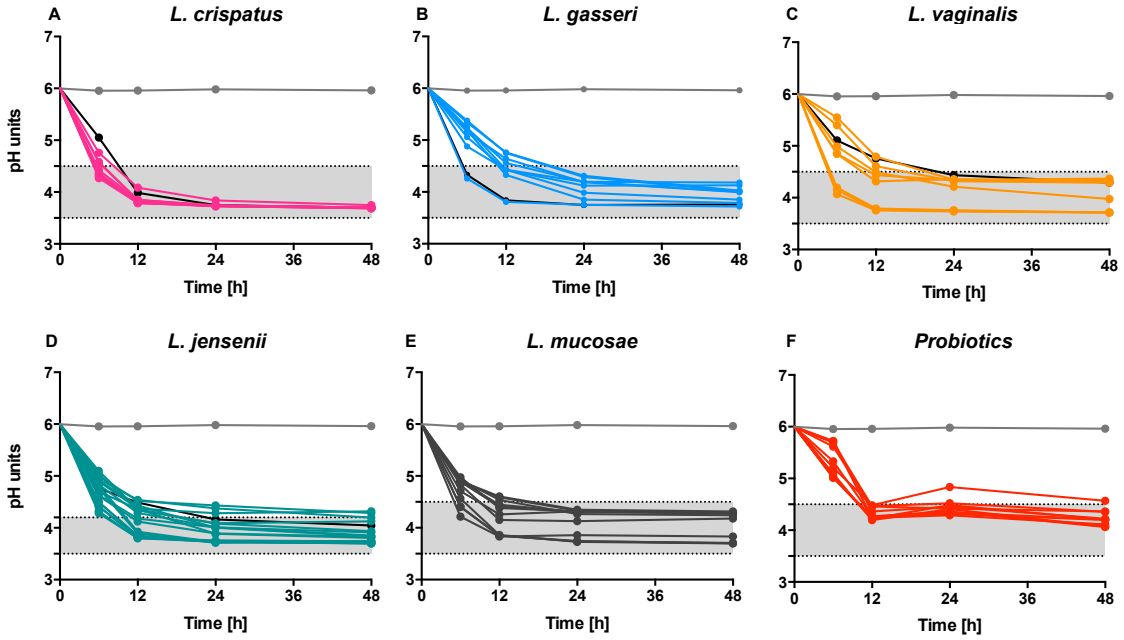


Figure 5.4. Comparing the ability of vaginal and probiotic *Lactobacillus* isolates to lower culture pH. Overnight cultures of vaginal *L. crispatus* (A, pink), *L. gasseri* (B, blue), *L. vaginalis* (C, orange), *L. jensenii* (D, green), *L. mucosae* (E, grey) and the probiotic (F, red) isolates were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS an incubated at 37 °C, and the culture pH was measured over 48 hours at 0, 6, 12, 24 and 48 hours. Probiotics include *L. reuteri, L. rhamnsus* und *L. acidophilus* strains. As control, the pH of abiotic MRS was measured (shown in light grey). ATCC® reference strains are visualised using black lines. Physiological vaginal pH of healthy women (ranging from 3.5 to 4.5) is shaded it grey.

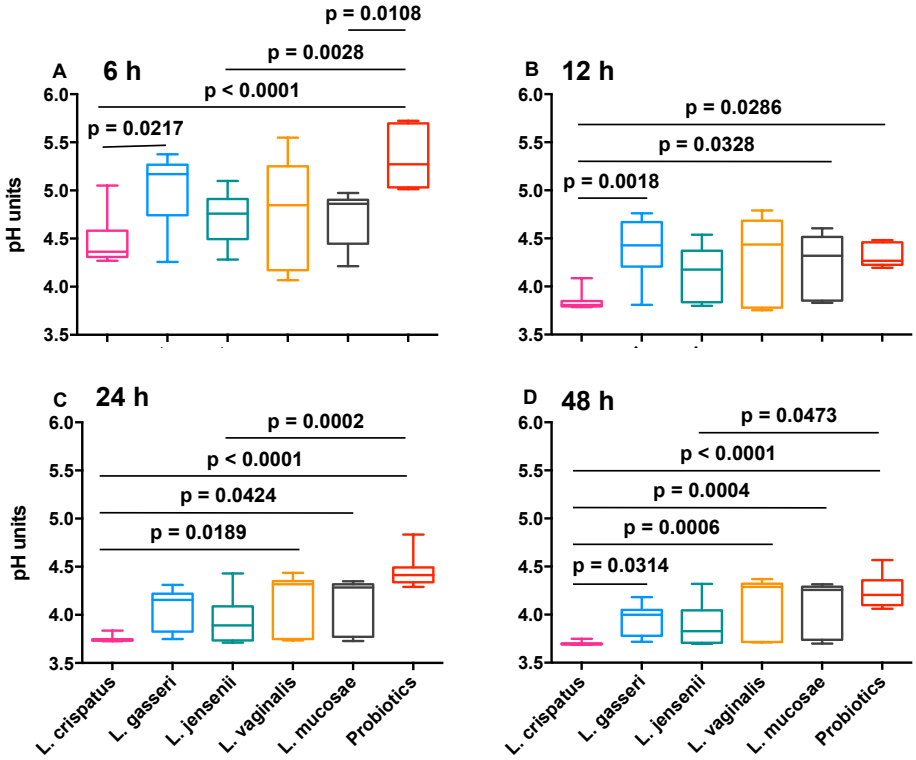


Figure 5.5. Summary analysis of the ability of vaginal and probiotic *Lactobacillus* spp. to lower pH at species level. Overnight cultures of *L. crispatus* (pink), *L. gasseri* (blue), *L. jensenii* (green), *L. vaginalis* (orange), *L. mucosae* (grey) and the probiotic (red) isolates were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS, and the culture pH was measured over 48 hours at 0, 6, 12, 24 and 48 hours. Probiotics include *L. reuteri, L. rhamnosus* und *L. acidophilus* strains. The pHs measured at 6 (A), 12 (B), 24 (C) and 48 (D) hours were grouped on species level (excluding ATCC® reference strains), and non-parametric multiple comparisons were done using the Kruskal Wallis test. P-values adjusted for multiple comparisons are shown.

5.3.6 Production of biologically-active compounds

5.3.6.1 L- and D- lactate production by vaginal Lactobacillus strains

The production of L- and D-lactate was measured in 46/57 vaginal *Lactobacillus* strains in anaerobic cultures, including 10/10 *L. crispatus*, 9/9 *L. gasseri*, 8/8 *L. vaginalis*, 10/18 *L. jensenii* and 9/12 *L. mucosae* strains, and the ATCC® reference strains. The remaining 11/57 were not investigated due to restrictions in numbers of tests provided in kit used and funding restrictions for this analysis. There was a lot of variability in the ability of these vaginal isolates to produce D- and L-lactate, both between and within *Lactobacillus* spp. (**Figure 5.6** and **Figure 5.7**). Vaginal *Lactobacillus* spp. generally produced lower levels of L-lactate than D-lactate (median 10.6 ng/uL [IQR 5.6–19.4] versus 35.8 ng/uL [30.4–41.9], $p<0.0001$), although all produced a bit of both. Further, the majority of vaginal *Lactobacillus* isolates produced more of both isomers of lactate than their respective ATCC® reference strains. Some vaginal strains produced high levels of L- but almost no D-lactate (such as *L. crispatus* 95.34 PA), while others produced a large amount of D- but little L-lactate (such as *L. jensenii* 96.45 PA). With the exception of the probiotic *L. acidophilus* isolates (which produced similar levels of both isomers), all probiotic strains produced higher levels of L- than D-lactate, although concentrations of both isomers were significantly lower than those of the vaginal strains ($p<0.0001$).

Among the vaginal strains that produced the highest levels of L-lactate (above 75th percentile) were three *L. jensenii* strains (72.14 PA, 95.1 PA, 95.31 PA), two *L. gasseri* strains (100.46 PA and 107.10 PA), one *L. vaginalis* (91.8a) and one *L. crispatus* (95.34 PA) strain, which were all derived from BV- women. Similarly, four *L. jensenii* strains (72.14 PA, 96.45 PA, 95.1 PA, 95.31 PA), three *L. vaginalis* strains (91.8a, 88.5b, 100.13 PA), three *L. crispatus* strains (80.3 a, 94.77 PA, 70.1 PA) and two *L. mucosae* strains (99.1 PA, 85.30 PA) were among the vaginal strains that produced the highest levels of or D-lactate, and all were from BV- women.

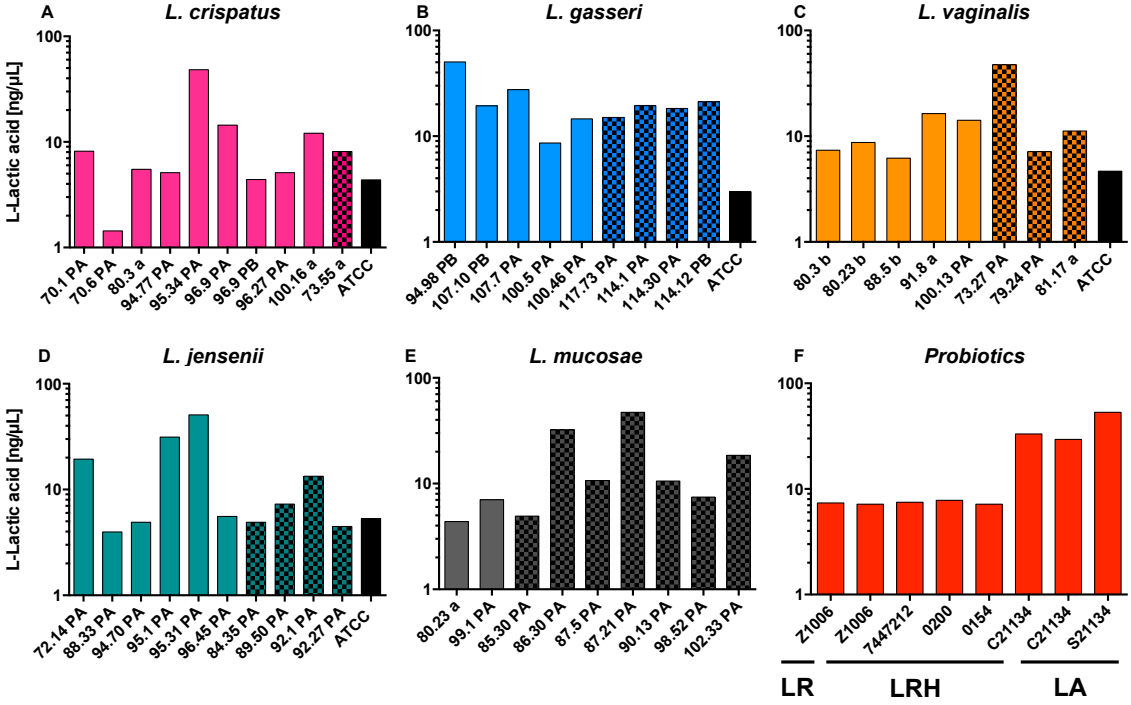


Figure 5.6. Comparing the ability of vaginal and probiotic *Lactobacillus* strains to produce L-lactate. Overnight cultures of *L. crispatus* (A, pink bars), *L. gasseri* (B, blue bars), *L. vaginalis* (C, orange bars), *L. jensenii* (D, green bars), *L. mucosae* (E, grey bars) and the probiotic (F, red bars) isolates were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS and incubated for 24 hours at 37 °C under anaerobic conditions, and the consenctration of L –lactate was measured using colorimetric assays (Sigma-Aldrich®, USA). Probiotics include *L. reuteri* (LR), *L. rhamnosus* (LRH) and *L. acidophilus* (LA) strains. ATCC® reference strains are shown by black bars. Solid bars represent isolates that were obtained from BV-women (n=12), while patterned bars indicate isolates that were obtained from women with a Nugent Score above 4-10 (BV intermediate n=4 and BV+ n=10).

There were no significant differences in L-lactate production between vaginal species (ANOVA p=0.0651), although *L. gasseri* (median 19.4 ng/µL, IQR 14.8–24.4) tended to produce higher amounts than other vaginal *Lactobacillus* spp., while comparable concentrations of D-lactate were measured for all vaginal species, ranging from a median of 32.8 ng/µL (IQR 26.8–35.9) for *L. gasseri* to 39.5 ng/µL (IQR 33.6–46.3) for *L. jensenii*. In contrast, the probiotic strains (described in Chapter 3, section 3.3.3.7) produced similar amounts of L-lactate, but less D-lactate than the vaginal species (ANOVA p=0.0237, adjust. p=0.0198 vs. *L. jensenii*).

Overall, however, the ability of *Lactobacillus* isolates (including vaginal and probiotic strains) to produce L- and D-lactate did not correlate (Spearman rho=0.00, p=0.9592), neither did their growth kinetics correlate with this ability (Spearman rho=-0.23, p=0.0838 for L-lactate; Spearman rho=0.05, p=0.7250 for D-lactate). As

the ability to produce lactate is thought to contribute to the ability of *Lactobacillus* strains to lower pH, it was interesting to note that there was no correlation between the amount of lactate produced and culture pH for either L-lactate (Spearman rho=0.15, p=0.2604) or D-lactate (Spearman rho=-0.02, p=0.8960), indicating that lactate production by these isolates was not the primary cause of pH decrease.

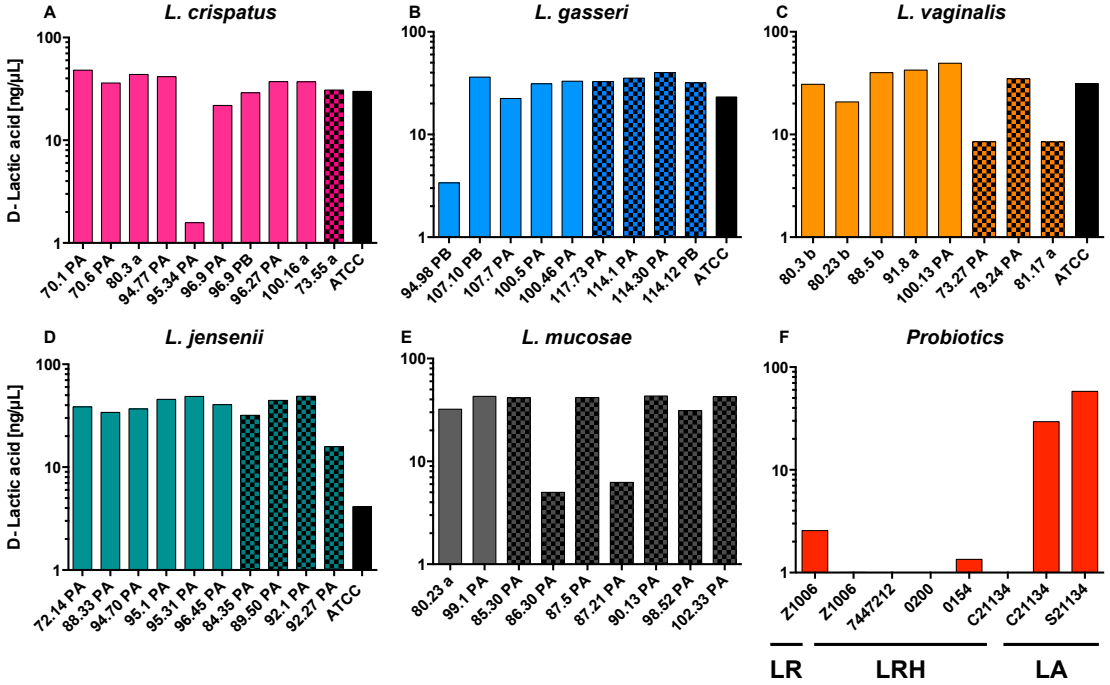


Figure 5.7. Comparing the ability of vaginal and probiotic *Lactobacillus* strains to produce D-lactate. Overnight cultures of *L. crispatus* (A, pink bars), *L. gasseri* (B, blue bars), *L. vaginalis* (C, orange bars), *L. jensenii* (D, green bars), *L. mucosae* (E, grey bars) and the probiotic (F, red bars) isolates were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS and incubated for 24 hours at 37°C under anaerobic condtions, and the concentration of D–lactate was measured using colorimetric assays (Sigma-Aldrich®, USA). Probiotics include *L. reuteri* (LR), *L. rhamnosus* (LRH) and *L. acidophilus* (LA) strains. ATCC® reference strains are shown by black bars. Solid bars represent isolates that were obtained from BV- women (n=12), while patterned bars indicate which isolates were obtained from women with Nugent scores >3 (BV intermediate n=4 and BV+ n=10).

5.3.6.2 Production of H_2O_2 by vaginal Lactobacillus isolates

Earlier studies in this field proposed that H_2O_2 was the major metabolite of *Lactobacillus* spp. that contribute to pathogen inhibition in the FGT (Hawes *et al*. 1996; Hillier *et al*. 1993; Cherpes *et al*. 2008), although several recent studies have suggested that this metabolite is produced by *Lactobacillus* strains only during aerobic metabolism and not under hypoxic conditions in which they would exist in the FGT (O'Hanlon *et al*. 2011; María Silvin Juárez Tomás *et al*. 2003; Shalev *et al*.

1996). All experiments in this Chapter were performed under anaerobic conditions, to mimic *in vivo* FGT conditions.

While all *L. gasseri* and *L. vaginalis* strains produced small amounts of H_2O_2 under anaerobic conditions, less than half of *L. jensenii*, *L. crispatus*, and *L. mucosae* strains produced detectable H_2O_2 (**Figure 5.8**). Neither the number of strains producing H_2O_2, nor the amount measured differed between isolates from BV- and BV+ women. However, the levels of H_2O_2 detected tended to be lower for *L. vaginalis* and *L. mucosae* strains from BV- than BV+ women. Vaginal *L. gasseri* isolates tended to produce higher concentrations of H_2O_2 than *L. crispatus* ($p=0.0168$) and *L. jensenii* ($p=0.0705$), after adjusting for multiple comparisons. Furthermore, all *L. gasseri* and the majority of *L. vaginalis* strains produced more H_2O_2 than the ATCC® reference strains (**Figure 5.8**). Considering that the microbicidal activity of H_2O_2 has only been demonstrated at concentrations ≥1000 mM (O'Hanlon *et al*. 2011), it is notable that no isolate tested in this study produced more than 20 μM (which is 50,000 times lower) under anaerobic conditions, in agreement with the probiotic *Lactobacillus* isolates (described in Chapter 3, section 3.3.3.8).

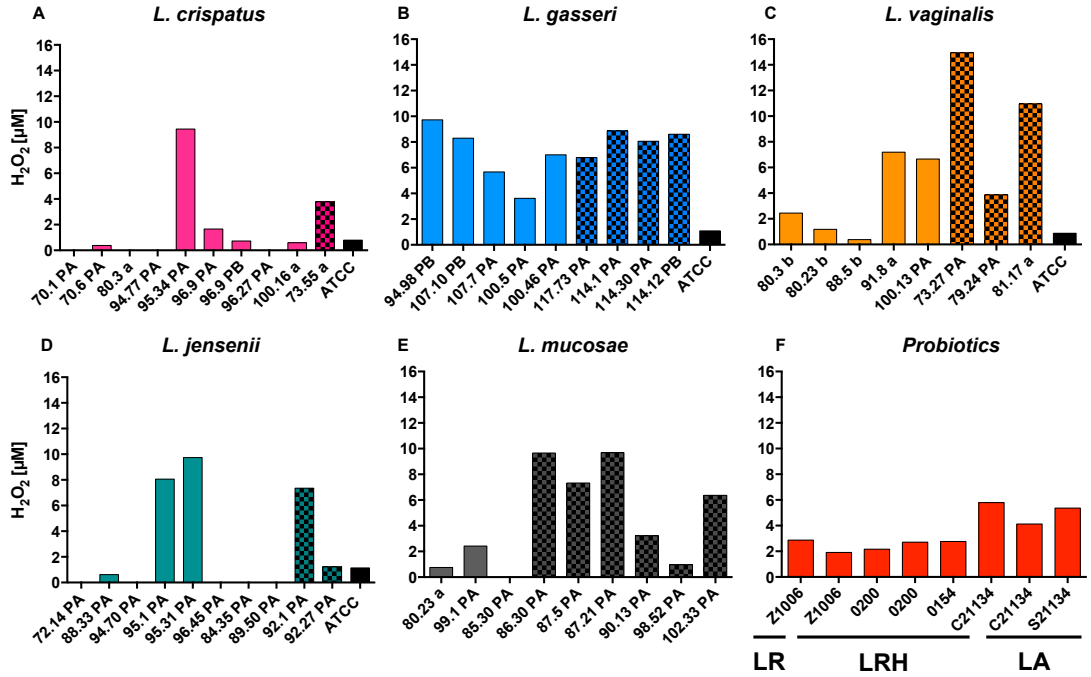


Figure 5.8. Comparing the ability of vaginal and probiotic *Lactobacillus* strains to produce H_2O_2 under anaerobic conditions. Overnight cultures of *L. crispatus* (A, pink bars), *L. gasseri* (B, blue bars), *L. vaginalis* (C, orange bars), *L. jensenii* (D, green bars), *L. mucosae* (E, grey bars) and the probiotic (F, red bars) isolates were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS and incubated for 24 hours at 37°C under anaerobic conditions. The supernatant was harvested and filtered (0.2 µM) to remove bacterial cells. Probiotics include *L. reuteri* (LR), *L. rhamnosus* (LRH) and *L. acidophilus* (LA) strains. The concentration of hydrogen peroxide (H_2O_2) was measured using a fluorometric assays (Sigma-Aldrich®, USA). ATCC® reference strains are shown by black bars. Solid bars represent isolates that were obtained from BV- women (n=12), while patterned bars indicate which isolates were obtained from women with Nugent scores >3 (BV intermediate n=4 and BV postive n=10).

There was no correlation between the ability of isolates to produce H_2O_2 and their abilities to lower pH at 24 hours or their growth kinetics (AUC). However, there was a positive correlation between levels of H_2O_2 and L-lactate produced (Spearman

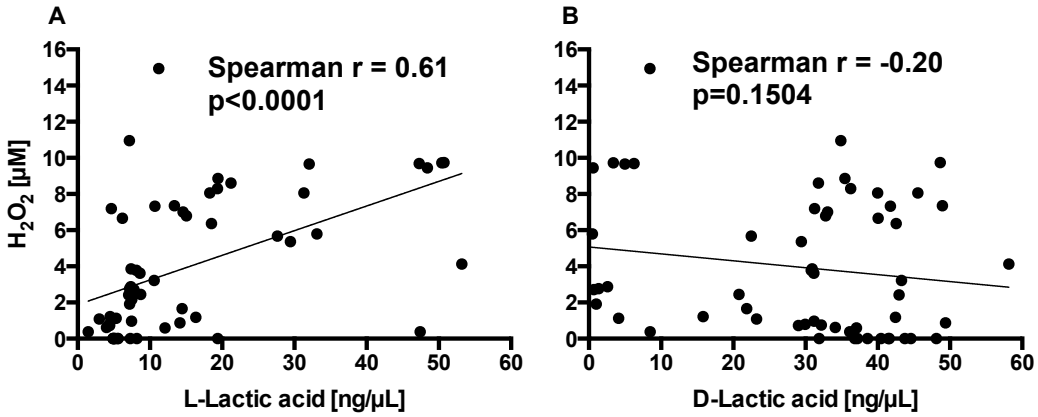


Figure 5.9. Correlation between ability to produce H_2O_2 and either (A) L-lactate or (B) D-lactate concentrations measured in vaginal *Lactobacillus* culture supernatants. The Spearman rank correlation test was used to statistically test correlations between the measured concentrations of H_2O_2 [µM] and lactate [ng/µL]. Each dot represents one *Lactobacillus* isolate. P-values<0.05 were considered significant.

rho=0.61, p<0.0001), but not with the amount of D-lactate produced (Spearman rho=-0.20, p=0.1504, **Figure 5.9**).

5.3.7 Factors predicting the ability of vaginal Lactobacillus strains to lower culture pH

To evaluate which of the characteristics that were measured for the vaginal isolates was most influential in lowering culture pH, a multivariate linear regression model was used to evaluate the relative contribution of growth kinetics, production of lactate and H_2O_2. None of these variables significantly influenced the ability to lower pH in this model (adjusted R-squared=-0.0104; **Figure 5.10**).

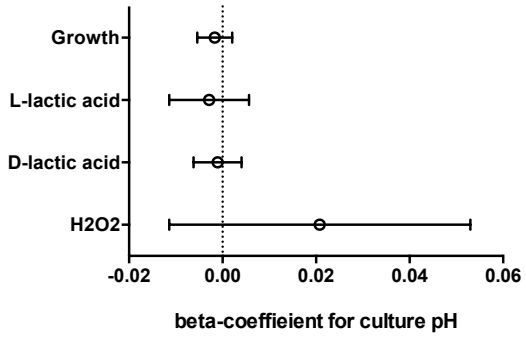


Figure 5.10. Multivariate regression model to explain change in culture pH. Multivariate linear regressions were performed, using culture pH as dependant variable and growth, L- and D-lactate and H_2O_2 production as exploratory variables. The β-coefficient represent the change in pH with every unit increase of the explanatory variable, adjusting for all other parameters. 95% CI are shown. Non-significant β-coefficients are presented using empty circles.

5.3.8 Antimicrobial properties of Lactobacillus isolates

The ability of *Lactobacillus* abiotic culture supernatants to inhibit the growth of a panel of *P. bivia* and *G. vaginalis* strains (commonly associated with BV), as well as GBS strains (commonly associated with aerobic vaginitis and adverse pregnancy outcomes) was determined (**Figure 5.11**). This analysis focused on 25/57 vaginal *Lactobacillus* [representing at least five strains from each of the vaginal *Lactobacillus* spp. isolated], of which the majority (16/25) were from BV- women. Of these, all showed some degree of inhibitory activity on all *P. bivia* strains tested (besides *L. gasseri* 94.98 PB), although the extent of inhibition differed by both *Lactobacillus* and *P. bivia* strains (**Figure 5.11**; left panel). *L. mucosae* isolates 86.30 PA and 87.21 PA (from BV+ women), similarly to *L. gasseri* 94.98 PB, did not inhibit the full panel of *P. bivia* strains tested. Of all the *Lactobacillus* spp. evaluated, *L. crispatus*

inhibited the growth of *P. bivia* strains the best, inhibiting all six *P. bivia* strains by more than 90%. The vaginal *Lactobacillus* strains inhibited *P. bivia* to a similar extent as their respective ATCC® strains (**Figure 5.11**; left panel), and the inhibition was comparable to what was previously measured for the probiotic *Lactobacillus* strains (Chapter 3, section 3.3.3.9).

Culture supernatants from almost all vaginal *Lactobacillus* strains (besides *L. gasseri* 94.98 PB) inhibited all GBS strains by >80% (**Figure 5.11**; right panel). The two *L. mucosae* isolates 86.30 PA and 87.21 PA (from BV+ women) that did not inhibit most *P. bivia* strains (**Figure 5.11**; left panel) inhibited the growth of the full GBS panel but more modestly (<50%) than other vaginal *Lactobacillus* strains. The inhibitory effect of supernatants from vaginal *Lactobacillus* isolates was similar to those previously measured for the probiotic strains (Chapter 3, section 3.3.3.9) and ATCC® *Lactobacillus* strains (**Figure 5.11**; right panel).

In contrast to the activity against the *P. bivia* and GBS pathogen panels, the ability of *Lactobacillus* culture supernatants to inhibit the growth of *G. vaginalis* isolates was generally more variable and significantly lower ($p<0.0001$), similarly to what was seen for the probiotic strains in Chapter 3, section 3.3.3.9. Of all the *Lactobacillus* isolates tested, only three vaginal *Lactobacillus* strains (*L. crispatus* 100.16a, *L. jensenii* 88.33PA, and *L. jensenii* 92.27 PA) and the *L. crispatus* ATCC® strain showed some ability to inhibit the growth of the full panel of *G. vaginalis* (**Figure 5.11**; middle panel). However, the ability of *Lactobacillus* culture supernatant to inhibit *G. vaginalis* appeared to be highly strain specific, as almost all of the *Lactobacillus* strains inhibited the vaginal *G. vaginalis* isolate 3H1 (**Figure 5.11**; middle panel; bottom row), while only 9/25 inhibited the *G. vaginalis* ATCC® strain (**Figure 5.11**; middle panel; top row). Overall, *L. crispatus* strains tended to exhibit the strongest inhibitory activity against *G. vaginalis*, although this was not statistically significant. The ability of other vaginal and probiotic *Lactobacillus* spp. to inhibit *G. vaginalis* varied depending on the *G. vaginalis* strain being used (Figure 5.11 and Chapter 3, section 3.2.10, respectively). The ATCC® reference *Lactobacillus* strains generally showed broader and better inhibitory activity against *G. vaginalis* strains than the vaginal *Lactobacillus* isolates (**Figure 5.11**; middle panel).

Notably, there was a very strong correlation between the extent to which vaginal *Lactobacillus* strains were able to inhibit both *G. vaginalis* and *P. bivia* (Spearman rho=0.814, p<0.0001), *G. vaginalis* and GBS (Spearman rho=0.635, p<0.0001); and *P. bivia* and GBS panels (Spearman rho=0.670, p<0.0001), indicating that individual strains have a similar capacity to inhibit various genital pathogens. In conclusion, *Lactobacillus*-conditioned supernatants inhibited a panel of *P. bivia* and GBS strains much more effectively than the *G. vaginalis* strains, which was both *Lactobacillus*- and pathogen strain-dependant.

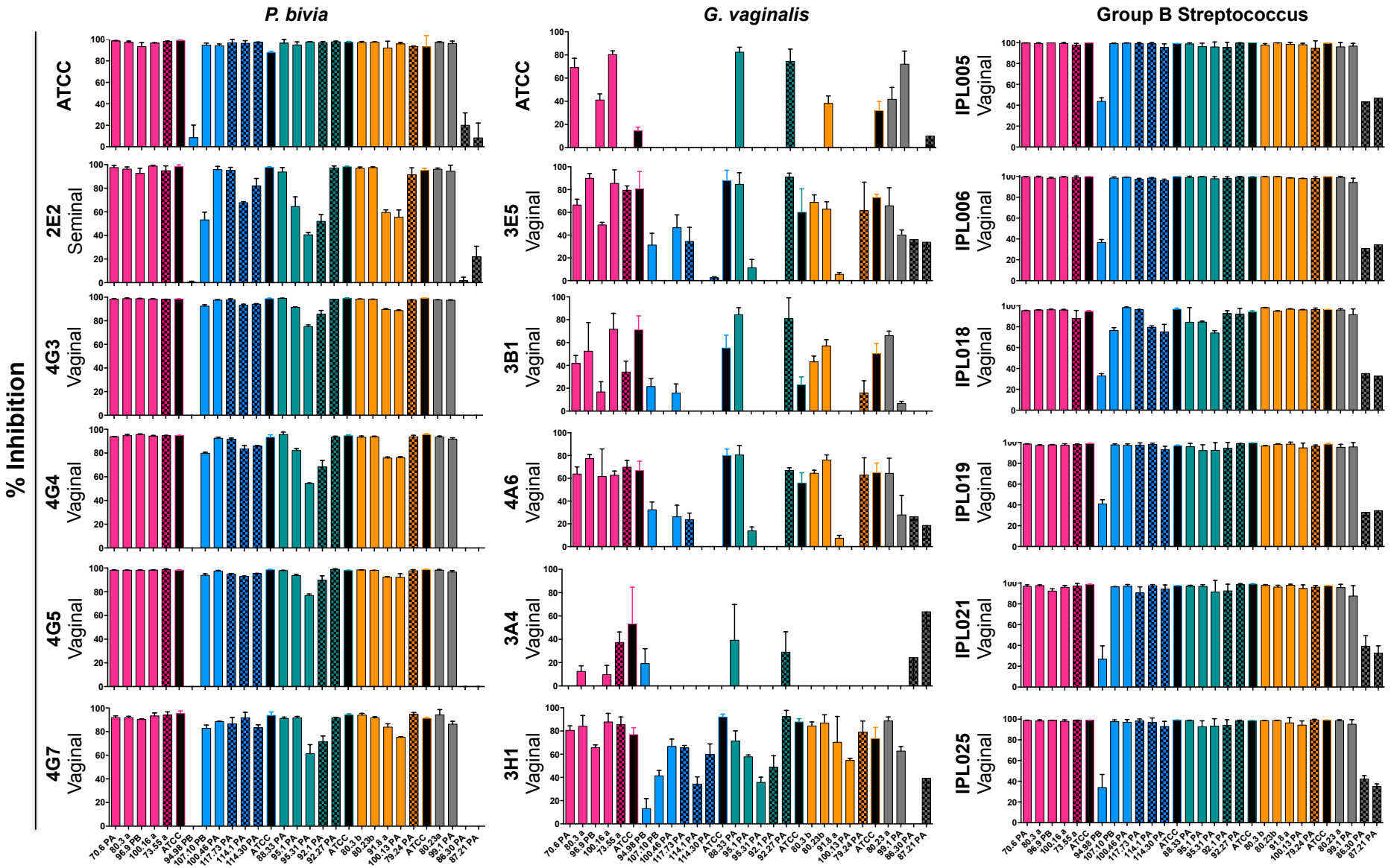


Figure 5.1. Inhibitory effect of *Lactobacillus*-conditioned media on the growth of common BV-associated organisms (like *P. bivia* and *G. vaginalis*) and other FGT pathogens (like GBS). Overnight cultures of *L. crispatus* (pink bars), *L. gasseri* (blue bars), *L. jensenii* (green bars), *L. vaginalis* (orange bars), and *L. mucosae* (grey bars) isolates were adjusted to OD_{600nm} of 0.1 (±0.01) in MRS and cultured for 24 hours at 37°C under anerobic conditions. The supernatant was sterile-filtered (0.2 µM) and added in a 1:1 ratio to overnight cultures of ATCC® and genital *P. bivia*, *G. vaginalis* and GBS strains (left, middle and right panel, respectively). The absorbance of FGT pathogen cultures in *Lactobacillus*-conditioned and –unconditioned media (control) was measured at 3, 6, 12, 24, 30 and 48 hours, and the the AUC was calculated for each strain. The percentage of inhibition was determined by comparing the growht of the FGT pathogen strains in the presence of non-conditioned media (control) to *Lactobacillus*-conditioned media. Pathogen strains names and their origin (ATCC®, vaginal or seminal) are shown in the Y-axis. ATCC® reference strains are shown by black bars, and border color refers to species. Solid bars represent isolated that were obtained from BV- women while patterned bars indicate isolates that were obtained from women with Nugent scores 4-10.

5.3.8.1 Predictors of Lactobacillus strains' ability to inhibit the grown of G. vaginalis, P. bivia and GBS growth

The ability of *Lactobacillus* spp. to lower pH or produce lactate, or H_2O_2 metabolites, have previously been proposed to inhibit pathogens (O'Hanlon *et al*. 2011; Hutt *et al*. 2016). However, in an exploratory univariate correlation analysis, the ability of *Lactobacillus* supernatants to inhibit *P. bivia* strains in this study correlated inversely with their ability to produce L-lactate (Spearman rho ranging from -0.4 [adjust. p=0.0221] for *P. bivia* ATCC® to -0.8 [adjust. p=0.0002] for *P. bivia* 4G4), H_2O_2 (same Spearman rho as for L-lactate), and positively with D- lactate for some *P. bivia* strains (Spearman rho=0.4 for *P. Bivia* 4G4 [adjust. p=0.0214], 4G5 [adjust. p=0.0092] and 4G3 [adjust. p=0.0182]). It was determined that *Lactobacillus* strains' abilities to produce L-lactate and H_2O_2 metabolites were co-linear in this study (Spearman rho=0.61; section 5.3.6.2), thus it is expected to see similar associations between these two and other variables in the data set. Using a step-up multivariate linear regression model (which omitted H_2O_2 due to its co-linearity with L-lactate) the ability to produce L-lactate most strongly accounted for the inhibitory effect on *P. bivia* ATCC®, followed by D-lactate, culture pH and growth (AUC), which explained 72.98% of the variability in the model overall (**Table 5.2**).

Using these influential variables, further multivariate linear regressions showed that culture pH measured (beta-coefficient (β) 22.57; 95% CI 10.95-34.17; unadjust. p<0.0001, adjust p=0.0003), D-lactate (β=0.57; 95% CI 0.34-0.79; unadjust. p<0.0001, adjust p=0.0003) and growth kinetics (AUC; β=0.006; 95% CI 0.003-0.01; unadjust. p=0.001, adjust p=0.003) were positively associated with increased inhibition, while the production of L-lactate was negatively associated with inhibition of *P. bivia* ATCC growth (β=-1.55; 95% CI -1.83- -1.27; unadjust. p<0.0001, adjust p=0.0003). Similar observations were made for all *P. bivia* strains, as shown in **Figure 5.12**, although growth was not significant in the models for any other *P. bivia* strain (**Table 5.2**).

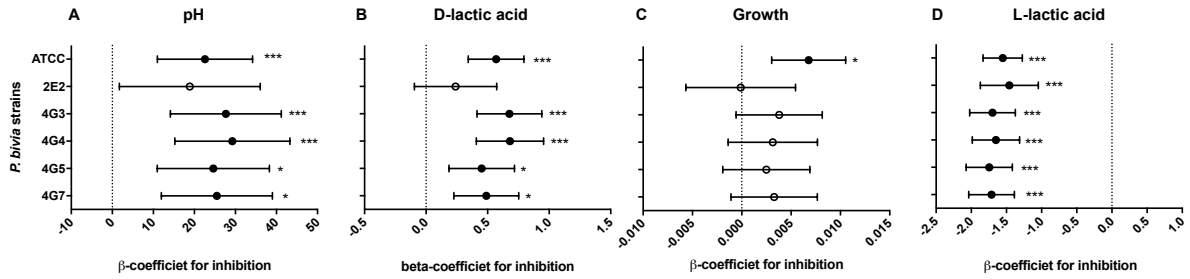


Figure 5.12. Modelling the most significant predictors of *P. bivia* inhibition by *Lactobacillus* strains. Multivariate linear regressions were performed for each strains separately, using the pathogen as dependant variable and the ability to lower pH, D-lactic production, growth and L-lactic production as exploratory variables. The β-coefficient represent the change in *P. bivia* inhibition with every unit increase of the explanatory variable, adjusting for all other parameters. H_2O_2 was not included due to its co-linearity with L-lactate Graphs show β-coefficients with 95% CI. Full circles indicate statistical significance, while empty circles indicate non-significance. Adjusted p-values are displayed. Stars indicate level of significance: p<0.001 ***, <0.01 **, <0.05 *.

Similar to their ability to inhibit *P. bivia*, the ability of *Lactobacillus* strains to produce L-lactate and H_2O_2 significantly negatively correlated with their ability to inhibit GBS in the univariate exploratory analysis (data not shown). Using the same step-up model approach, the ability of *Lactobacillus* isolates to produce L- and Dlactate and pH of the abiotic supernatant explained more than 70% of variability in the models for GBS inhibition. Addition of *Lactobacillus* growth kinetics (AUC) improved the step-up models slightly for some strains (e.g. for IPL019, then explaining 76.3% of the variability in the model, **Table 5.2**). Factors that were determined to be influential for GBS inhibition using the step-up model were included in multivariate regression models, and the β-coefficients for individual GBS strains are shown in **Figure 5.13**. These were comparable to those for *P. bivia* inhibition as shown **Figure 5.12**.

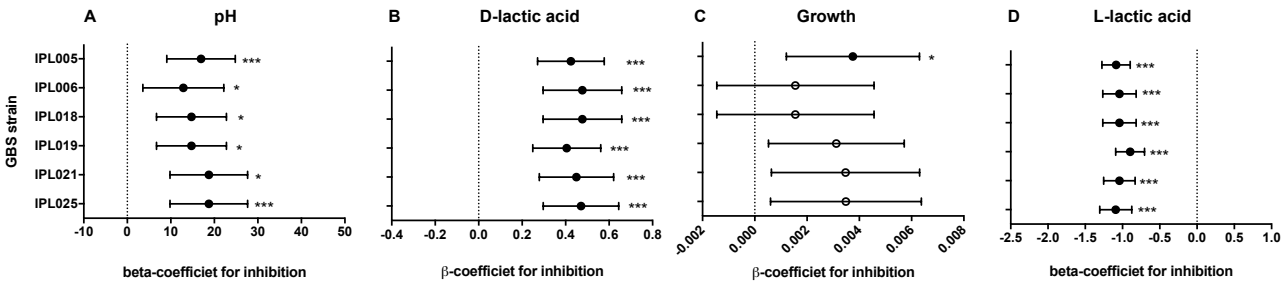


Figure 5.13. Modelling the most significant predictors of GBS inhibition by vaginal *Lactobacillus* strains. Multivariate linear regressions were performed for each strains separately, using the pathogen as dependant variable and the ability to lower pH, D-lactic production, growth and L-lactic production as exploratory variables. The β-coefficient represent the change in GBS inhibition with every unit increase of the explanatory variable, adjusting for all other parameters. H_2O_2 was not included due to its co-linearity with L-lactic acid. Graphs show β-coefficients with 95% CI. Full circles indicate statistical significance, while empty circles indicate non-significance. Adjusted p-values are displayed. Stars indicate level of significance: p<0.001 ***, <0.01 **, <0.05 *.

In the univariate exploratory analysis for *G. vaginalis* inhibition, there was a significant correlation between the ability of *Lactobacillus* to lower pH and their ability to inhibit some *G. vaginalis* strains (Spearman rho=-0.5 for ATCC® and 3E5, adjust. p=0.0080 and adjust. p=0.0092, respectively). There was also a trend towards a positive correlation between *Lactobacillus* growth kinetics and ability to inhibit *G. vaginalis* (Spearman rho ranging from 0.2 for *G. vaginalis* 3A4 to 0.4 for 3E5), indicating the importance of lowering pH and ability of *Lactobacillus* strains to grow well for *G. vaginalis* inhibition. L-lactate and H_2O_2 production by *Lactobacillus* strains correlated negatively with their ability to inhibit *G. vaginalis* strains (data not shown).

In the step-up model, the variable that had the strongest impact on the model for *G. vaginalis* ATCC® inhibition was the ability of *Lactobacillus* strains to produce H_2O_2, although addition of lower pH did improve the model. Addition of the other variables did not meaningfully improve the model that included H_2O_2 production and ability to lower pH, which explained 28.4% (adjusted R-squared) of the variability within this model. The same two variables explained 68.8% of the variability for the models using *G. vaginalis* 3B1, indicating that this model is more robust than the model for *G. vaginalis* ATCC® inhibition. Similarly, the step-up model for *G. vaginalis* 3E5 founds that again H_2O_2 was the strongest predictor, but production of D-lactate (and not pH) improved the model, that explained 77.2% of the variability for this strain. The step-up models for all *G. vaginalis* strains are summarized in **Table 5.2**. β-coefficients are shown in **Figure 5.14**; growth was not included in these multivariate regressions, as it did not improve step-up models of any *G. vaginalis* strain.

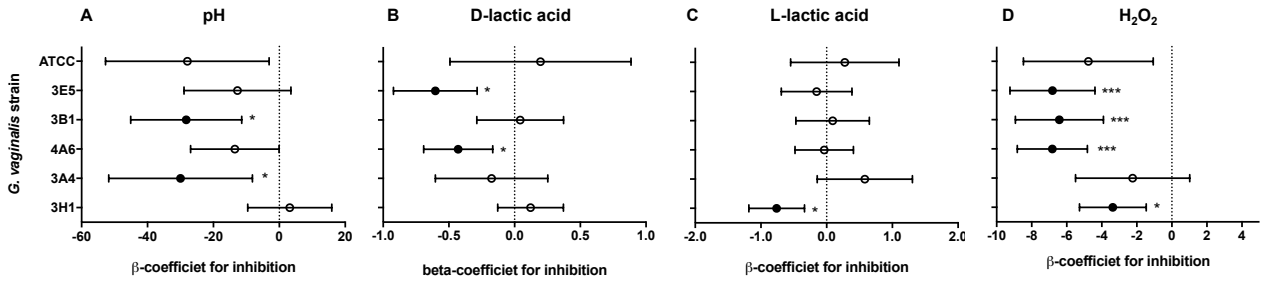


Figure 5.14. Modelling the most significant predictors of *G. vaginalis* inhibition by vaginal *Lactobacillus* strains. Multivariate linear regressions were performed for each strains separately, using the pathogen as dependant variable and the ability to lower pH, D-and L-lactate production as well as H_2O_2 as exploratory variables. The β-coefficient represent the change in *G. vaginalis* inhibition with every unit increase of the explanatory variable, adjusting for all other parameters. H_2O_2 was included as it was the strongest predictor in most step-up models. Growth was not included as is did not improve step-up models. Graphs show beta-coefficients with 95% CI. Full circles indicate statistical significance, while empty circles indicate non-significance. Adjusted p-values are displayed. Stars indicate level of significance: p<<0.001 ***, <0.01 **, <0.05 *.

Overall, these analyses show that the pH, D- and L-lactate levels measured in the added *Lactobacillus*-conditioned media best predicted *P. bivia* and GBS inhibition. The findings that a high culture pH was associated with increased *P. bivia* inhibition and high L-lactate levels with lower inhibition were unexpected and require further investigation. In contrast, as expected, a low pH was associated with stronger *G. vaginalis* inhibition. However, the strongest predictor for *G. vaginalis* inhibition was H_2O_2, with higher concentrations being associated with a weaker ability to inhibit *G. vaginalis*.

Table 5.2. Summary of step-up multivariate linear regression models for pathogen inhibition

Pathogen	Significant factors in step-up model	R^2 value
***P. bivia* strain**		
ATCC©	L- lactate, D-lactate, pH, growth	72.98%
2E2	L- lactate, pH	62.10%
4G3	L- lactate, D-lactate, pH	78.40%
4G4	L- lactate, D-lactate, pH	77.33%
4G5	L- lactate, D-lactate, pH	78.76%
4G7	L- lactate, D-lactate, pH	77.85%
GBS strain		
IPL005	L- lactate, D-lactate, pH, growth	82.35%
IPL006	L- lactate, D-lactate, pH	72.72%
IPL018	L- lactate, D-lactate, pH	76.01%
IPL019	L- lactate, D-lactate, pH, growth	76.31%
IPL021	L- lactate, D-lactate, pH, growth	78.11%
IPL025	L- lactate, D-lactate, pH, growth	79.36%
***G. vaginalis* strain**		
ATCC©	H_2O_2, pH	28.4 %
3B1	H_2O_2, pH	68.82%
3E5	H_2O_2, D-lactate	77.19%
4A6	H_2O_2, D-lactate, pH	81.61%
3A4	pH	15.48%
3H1	H_2O_2, L- lactate	74.2%

5.3.9 Effect of Lactobacillus strains on inflammatory responses by CaSki cells

To be considered for the development of vaginally-delivered probiotic for reproductive health, the *Lactobacillus* isolates should not exert inflammatory effects on the mucosal epithelium of the host, as this may increase risk of HIV acquisition in women (Masson *et al*. 2016; Liebenberg *et al*., 2017). For a subset of the vaginal *Lactobacillus* isolates (including three strains per *Lactobacillus* spp.) and six probiotic strains, the effect on viability of and cytokine expression by cervical epithelial Ca Ski cells was determined (**Figure 5.15**). For this analysis, the following vaginal strains were assessed: *L. crispatus* 100.16a, 96.9PB, and 95.34PA; *L. gasseri* 100.46PA, 117.73PA and 114.30PA; *L. jensenii* 88.33PA and 95.31PA, 92.27PA; *L. vaginalis* 80.3b and 100.13PA, 79.24PA; and *L. mucosae* 80.23a, 86.30PA, and 102.33PA.

None of the vaginal *Lactobacillus* strains compromised the viability to Ca Ski cells (data not shown), but all induced the secretion of the anti-inflammatory cytokine IL-1RA [1.6–14.5-fold compared to control (no *Lactobacillus* strain added); **Figure 5.15**A], and IL-10 to a lesser extent (0.4 – 1.3-fold compared to the control, **Figure 5.15**B). IL-10 concentrations produced by Ca Ski cells co-cultured with *Lactobacillus* strains were generally lower than IL-1RA levels measured (median 7.3 [IQR 5.8-8.2] pg/mL vs. 239.3 (IQR 91.1–540.4], respectively; p=<0.0001). The same trends were observed for probiotic isolates (**Figure 5.15**A-B), although these induced IL-1RA to a greater extent than the vaginal *Lactobacillus* strains [median 10.2-fold (IQR 7.4-11.5-fild) vs. 3.0-fold (IQR 1.9-11.0-fold), respectively; p=0.2346).

Chemokine responses by Ca Ski cells co-cultured with vaginal *Lactobacillus* strains were more variable. Of the chemokines measured, IL-8 was detected at the highest concentrations (median 819.2 pg/mL, IQR 128.8-1059 for vaginal isolates, **Figure 5.15**C). However, about two thirds of the *Lactobacillus* isolates tested increased production of IL-8 by Ca Ski cells, while the other third decreased the concentration of IL-8 produced 1.7- to 9.9-fold compared to the Ca Ski cell alone controls. For MIP-1α (**Figure 5.15**D), similar cytokine response patterns were seen, although the concentrations measured were much lower (<8 pg/mL). In contrast to IL-8 and MIP-1 α, this study found that IP-10 was generally downregulated by co-culture of *Lactobacillus* strains with Ca Ski cells (up to 764-fold, **Figure 5.15**E). *Lactobacillus*

isolates did not induce or suppress production of MCP-1 by Ca Ski cells. No differences in ability to induce cytokines or chemokines were observed between vaginal and probiotic *Lactobacillus* isolates (**Figure 5.15**C-F).

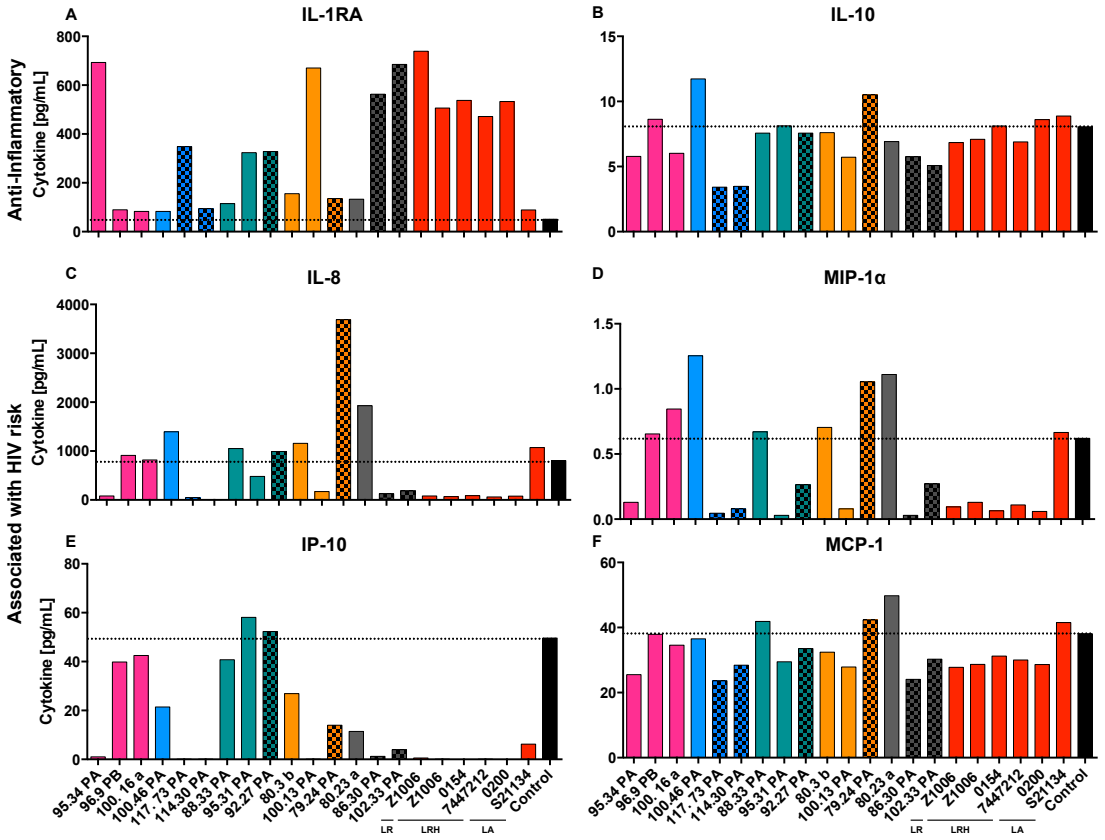


Figure 5.15. Vaginal Ca Ski cell cytokine responses following co-culture with vaginal and probiotic *Lactobacillus* isolates. 4.18x10^6 CFUs of *Lactobacillus* cultures (*L. crispatus* [pink], *L. gasseri* [blue], *L. jensenii* [green], *L. vaginalis* [orange], *L. mucosae* [grey] and the probiotic isolates [red], including *L. reuteri* [LR], *L. rhamnosus* [LRH] and *L. acidophilus* [LA]) were added to Ca Ski cells grown to 80-90% confluence and incubated for 24 hours. The concentrations of 27 cytokines were measured in culture supernatants by multiplex luminex®, and a selection of anti-inflammatory (including IL1-RA [A] and IL-10 [B]) and chemokines related to HIV risk (including IL-8 [C], MIP-1α [D], IP-10 [E] and MCP-1 [F]). Abiotic cell culture supernatant was used as control (black bar). The stack bars show the Ca Ski cytokine response caused by each bacterial strain and the control. The dotted line displays the cytokines levels measured in the control. Solid bars represent isolated that were obtained from BV- women, while patterned bars indicate isolates that were obtained from women with Nugent scores >3.

5.3.10 Antibiotic sensitivity patterns of Lactobacillus isolates

The susceptibility of vaginal *Lactobacillus* isolates (n=57) to antibiotics commonly used to treat BV (metronidazole and clindamycin) and other common infections in South Africa (including penicillin, amoxicillin, rifampicin and rifabutin) were measured. All vaginal *Lactobacillus* isolates were resistant to metronidazole (**Figure**

5.16A). In contrast, the susceptibility to clindamycin (**Figure 5.16**A) varied considerably within and between *Lactobacillus* spp. *L. vaginalis* and *L. jensenii* isolates were the most sensitive to clindamycin (median zones of inhibition of 29.5 mm [IQR 25.5 – 30.0] and 28.0 mm [IQR 25.5 – 30.0], respectively), while *L. gasseri* was the least susceptible (median 0.0 mm, IQR 0.0 – 6.8). *Lactobacillus* isolates from BV- and BV+ women did not shown any significant differences in sensitivity to metronidazole and clindamycin. While the variability of inhibition zones measured between strains of the same *Lactobacillus* spp. was quite small for *L. vaginalis* (CV 9.2%), it was moderate for the remaining *Lactobacillus* spp., ranging from 20% for *L. mucosae* to 106.7% for *L. gasseri*. The inhibition zones of the probiotic *L. rhamnosus, L. acidophilus* and *L. reuteri* strains (described in detail in Chapter 3, section 3.2.8) were smaller than most clinical strains (with the exception of *L. gasseri*), indicating that they might be less susceptible to clindamycin than the majority of vaginal *Lactobacillus* strains.

All vaginal *Lactobacillus* isolates showed similar sensitivity to penicillin, with the exception of *L. gasseri* isolates that tended to be less susceptible than the other *Lactobacillus* spp. (**Figure 5.16**B). There was little variation between strains in their susceptibility to penicillin (CVs ~15%), with the exception of *L. gasseri,* which had a CV of 60.8%. In comparison to the clinical strains, probiotic *L. acidophilus* strains appeared to be more sensitive to penicillin (median inhibition zone 39.0 mm, IQR 31.5-41.5). However, *L. rhamnosus* isolates tended to be similarly susceptible to penicillin (median zones of 26.0 mm, IQR 15.0-33.5) as the vaginal isolates, while *L. reuteri* tended to exhibit lower levels of susceptibility (median zones of 8.0 mm, IQR 7.0-28.0; Chapter 3, section 3.2.8).

The inhibition zones for amoxicillin were highly comparable to those measured for penicillin (**Figure 5.16**B), as was the intra-species variability. Compared to the probiotic isolates, *L. crispatus* were significantly more sensitive to amoxicillin than probiotic *L. rhamnosus* strains (median zones of 27.0mm, IQR 22.0-31.0; adj. p=0.0497). Similarly, vaginal *L. mucosae* isolates were more sensitive to amoxicillin than probiotic strains of *L. reuteri* (median 20.0 mm, IQR 15.0-26.0; adj. p=0.0128) and *L. rhamnosus* (adj. p=0.0033), indicating that *L. reuteri* and *L. rhamnosus* but not *L. acidophilus* may be less sensitive to amoxicillin than the vaginal isolates.

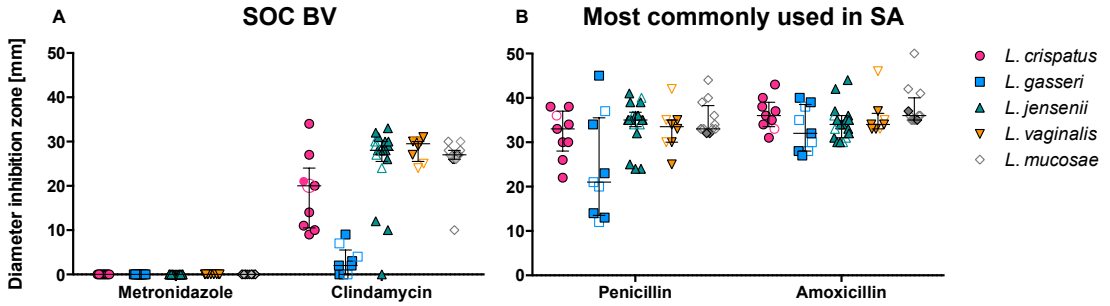


Figure 5.16. Evaluation of vaginal *Lactobacillus* isolates' susceptibility to antibiotices used for BV treatment (A) and those most commonly used in South Africa (SA, B). The susceptibility of *L. crispatus* (pink), *L. gasseri* (blue), *L. jensenii* (green), *L. vaginalis* (orange), and *L. mucosae* (grey) isolates to metronidazole (5 µg), clindamycin (2 µg), penicillin (2 µg) and amoxicillin (10 µg) was determined using disc diffusion assayss. The graph includes median with IQR. Isolates from BV-women are shown using filled symbols, while isolates from women with Nugent>3 have clear symbols.

Rifampicin and rifabutin are commonly used to treat TB in South Africa (Nahid *et al*. 2016) and generally form part of the six-month antibiotic regime administered to ~500,000 people in South Africa who develop TB annually (World Health Organization 2018). Since rifampicin was evaluated in the 1990's as a treatment option for BV (Hoover *et al*. 1993; Kharsany *et al*. 1993), it was important to evaluate the susceptibility of vaginal *Lactobacillus* isolates to this class of antibiotics. All vaginal *Lactobacillus* isolates were generally highly susceptible to rifampicin (<12.5 µg/ml) and rifabutin (<10.0 µg/mL; **Figure 5.17**). *L. vaginalis*, followed by *L. gasseri*, then *L. mucosae,* had the lowest MICs to rifampicin. Although *L. jensenii* and *L. crispatus* tended to have the highest MICs (medians 2.8 [IQR 0.4–7.8] and 6.3 [IQR 3.1 – 12.5] µg/mL, respectively) of the vaginal isolates, these MIC values would still indicate high susceptibility to these antibiotics. BV status of the donor did not impact sensitivity of the vaginal *Lactobacillus* spp. to rifamycins. The sensitivity varied within clinical *Lactobacillus* spp., ranging from a CV of 56% for *L. gasseri* to 198% for *L. mucosae*. For rifabutin, the same low susceptibilities were observed (**Figure 5.17**). Sensitivities of the probiotic isolates to these rifamycins were similar to the clinical strains (medians <0.13 µg/mL, described in Chapter 3, section 3.3.3.5).

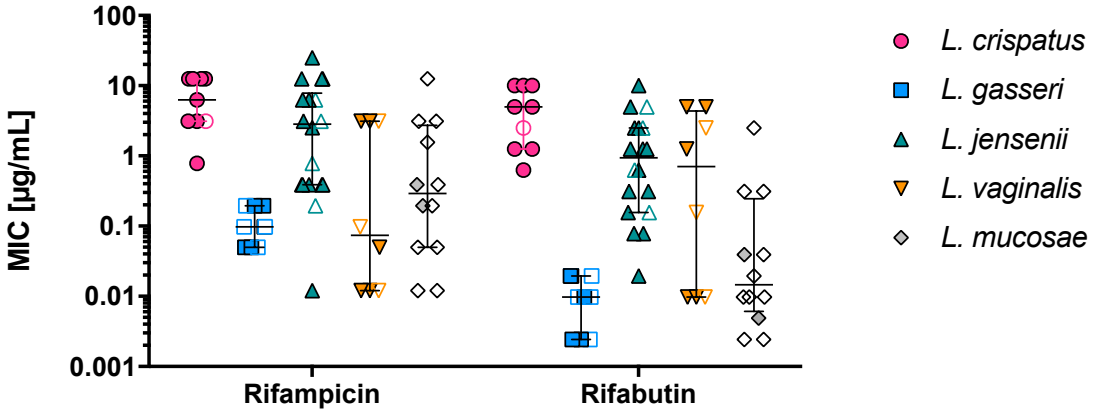


Figure 5.17. Evaluation of clinical *Lactobacillus* isolates' susceptibility to rifampicin and rifabutin. The susceptibility of *L. crispatus* (pink), *L. gasseri* (blue), *L. jensenii* (green), *L. vaginalis* (orange), and *L. mucosae* (grey) isolates to rifampicin (25µg/ml – 0.024µg/ml), and rifabutin (5µg/ml – 0.00488µg/ml) was determined using MIC assayss. If MICs were below the lowest or above the highest concentration tested, they were asigned a MIC value half of the lowest or twice the highest concentration tested, respectively. The graph includes median with IQR. Isolates from BV- women are shown using filled symbols, while isolates from women with Nugent>3 have clear symbols.

Broader antibiotic resistance profiling was conducted on 14 vaginal and six probiotic *Lactobacillus* isolates, including inhibitors of protein synthesis (chloramphenicol, linezolid, tetracylince, tigecycline, clindamycin, erythromycin, streptomycin, gentamycin, quinupristin/dalfipristin), cell wall and membrane (penicillin, vancomycin, nitrofurantoin, oxacillin, ampicillin, daptomycin), and DNA synthesis (moxifloxacin, ciprofloxacin, levofloxacin) using Sensititre™ plates. *L. gasseri* strains appeared to be sensitive to a wider range of antibiotics than the other *Lactobacillus* spp. tested (**Figure 5.18**). *L. crispatus* strains showed exactly the same antibiotic sensitivity to inhibitors of protein synthesis (chloramphenicol, linezolid, tetracycline, tigecycline, streptomycin, gentamycin, quinupristin/dalfipristin), cell wall/membrane (penicillin, vancomycin, daptomycin), and DNA synthesis (moxi, -cipro- and levofloxacin, **Figure 5.18**). In contrast, the other vaginal *Lactobacillus* strains were more variably sensitive (**Figure 5.18**). Further, there was neither a clear difference between isolates from BV- and BV+ women, nor between clinical and probiotic *Lactobacillus* spp. in antibiotic sensitivity profiles. The majority of *Lactobacillus* isolates tested (>18/20) had MICs above the highest concentration

tested for daptomycin (>2µg/mL), vancomycin (>16µg/mL), gentamycin (>8µg/mL), ciprofloxacin (>1µg/mL) and levovfloxacin (>2µg/mL).

Based on the clinical MIC breakpoints defined by the EUCAST (EUCAST 2018), all of the clinical isolates would be considered sensitive to ampicillin (MIC ≤4 µg/mL), and the majority were sensitive to chloramphenicol (MIC ≤8 µg/mL). For penicillin, (sensitivity defined as ≤0.25 µg/mL, resistance as >0.5µg/mL), all clinical *L. gasseri*, *L. mucosae* and *L. vaginalis* strains were sensitive, all *L. crispatus* and one of the *L. jensenii* strains (88.33 PA, from BV- woman; MIC 0.5 µg/mL) had an intermediate sensitivity, and one *L. jensenii* strain (92.27 PA, from BV+ woman) was resistant (MIC > 0.5 µg/mL). *L. crispatus* 100.16 a, *L. gasseri* 117.73 PA and 100.46 PA, *L. jensenii* 92.17 PA and 95.31 PA, *L. vaginalis* 79.24 PA and *L. mucosae* 102.33 PA were susceptible to clindamycin (MIC ≤1 µg/mL), while all other isolates had MICs >1 µg/mL. Since the resistance breakpoint for clindamycin has been defined as > 4 µg/mL (EUCAST 2018), it was not possible to conclude whether these isolates were resistant with the current data. However, the disc diffusion assay showed that *L. gasseri* 114.30 PA and *L. rhamnosus* 0200 and 0154 (from Gynophilus® and Provacare™) are resistant to clindamycin (described in Chapter 3, section 3.3.3.5). For all other antibiotics tested, no breakpoints have been defined yet. It was interesting to note that antibiotic susceptibility patterns appeared to be *Lactobacillus* strain-specific, with some strains being generally more susceptible than others (no overlap in radar plot, **Figure 5.18**), although this did not depend on the BV status of the donor.

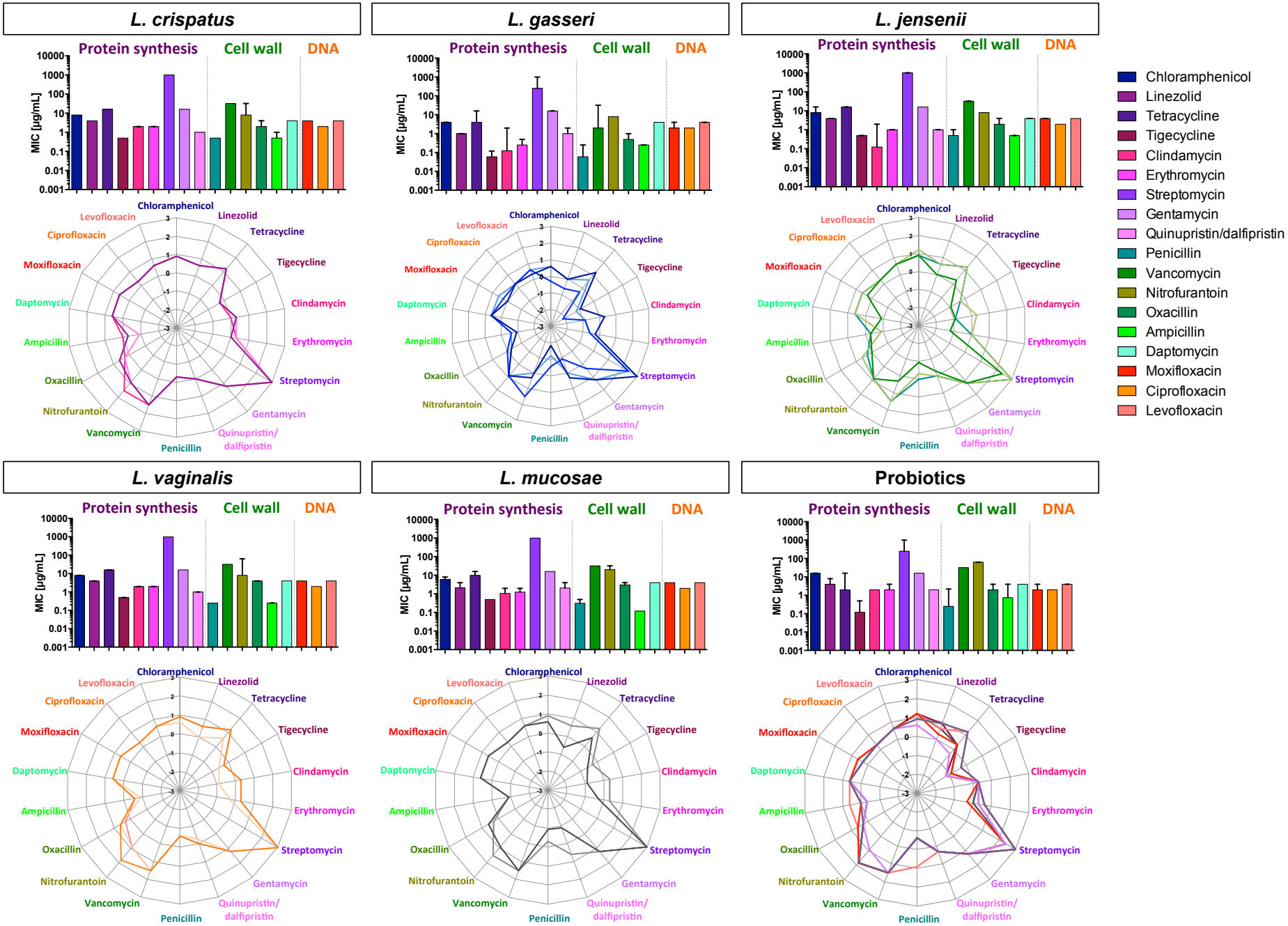


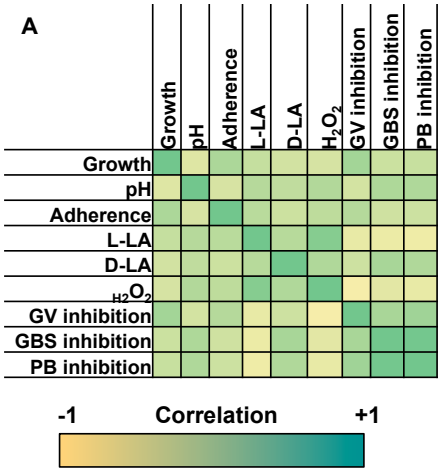
Figure 5.18. Susceptibility of vaginal and probiotic *Lactobacillus* isolates to various classes of antibiotics, including 21 protein synthesis, cell wall/membrane and DNA inhibitors. For a subset of 20 *Lactobacillus* isolates, the MIC to antibiotics interfering with the protein synthesis (shades of purple), cell wall and membrane (shades of green) and DNA synthesis (shades of red) was determined using Sensititre™ plates. The bar graphs shows the median and IQR for each species, while each line in the radar plot represent the susceptibility of individual isolates.

5.3.11 Selection of the top performing vaginal Lactobacillus strains

To identify the most promising vaginal *Lactobacillus* strains for the development of a probiotic for vaginal health for South African women, a weighted scoring system was developed based on PPP characteristics, including ability to grow well *in vitro* [described in section 5.3.4], lower pH [described in section 5.3.5], adhere to cervical epithelial cells [described in section 5.3.3], produce L- and D-lactate [described in section 5.3.6.1], and H_2O_2 [described in section 5.3.6.2], and inhibit growth of pathogens [described in section 5.3.8]. The cytokine and antibiotic sensitivity profiles were not considered, as these data were available for only a few vaginal *Lactobacillus* strains.

For this ranking, a total of 36/57 *Lactobacillus* strains (for which complete characteristic evaluations were available) were included. The four ATCC® reference strains and seven probiotic isolates were included in the ranking for comparison. Because some of the characteristics included in the ranking may correlate (for example production of L- lactate and H_2O_2), a weighted scoring system was developed to account for co-linearity between characteristics using a Spearman rank correlation co-efficient (described in **Figure 5.19**).

A



B	Weighting of each characteristic in:			
Characteristic	**Model A**	**Model B**	**Model C**	**Model D**
Growth	0.813	0.762	0.891	0.847
Culture pH	0.899	0.737	0.775	0.780
Adherence	0.886	0.775	0.913	0.907
L-lactic acid	0.778	0.270	0.690	0.816
D-lactic acid	1.003	0.936	0.879	0.882
H_2O_2	0.752	-	-	-
GV inhibition	0.843	1.009	0.931	-
GBS Inhibition	1.024	1.260	-	-
PB inhibition	1.003	1.250	0.922	-
GV & PB inhibition	-	-	-	0.768

Figure 5.19. Summary of (A) correlation matrix between measured characteristics; and (B) weighting of each characteristcs in the four different ranking models (models A to D) that were considered. A correlation matrix (including growth, ability to lower pH, adherence to cervical cells, production of L- and D-lactate as well as H_2O_2, inhibitory effect on *G. vaginalis, P. bivia* and GBS for Model A (A) was calculated to determine the weight of each characteristic in each model (B). Model A included growth, ability to lower pH, adherence to cervical cells, production of L- and D-lactate as well as H_2O_2, inhibitory effect on *G. vaginalis*, *P. bivia* and GBS. Model B excluded H_2O_2 production. Model C excluded H_2O_2 production and GBS inhibition, and Model D excluded H_2O_2 production and GBS inhibition, in addition to grouping *G. vaginalis* and *P. bivia* inhibition into one category. Dark green indiciates a strong positive correlation, while dark yellow indicates a strong negative correlation.

Considering all these characteristics in the scoring matrix (**model A**), *L. mucosae* 90.13 PA (18.7 points), *L. crispatus* 70.6 PA (score: 17.7 points) and 100.16 a (16.3 points), *L. vaginalis* 80.23 b (16.1 points), and *L. jensenii* 88.33 PA (15.3 points) and 92.27 PA (15.3 points) were the six highest scoring *Lactobacillus* strains, with similar points as the ATCC® *L. crispatus* (16.5 points) and ATCC® *L. gasseri* (15.4 points) strains, while all commercially available probiotics ranked lower (scores < 12.5; **Figure 5.20**).

Further step-wise sensitivity analyses were performed to test the robustness of the selection:

1. Because the concentrations of H_2O_2 produced by *Lactobacillus* were all below the level found to be microbicidal (O'Hanlon *et al*. 2011), a ranking model **(model B)** was considered in which H_2O_2 was excluded from the scoring system. Excluding H_2O_2 did not change the ranking of the top six strains [*L. crispatus* 70.6 PA (18.4 points), *L. mucosae* 90.13 PA (16.7 points), *L. crispatus* 100.16 a (16.2 points), *L. jensenii* 88.33 PA (15.5 points) and 92.27 PA (15.4 points), and *L. vaginalis* 80.23 b (16.2 points), **Figure 5.20**].
2. Because a new probiotic *Lactobacillus* strain would be marketed primarily for the treatment of BV and not GBS, a model was considered in which the inhibitory effect of the *Lactobacillus* strains against GBS were excluded, in addition to H_2O_2 production (**model C**), and again all previous strains remained in the top six (**Figure 5.20**).
3. In addition to excluding H_2O_2 production and GBS inhibition, a model was considered in which the inhibitory effect on *G. vaginalis* and *P. bivia* were grouped into one category (**model D**) to avoid skewing the ranking towards strains that perform generally well in the inhibition assays (**Figure 5.20**). However, all strains besides *L. vaginalis* 80.23b (now on rank 7) remained in the top six, in addition to *L. crispatus* 96.9 PB (previously on rank 7).
4. Grouping L- and D-lactate, which might be questionable as the isomers have different biological functions, antimicrobial vs. virucidal, did not influence ranking of top-performing strains (data not shown).

The sensitivity analysis outlined in Models A to D revealed that the scoring system was quite robust: *L. crispatus* 70.6 PA and 100.16 a, *L. jensenii* 88.33 PA and 92.27

PA, and *L. mucosae* 90.13 PA remained in the top five isolates in all scoring models considered. In addition, *L. vaginalis* 80.23 b and *L. crispatus* 96.9 PB remained in the top ten (**Figure 5.20**). The top six *Lactobacillus* strains included representatives from all the major vaginal species, with the exception of *L. gasseri. L. crispatus* and *L. jensenii* appeared to be the most highly ranked. Interestingly, according to these ranking models, more than half of the top strains originated from women with no BV or STIs (with the exception of *L. mucosae* 90.13 PA [derived from a woman who was BV+ and STI+] and *L. jensenii* 92.27 PA [derived from a woman who was BV+ but STI-].

As a comparison, the commercially available probiotic strains were included in the scoring system. However, none of these scored in the top ten in any of the models considered. The *L. crispatus* and *L. gasseri* ATCC® strains achieved comparable scores to the top ten strains, although were not considered for probiotic potential in this study nor included in ranking.

In conclusion, *L. crispatus* 70.6PA and 100.16a, *L. jensenii* 88.33PA and 95.1PA, *L. vaginalis* 80.23b and *L. gasseri* 94.98PB will be considered for the development of a locally relevant probiotic for vaginal health, all of which originated from BV- and STI- South African women.

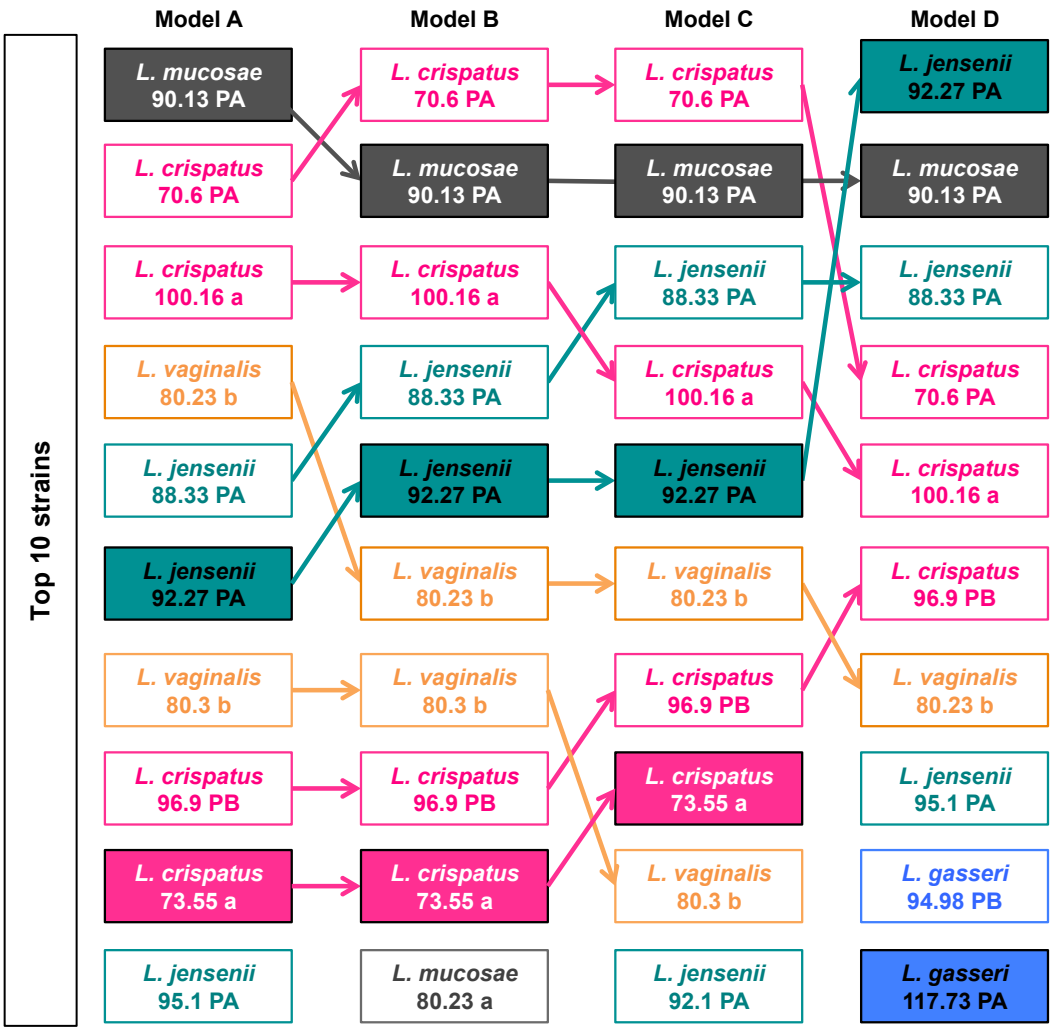


Figure 5.20. Relative ranking of vaginal *Lactobacillus* isolates according to the four different models that were considered. A weighted scoring system including growth, ability to lower pH, adherence to cervical cells, production of L- and D-lactic acid as well as H_2O_2, inhibitory effect on *G. vaginalis, P. bivia* and GBS (**model A**), excluding H_2O_2 production (**model B**), exlcuding H_2O_2 production and GBS inhibition (**model C**) and exlcuding H_2O_2 production and GBS inhibition, in addition to grouping *G. vaginalis* and *P. bivia* inhibition into one category (**model D**) was used to determine the best performing *Lactobacillus* strains. ATCC® strains were not included in this figure. Strains that originate from BV- women are shown using empty boxes, while those from women with a Nugent Score >3 have filled boxes. Species are color-codes (*L. crispatus*=pink, *L. gasseri*=blue, *L. jensenii*=green, *L. vaginalis*=orange, and *L. mucsaoe*=grey).

5.4 Discussion

BV is the most common vaginal condition of reproductive-aged women, which is characterised by a depletion of commensal *Lactobacillus* spp., particularly *L. crispatus*, *L. gasseri, L. jensenii*, *L. vaginalis* and *L. mucosae*, and an intense overgrowth of pathogenic bacteria, including *G. vaginalis* and *P. bivia* (Srinivasan *et al*. 2012; Africa *et al*. 2014; Lennard *et al*. 2017). The standard of care for BV, antibiotics, show some improvement in the short term, but not in the long term (Barrons & Tassone 2008). Probiotics in combination with antibiotics may improve efficacy and durability of BV treatment but the outcomes of clinical trials are heterogenetic (Tan *et al*. 2017). Probiotics retailed for vaginal health in South African predominantly contain GI and not FGT commensals (Chapter 2 and 3). The aim of this Chapter was therefore to evaluate key probiotic characteristics of vaginal *Lactobacillus* strains isolated from healthy, BV- South African women that could be considered for the development of a probiotic for vaginal health. Overall, this study showed that clinical *Lactobacillus* strains, and *L. crispatus* isolates in particular, performed better according to the PPP criteria (ability to adhere to cervical cells, growth under various pH conditions, ability to decrease culture pH, produce lactic acid and/or H_2O_2, inhibit growth of vaginal pathogens, and not induce inflammatory responses) than commercially available probiotic strains currently available in South Africa.

Adherence to human cells is an essential feature for any potential probiotic candidate, as it is necessary for the probiotic strain to remain in the environmental niche to be able to confer any health benefits, including preventing attachment of pathogens (Atassi *et al*. 2006; Coudeyras *et al*. 2008; Mastromarino *et al*. 2002). In this study, the majority of vaginal *Lactobacillus* strains adhered to cervical Ca Ski cells in a strain-specific manner, with about a quarter (13/57) showing superior adherence (defined as having >2% of added bacterial cells adhering). In contrast, none of the probiotic *Lactobacillus* isolates adhered as strongly to Ca Ski cells as these vaginal *Lactobacillus* isolates, but instead only showed intermediate levels of adhesion, suggesting that the vaginal *Lactobacillus* strains might be superior to the probiotics in term of remaining at the site of action and inhibiting pathogen adhesion.

The growth of clinical *Lactobacillus* strains was measured at low culture pH considered to reflect healthy (pH <4.5), as well as at higher pHs associated with dysbiotic vaginal conditions (pH >4.5), as strains that tolerate both conditions will have a growth advantage (Hossein Nezhad *et al*. 2010; Huang & Adams 2004; Silva *et al*. 2005). All strains grew best at pH 6.0 in MRS, as described previously (Hossein Nezhad *et al*. 2010). *L. crispatus* strains appeared to be the most tolerant of low pH, although this was highly strain-specific. In contrast, the probiotic isolates showed more moderate growth at pH 6.0 than any of the vaginal isolates, and did not grow as well at lower pHs as vaginal *L. crispatus* strains. These results suggest that *L. crispatus* possibly should be given priority over other vaginal *Lactobacillus* spp. in the development of a vaginal probiotic. None of the *Lactobacillus* strains grew at pH 2.0, suggesting that these probiotic candidates would have to be encapsulated to survive the gut passage if they were to be administered orally (Chávarri *et al*. 2012).

A low vaginal pH inhibits the growth and proliferation of viral and bacterial pathogens (O'Hanlon *et al*. 2011; Valore *et al*. 2002; Graver & Wade 2011; Nardini *et al*. 2016). In this study, all vaginal *L. crispatus* strains lowered the pH *in vitro* to 3.7, while only some strains of the other vaginal *Lactobacillus* spp. achieved pHs <4.0. This degree of acidification is in agreement with pH ranges observed in healthy woman, as described previously (Boskey *et al*. 1999; María Silvina Juárez Tomás *et al*. 2003). *In vivo*, women with an *L. crispatus*-dominated vaginal microbiota have been shown to have a lower pH than women with a *L. gasseri*- or *L. jensenii*-dominated microbiota, which was shown to be independent of ethnic group (Ravel *et al*. 2011). This suggests that ability to lower pH *in vitro* might be a good biomarker for what is happening *in vivo*, and that *L. crispatus* strains indeed have a higher capacity to acidify the environment than other vaginal *Lactobacillus* spp. None of the probiotic strains decreased the pH to <4.0, again emphasising that the commercially available strains might not be the best suited for treatment of BV.

The production of either L- or D-lactate by the vaginal *Lactobacillus* strains was quite variable, although they tended to produce higher levels of D- than L-lactate. Although not significant, *L. gasseri* strains tended to produce the highest amounts of L-lactate while *L. jensenii* strains tended to produce the least. This is in agreement with other studies showing that most strains of *L. jensenii* only produce D-lactic acid. In

contrast, *L. jensenii* strains produced significantly more D-lactate than the probiotic isolates. It has been previously described that *L. gasseri* produced significantly more lactate than *L. jensenii* and *L. crispatus* (Hutt *et al*. 2016), which is in agreement with these results. There was no difference in the lactate production by homo-fermenters (including *L. crispatus*, *L. gasseri*, and *L. jensenii,* which ferment glucose with lactate as the primary by-product) and hetero-fermenters (including *L. vaginalis* and *L. mucosae,* which ferment glucose to either lactate, ethanol/acetic acid or CO_2 as by-products; Zheng *et al*. 2015). In contrast to the *in vivo* findings by O'Hanlon *et al.* (2013) in cervicovaginal fluid samples from women with *Lactobacillus*-dominated microbiota, no correlation was observed between the ability of vaginal *Lactobacillus* isolates to produce lactic acid and their ability to lower culture pH, although it has been suggested that other acids, such as acetic acid, might contributed to the decrease in pH (Tachedjian *et al*. 2017).

H_2O_2 production by *Lactobacillus* spp. generally only occurs under aerobic conditions, and as such, the contribution of H_2O_2 to vaginal health, which is generally considered an anaerobic environment, is questionable. In agreement with previous studies measuring H_2O_2 levels under hypoxic conditions (O'Hanlon *et al*. 2011; María Silvin Juárez Tomás *et al*. 2003; Shalev *et al*. 1996), less than 15µM H_2O_2 was present in the cultures of the clinical *Lactobacillus* strains evaluated in this study under anaerobic growth conditions. These levels where similar to physiological concentrations of H_2O_2 (<100 µM) measured in vaginal fluids *in vivo* (O'Hanlon *et al*. 2011), which were >10.000-fold lower than concentrations shown to inhibit BV-associated bacteria *in vitro* (O'Hanlon *et al*. 2011; O'Hanlon *et al*. 2010). These results suggest that H_2O_2 production by vaginal *Lactobacillus* spp. most likely is not biologically relevant for pathogen inhibition *in vivo*. Interestingly, there was a strong correlation between production of H_2O_2 and L-lactate by the vaginal *Lactobacillus* isolates. In support of this, it has previously been noticed that vaginal *Lactobacillus* strains that produced H_2O_2 *in vitro* also produced considerably more lactate than those not producing H_2O_2 at all (Tomás *et al*. 2005), suggesting that *Lactobacillus* strains that are able to produce H_2O_2 despite hypoxic conditions might have a superior metabolic capacity.

G. vaginalis and *P. bivia* are common BV-associated pathobionts of the FGT (Lennard *et al*. 2017; J. Ravel *et al*. 2011; Gajer *et al*. 2012), so the ability of vaginal *Lactobacillus* strains to inhibit these organisms was important to evaluate in this study. However, less that one third of the vaginal *Lactobacillus* strains inhibited the ATCC® *G. vaginalis* strain and one of the vaginal *G. vaginalis* isolates, while the inhibitory effect on the remaining vaginal *G. vaginalis* strains was highly dependent on both *Lactobacillus* as well as *G. vaginalis* strains. *L. crispatus* strains tended to exhibit the strongest inhibitory activity against *G. vaginalis*, and only four vaginal *Lactobacillus* strains inhibited the growth of all six *G. vaginalis* isolates. Previously, vaginal *Lactobacillus* strains from healthy women have been shown to have a stronger inhibitory effect against various *G. vaginalis* strains *in vitro* than *Lactobacillus* strains isolated BV+ women (Teixeira *et al*. 2012). This was only observed for *L. mucosae* in this study; however, it needs to be considered that not many *Lactobacillus* strains from BV+ women were included in these assays. It was interesting to note that the inhibition of some *G. vaginalis* strains was generally lower than others, indicating that the *G. vaginalis* genotype may be an important determinant on their susceptibility to inhibition by clinical *Lactobacillus* strains. Previous studies have shown that metronidazole-resistant *G. vaginalis* strains as well as those originating from BV+ women were less susceptible to inhibition by *Lactobacillus* spp. (Teixeira *et al*. 2012; McLean & McGroarty 1996). It also has been described that biofilm-producing *G. vaginalis* strains may be more tolerant to H_2O_2 and lactic acid than non-biofilm producing strains (Patterson *et al*. 2007). In this study, the ability of vaginal *Lactobacillus* strains to lower pH correlated with their ability to inhibit *G. vaginalis*, suggesting that an acidic environment was influential in this inhibition. While some showed that a low pH accounted for the majority of the inhibitory activity against *G. vaginalis* (McLean & McGroarty 1996), others did not (Atassi *et al*. 2006; Saunders *et al*. 2007). Instead, some have found that bacteriocins (Saunders *et al*. 2007) or an undefined by compound that was resistant to proteolytic enzymes (Atassi *et al*. 2006) accounted for the ability to inhibit *G. vaginalis*.

In was not expected that the ability of vaginal *Lactobacillus* isolates in this study to produce L- lactate and H_2O_2 was negatively associated with their ability to inhibit *G. vaginalis*. Since ability to produce L- lactate and H_2O_2 were co-linear in this study, it is expected that both would correlate with the ability of *Lactobacillus* strains to inhibit

Gardnerella isolates, although the H_2O_2 concentrations measured in the culture supernatant used for these experiments were too low to exhibit an antimicrobial effect (O'Hanlon *et al*. 2011). However, it still cannot be explained why L- lactate correlated negatively with the ability of *Lactobacillus* strains to inhibit *G. vaginalis.* It is possible that the vaginal *Lactobacillus* strains in this study that produced low levels of L- lactate might have produced higher levels of other metabolites that facilitate the inhibition of *G. vaginalis,* which were not measured in this study. Parente *et al.* (1994) found that higher lactic acid levels were associated with lowered bacteriocin production during batch fermentation of *Lactobacillus* strains (Parente *et al*. 1994). It could therefore be hypothesized that, at the time point at which *Lactobacillus*-conditioned media was harvested, media that had high L- lactate concentrations also had lower levels of bacteriocins, and therefore inhibited *G. vaginalis* poorly. Hutt *et al*. (2016) previously reported that the amount of lactic acid produced by *Lactobacillus* spp. did not predict their antagonistic activity against *G. vaginalis*, suggesting that lactic acid might not be the primary mechanism by which *Lactobacillus* strains inhibit vaginal *Gardnerella* spp. Although this study was not specifically designed to determine the mechanisms of actions of *Lactobacillus* strains against pathogens like *G. vaginalis*, it is likely that this inhibition was partly facilitated via the acidification of the media, but that metabolites other than lactate, such as bacteriocins and/or biosurfactant, contributed.

In contrast to the ability of *Lactobacillus* strains to inhibit *G. vaginalis,* their ability to inhibit growth of *P. bivia* and GBS was much stronger and less variable. Again, L-lactate and H_2O_2 correlated inversely with the inhibition, possibly due to the same reasons as discusses above. Furthermore, the inhibitory effect of vaginal and probiotic *Lactobacillus* isolates was comparable, confirming that microbicidal effects are strain-and not species-specific. It is unclear why the inhibitory effect of *Lactobacillus*-conditioned media was weaker against *G. vaginalis* than *P. bivia* and GBS. Anaerobic versus aerobic growth conditions cannot explain the difference as both *G. vaginalis* and *P. bivia* were grown anaerobically while GBS was grown aerobically. Although there was no correlation between the ability of *Lactobacillus* isolates to lower pH and their ability to inhibit *P. bivia* and GBS growth, these pathogens might still be more susceptible to low pH. It has been shown that *G. vaginalis* lowers pHs to ~4.5 *in vitro* (Saunders *et al*. 2007), which suggests that *G.*

vaginalis is still viable at this pH. In contrast, a decrease in pH to less than 5.5 *in vitro* was associated with microbicidal activity against *P. bivia* in the Saunders (2007) study. Previously, vaginal *P. bivia* absolute concentrations correlated positively with vaginal pH *in vivo* (Pybus & Onderdonk 1996), confirming that *P. bivia* might be susceptible to lower pH. Similarly, GBS was found to have reduced growth at pH 5.0, and failed to persistently grow at pH <4.5 (Yang *et al*. 2012; Wood *et al*. 2005; Borges *et al*. 2012). In future studies, adjusting the pH of the *Lactobacillus*-conditioned media to pH 6.0, and repeating the assay would allow determination of the relative contribution of low pH to pathogen inhibition. Furthermore, the addition of catalase to inactivate H_2O_2 (Stewart *et al*. 2000); or proteolytic enzymes to degrade bacteriocins (Cotter *et al*. 2005) would allow elucidating the mechanism/s responsible for the inhibition of *G. vaginalis*, *P. biva* and GBS growth by these vaginal isolates.

Because increased levels of genital inflammatory cytokines were shown to increase HIV risk in women (Masson *et al*. 2016; Liebenberg *et al*. 2017), cytokine responses by cervical epithelial Ca Ski cells following exposure to the vaginal *Lactobacillus* isolates from this study were measured. All clinical *Lactobacillus* strains up-regulated the regulatory cytokine IL-1RA, which has previously been found to inhibit the pro-inflammatory cytokines IL-1α and IL1-β (Sims & Smith 2010). In addition, the majority of *Lactobacillus* strains down-regulated IP-10 production by Ca Ski cells. IP-10 concentrations in cervico-vaginal fluid and the gradient between blood and the genital tract has also been associated with risk of HIV acquisition of South African women (Liebenberg *et al*. 2017). Down-regulation of IP-10 may be mediated by the production of lactic acid by *Lactobacillus* strains, as other studies have shown that lactic acid can suppress inflammatory responses *in vitro* (Gong *et al*. 2014; Aldunate *et al*. 2013; O'Hanlon *et al*. 2013). Together, the findings that IL-1RA is upregulated while IP-10 production is lowered indicates that the administration of these clinical *Lactobacillus* strains might be considered generally safe, as is does not increase important markers of genital inflammation. However, a few of the *Lactobacillus* strains appeared to cause increased production of IL-8 by Ca Ski cells, which is important to consider in settings with high HIV rates. IL-8 attracts and activates neutrophils in inflammatory tissue, and can stimulate HIV replication in lymphocytes and macrophages (Pananghat *et al*. 2016; Lane *et al*. 2001). It should be acknowledged that studies in which bacteria are added in co-culture with FGT cells

are challenging, as great care needs to be taken in selecting the appropriate medium and grown conditions for both cells and bacteria to survive. In this study, cells were cultured on tissue culture plates rather than transwell plates, and the lowering of culture pH by *Lactobacillus* strains could have been toxic to Ca Ski cells, as the approach used in this study does not allow diffusion of the acid into the basolateral medium to minimize toxicity. Although lactate levels and pH achieved in these co-cultures were not measured in this study, the viability of CaSki cells was determined after co-culture to ensure that cytokine responses were not influenced by bacteria-induced cell toxicity.

Susceptibility testing of clinical *Lactobacillus* strains to antibiotics commonly used for BV treatment (such as metronidazole and clindamycin) showed that all strains were resistant to metronidazole, while only a few strains were resistant to clindamycin, which is similar to what has previously been described (Ocaña *et al*. 2006; Charteris *et al*. 1998; Delgado *et al*. 2005). The selection of clindamycin resistant or sensitive strains for the development of a product will have an impact on whether the probiotic could be administered concurrently with antibiotic clindamycin treatment for BV, as co-prescriptions of antibiotics and probiotics should only be done when the probiotic strain is not susceptible to the administered antibiotic (Neut *et al*. 2017). The susceptibility of clinical *Lactobacillus* strains to other commonly used antibiotics in South Africa (such as penicillin and amoxicillin) was also evaluated in this study. All clinical *L. gasseri*, *L. mucosae* and *L. vaginalis* strains tested appeared to be sensitive to penicillin, all *L. crispatus* and one of the *L. jensenii* strain had intermediate sensitivity, and one *L. jensenii* strain was resistant. This assay also showed that all of the clinical isolates would be considered sensitive to ampicillin and the majority sensitive to chloramphenicol. These results indicate that antibiotic resistance to a broad panel of antibiotics might not be common among vaginal *Lactobacillus* isolates but still present. It is therefore imperative to perform broad antibiotic sensitivity profiling before formulating probiotic strains, due to the threat of antibiotic resistance (Stecher *et al*. 2012; Van Reenen & Dicks 2011; Gueimonde *et al*. 2013). Further, whole genome sequencing should be performed to identify antibiotic resistance genes. No breakpoints have been established for *Lactobacillus* spp. for the majority of antibiotics that were screened for, making it impossible to confidently define resistance profiles for these isolates. As *Lactobacillus* spp. are

commonly included in probiotic products targeting gut health, and are regularly consumed by a huge proportion of the global and South African population, there is an urgent need for the EUCAST guidelines to establish these breakpoints.

To select the vaginal *Lactobacillus* strains with the best probiotic profile (most closely aligned with the PPP), a weighted scoring system was developed in this Chapter based on the following criteria: ability to grow well *in vitro*, lower pH, adhere to cervical epithelial cells, produce lactic acid and H_2O_2, and to inhibit growth of pathogens. The top performing strains included two *L. crispatus* isolates (70.6PA and 100.16a), two *L. jensenii* isolates (88.33PA and 95.1PA), and one isolate each of *L. vaginalis* (80.23b) and *L. gasseri* (94.98PB), all of which were from healthy young South African women without BV (Nugent 0-3) and with no other STIs. Despite testing the scoring system using four different models, it appeared robust, independently from which respective PPP characteristics were included, confirming that the top six vaginal *Lactobacillus* strains performed well in all probiotic traits tested. Although the commercially available probiotic *Lactobacillus* strains were included as independent controls in parallel with the vaginal isolates in the scoring system, none performed as well as these clinical strains, suggesting that there is a huge potential to improve probiotics for vaginal health, which in turn might improve the effect of these administered probiotics on BV cure and recurrence.

This study had several limitations that should be mentioned here. Not all probiotic characteristics included in this study were determined for all 57 clinical *Lactobacillus* strains, due to some assay and cost limitations, although rational selection of strains was done to choose the isolates with early promising PPP characteristics. In particular, detailed cytokine and antibiotic resistance profiling should be performed for the top-ranking clinical strains, as these are likely to be important *in vivo*. Furthermore, other important PPP characteristics were not measured in this study (such as ability to form biofilms, produce toxins, biosurfactants, and/or bacteriocins, their metabolic profiles and ability to metabolise prebiotics, and their safety in immune-compromised animals), although they should be considered in the selection of the clinical *Lactobacillus* strains going forward. Whole genome sequencing of the top ranking *Lactobacillus* isolates has not yet been performed, which would allow for the identification of virulence and antibiotic resistance genes, prophage sequences, as

well as genes involved in metabolic pathways relevant to their probiotic potential. Although the *Lactobacillus* isolates appeared to inhibit growth of various BV-associated bacterial strains and GBS, no broad mechanism of action has been determined for pathogen inhibition. This would help us to understand how probiotics confer their health benefits. Vinderola *et al*. (2017) recently suggested that *in vitro* and *in vivo* characteristics of probiotics do not correlate well, therefore the value of these *in vitro* tests as predictors of probiotic capacity may not provide the full story, but they are likely to be useful to narrow down potential candidates.

The question as to whether a single or multiple *Lactobacillus* species formulation should be considered, and whether a single or multiple strains of the same *Lactobacillus* spp. should be co-formulated in the vaginal probiotic, also needs to be addressed. From an ecological perspective, a higher diversity of healthy bacterial species promotes community stability and functionality, in addition to functional resilience after perturbation (Girvan *et al*. 2005; Lozupone *et al*. 2012; Eisenhauer *et al*. 2012), and more stable communities are also less susceptible to the invasion of pathogens (Dunstan & Johnson 2006). Further, inclusion of prebiotics also needs to be considered in the probiotic formulation, as this may be important for conferring a health benefit to the host. Although it would possibly be more difficult to deliver, it is desirable that vaginal pessaries should be considered, which could be administered over a long period of time. However, it has been suggested that oral probiotics can improve vaginal health (Reid *et al*. 2001), and that orally administered strains can be recovered vaginally although at much lower concentrations than vaginally applied strains (Reid *et al*. 2001; Reid *et al*. 2003; Morelli *et al*. 2004). Orally administered probiotics rely on passive ano-genital transfer (Reid *et al*. 2001), while vaginal administration will ensure that the probiotic bacteria are administered directly at the desired site for colonization.

Conclusion

This study evaluated probiotic characteristics of a large panel of vaginal *Lactobacillus* strains, isolated from healthy young South African women. The probiotic potential of these isolates were found to be highly strain-specific, and clinical *Lactobacillus* strains form BV- women appeared to perform better than commercially available probiotic strains currently used in probiotics for vaginal health available in South

Africa. The best-performing vaginal *Lactobacillus* isolates should be used for the development of a region-specific probiotic for vaginal health that may result in improved BV cure rates when being administered in adjunct to SOC in South Africa.

Chapter 6

The role of bacteriophages in the aetiology of BV

6. Evidences for the likely contribution of bacteriophages to the onset of BV

6.1 Introduction

Bacteriophages are amongst the most numerous organisms on earth (Labrie *et al*. 2010), and are thought to play an important role in shaping the microbiota of the human gut (Manrique *et al*. 2016; Wagner *et al*. 2013; Lepage *et al*. 2008; Parkes *et al*. 2014), oral cavity (Pride *et al*. 2012; Willner *et al*. 2011), skin (Marinelli *et al*. 2012; Liu *et al*. 2015), and lungs (Segura-Wang *et al*. 2018). Several groups have identified integrated bacteriophage genomes in vaginal *Lactobacillus* isolates and other vaginal bacterial species (Pavlova & Tao 2000; Kilic *et al*. 2001; Martín *et al*. 2009; Malki *et al*. 2016), which suggests that these common viruses might similarly play a role in shaping the microbiome of the lower FGT.

BV is defined by a rapid shift in the composition of vaginal bacterial communities (Gajer *et al*. 2012; Srinivasan *et al*. 2010). In other environmental niches, bacteriophages have been shown to be highly efficient predators that are able to quickly kill a population of sensitive bacterial hosts, and thereby rapidly alter the composition of microbial communities (Santos *et al*. 2014; Mitarai *et al*. 2016; Pantastico-Caldas *et al*. 1992). Although it has not yet been demonstrated *in vivo*, it has been hypothesized by several authors (Blackwell 1999; Pavlova *et al*. 1997; Pavlova & Tao 2000) that bacteriophage infection may be the underlying cause for the rapid alteration of the vaginal microbiota associated with BV. The depletion of *Lactobacillus* spp. associated with the onset of BV might occur due to the induction of temperate bacteriophages that are harboured in the genomes of commensal *Lactobacillus* strains themselves (or in probiotic *Lactobacillus* strains that are introduced into the FGT), and/or by introduction of virulent bacteriophages into the lower FGT that target commensal *Lactobacillus* strains. As such, the presence of prophage regions or CRISPR loci within vaginal *Lactobacillus* genomes can be used as a measure for the presence of temperate bacteriophages or previous exposure to virulent bacteriophages, respectively (Rho *et al*. 2012; Hobbs & Abedon 2016). While several studies have demonstrated that temperate bacteriophages can be induced from vaginal *Lactobacillus* isolates (Raya *et al*. 1989; Pavlova *et al*. 1997; Kilic *et al*. 2001; Damelin *et al*. 2011), no virulent bacteriophages have been isolated from secretions collected from the lower FGT yet, and no *in vivo* studies have shown a direct link between bacteriophage communities and the development of BV.

In this study, it was hypothesized that prophage sequences and CRISPR loci are common in the genomes of vaginal and probiotic *Lactobacillus* isolates. In addition, it was hypothesized that bacteriophages capable of infecting vaginal *Lactobacillus* strains will be present in genital tract secretions from BV positive women while being absent from BV negative women. The aim of this Chapter was therefore to: (1) determine how frequent prophage sequences or CRISPR loci are present in published *Lactobacillus* genome sequences, by performing *in silico* analyses; and (2) to determine whether bacteriophages might be involved in the pathogenesis of BV, by inducing temperate bacteriophages from vaginal *Lactobacillus* isolates and by isolating virulent bacteriophages from vaginal secretions from South African women and testing their host ranges.

6.2 Methods

6.2.1 Analysis of published Lactobacillus genomes for the presence of prophage sequences

Whole genome sequences for a number of vaginal and probiotic *Lactobacillus* spp. are publically available from NCBI genome[15], including *L. crispatus* [n=1; ST1], *L. gasseri* [n=; ATCC33323, 4M13, and DSM 14869], *L. jensenii* [n=2; SNUV360 and JV-V16], *L. vaginalis* [n=1, DSM5837], *L. mucosae* [n=1; LM1], *L. rhamnosus* [n=15; GG, Lc705, ATCC8530, LOCK900, LOCK908, HN001, BFE5264, Pen, 4B15, LR5, DSM14870, SCT-10-10-60, BPL5, ASCC290, WQ2], *L. reuteri* [n=9; DSM20016, JCM1112, SD2112, I5007, TD1, IRT, ZLR003, I049, and ATCC53608], *L. acidophilus* [n=5; NCFM, La-14, FS14, ATCC53544, LA1] and *L. helveticus* [n=13; CAUH18, DPC4571, R0052, H10, CNRZ32, H9, KLDS1.8701, MB2-1, D76, D75, FAM8627, FAM22155, and FAM8105]. PHASTER (PHAge Search Tool Enhanced Release; Zhou *et al*. 2011; Arndt *et al*. 2016)[16] was used to identify and annotate prophage sequences in these genomes. For each potential prophage sequence, its region length, completeness score, specific keywords (such as integrase, terminase, portal, capsid, tail, lysin), number or tRNAs, proteins, bacteriophage proteins, and bacterial proteins detected, the most common bacteriophage name, and the GC content were captured.

6.2.2 Identification of CRISPR sequences in published Lactobacillus genomes

The publically available CRISPR finder program[17] was used to identify CRISPR loci in publically available genome sequences of vaginal *Lactobacillus* spp. (including *L. crispatus* [n=13; JV-VOI, MV-1A-US, 125-2-CHN, MV-3A-US, SJ-3C-US, 214-1, CTV-05, FB049, FB077-07, 2029, EM-LC1, DSM20584, and ST1], *L. gasseri* [n=12; 497_LGAS, JV-V03, K7, 224-1, SV-16A-US, MV-22 SJ-9E-US, ATCC33323, 2016, CECT5714, 202-4, and 130918], *L. jensenii* [n=14; IM18-1, IM18-3, SJ-7A-US, 1153, 269-3, MDIIE-70(2), DSM20557, JV-V16, IM1, IM11, 115-3-CHN, 27-7-CHN, IM59, and IM3], *L. vaginalis* [n=1, DSM5837], *L. mucosae* [n=8, LM1, CRL573, DPC6426, Marseille, DSM13345, WCC8, KHPC15, and KHPX11], and *L. iners* [n=15; SPIN1401G, UPII60-B, LactinV 03V1-b/09V1-c/11V1-d/01V1-a, SPIN2503V10-D, UPII143-D, LEAF2052A-d/2053A-b/2062A-h1/3008A-a, AB-1,

[15] https://www.ncbi.nlm.nih.gov/genome
[16] http://phaster.ca/
[17] http://crispr.i2bc.paris-saclay.fr/Server/

DSM13335, and ATCC55195]) from NCBI genome. The number of confirmed CRISPR loci (defined by the presence of ≥3 motifs and ≥2 exactly identical directed repeats) and possible CRISPR loci (defined by the presence of <3 motifs and <2 identical directed repeats), CRISPR loci lengths, the length of directed repeats, and the number of spacers were recorded. To identify possible sequence similarities between the CRISPR loci and sequenced bacteriophages, the spacers of confirmed CRISPR loci were extracted and blasted against the Genbank bacteriophage database (not against the plasmid database) using CRISPRTarget[18].

6.2.3 Screening vaginal secretions for the presence of lytic bacteriophages against vaginal Lactobacillus isolates and E. coli MG1655

6.2.3.1 Description of the cohort from with vaginal samples were collected

As part of a larger study of the effects of hormonal contraception on HIV risk in adolescents (the uCHOOSE study), 151 adolescent girls and young women (16-19 years) were enrolled from the DTHF Youth Centre, which is based in an informal settlement called Masiphumulele, just outside of Cape Town, between July 2015 and February 2018. All adolescents were HIV-negative, sexually active, and considered healthy. At the time they were recruited, they were neither pregnant nor menstruating, had not taken any antibiotics in the previous two weeks and were counselled to abstain from sex, douching or using spermicides for 48 hours prior to study visits.

6.2.3.2 Collection and processing of vaginal samples

From each participant in the uCHOOSE study, a lateral vaginal wall ESwab™ was collected and placed into Amies medium (ESwab™ Liquid Amies Collection and Transport System, Copan Diagnostics Inc., USA) for bacterial culture and isolation of virulent bacteriophages. In addition, an endocervical swab was collected and placed into 500 µL of PBS for the isolation of virulent bacteriophages. Upon receipt in the laboratory, 400 µL of each sample (used for isolation of virulent bacteriophages) were added to an Eppendorf tube with 10% chloroform (288306, Sigma-Aldrich®, USA) to lyse bacteria. The tubes were inverted three times, and centrifuged at 3390g (Microcentrifuge, Labnet Inc., USA) for 5 min. The supernatant fraction was then transferred to a new Eppendorf tube, and stored at 4°C until serial enrichment assays (section 6.2.3.3) were performed. In addition, 50 µL of the Amies medium was added to 1.4 mL of each of the following enrichment media: (1) MRS (69966, Sigma-

[18] http://bioanalysis.otago.ac.nz/CRISPRTarget/crispr_analysis.html

Aldrich®, USA) to enrich for *Lactobacillus* spp. growth; (2) SB (CM0497B, ThermoFisher™, USA) supplemented with 5% horse blood to enrich for the growth of BV-associated bacteria; and (3) unselective TS (22092, Sigma-Aldrich®, USA) medium. These three different growth media were incubated at 37°C for 2 days under anaerobic conditions, in order to enrich for specific bacterial species and associated bacteriophages (if their host strains were present) that might be present in the FGT sample. The tubes were centrifuged at 589g (Microcentrifuge, Labnet Inc., USA) for 5 min, and 1 mL of the supernatant was transferred to a new Eppendorf tube. Chloroform (10%, Sigma-Aldrich®, USA) was added to each tube to lyse bacteria, and processed as described above. The uCHOOSE study was approved by UCT (HREC Ref 801/2014), and in addition UCT HREC approved this study as part of this PhD (HREC Ref 319/2016).

6.2.3.3 Serial transfer enrichment assay for isolation of virulent bacteriophages

For the detection of virulent *Lactobacillus* bacteriophages, five different vaginal sample types (lateral vaginal wall ESwab™ in Amies medium, and enriched in MRS, SB, and TS, and endocervical swab in PBS; section 6.2.3.2) from a convenient sample of 88 of the 151 uCHOOSE participants (n=37 BV positive [Nugent 7-10], n=7 BV intermediate [Nugent 4-6], n=44 BV negative [Nugent 0-3]) were screened for the presence of virulent *Lactobacillus* bacteriophages, while only the lateral wall swab in Amies medium from the same 88 participants was used for the detection of virulent *E. coli* bacteriophages.

A serial transfer enrichment assay was optimized in this study to enrich for lytic bacteriophages present in these genital tract samples. The following bacterial strains were included as hosts in the screen for bacteriophages: *E. coli* MG1655 (considered a highly susceptible host strain for bacteriophage infection; Allen *et al*. 2017), and five vaginal *Lactobacillus* strains previously described in Chapter 5 (including *L. crispatus* 73.55 a, *L. gasseri* 94.98 PB, *L. jensenii* 72.1 PA, *L. vaginalis* 81.17 a, and *L. mucosae* 80.23 a). To enrich for virulent bacteriophages, 600 µL of singe-strain *Lactobacillus* and *E. coli* overnight cultures adjusted to OD_{600nm} of 0.1 (± 0.1) in MRS (supplemented with 10mM $CaCl_2$) or Luria-Bertani (LB, L3522, Sigma-Aldrich®, USA, supplemented with 10mM MgSo4, 0.2% Maltose and 10mM $CaCl_2$) broth, respectively, were added per well to a 96-well deep well plate (Nunc™ 1.0 mL DeepWell™ Plates, ThermoFisher™, USA). The plates were incubated anaerobically

in the case of *Lactobacillus* strains or aerobically for *E. coli* MG1655 at 37°C. When OD_{600nm} of 0.3 (± 0.1) was reached, 50 µL of chloroformed FGT sample (section 6.2.3.2) was added to each well, and the plates were incubated at 37°C overnight (anaerobically for *Lactobacillus* and aerobically for *E. coli* strains). As negative control, no FGT sample was added to some wells. As bacteriophages are more stable at 4^oC than 37^oC, the 96-well plate was incubated at 4°C for >1 hour the next morning to allow bacteriophages to stabilize, before adding chloroform (10%, Sigma-Aldrich®, USA) to each well to lyse the host bacteria. The plate was centrifuged at 4500g (Eppendorf centrifuge 5810, Eppendorf AG, Germany) for 10 min at 4°C. Supernatants were transferred to a new 96-well deep well plate, and stored at 4°C. After each round of enrichment, a spot assay (section 6.2.3.4) was performed to test for the presence of bacteriophages targeting the bacterial host strains. To start the next round of enrichment, 500 µL of bacterial host overnight culture adjusted to OD_{600nm} of 0.1 (± 0.1) as described above was added per well to a 96-deep well plate and allowed to grow until an OD_{600nm} of 0.3 (± 0.1) at 37^oC. Then, 100 µL of enriched supernatant was added to each well, and the plates were incubated and processed as described above. For each FGT sample, 10 rounds of serial enrichment transfers were performed.

6.2.3.4 Testing for the presence of virulent bacteriophages

The spot assay was used after each round of serial enrichments (section 6.2.3.3) to test for the presence of virulent bacteriophages targeting *E. coli* MG1655 or *Lactobacillus* spp. Overnight cultures (200 µL) of vaginal and probiotic *Lactobacillus* or *E. coli* strains was inoculated into 3 mL of molten MRS (supplemented with 10mM $CaCl_2$) or LB (supplemented with 10mM MgSo4, 0.2% Maltose and 10mM $CaCl_2$) top agar (0.6 % agar), respectively, and overlaid onto MRS or LB agar plates (1.2 % agar), respectively. Each overlay was allowed to solidify for 10 min. Two µL of bacteriophage sample was spotted onto each bacterial overlay and incubated at 37°C for 48 hours under either anaerobic (*Lactobacillus* spp.) or aerobic conditions (*E. coli*). The next day, the plates were checked for the presence of spots and/or plaques. If lysis was observed, spots and plaques were harvested in SM buffer (50 µL of 100 mM NaCl, 10 mM $MgSO_4 \bullet 7H_2O$, 50 mM Tris-HCl, pH=7.4), and stored over night at 4°C to allow bacteriophages to diffuse from the agar into the buffer. To lyse remaining host bacteria, 10% chloroform (Sigma-Aldrich, USA) was added to each

tube, after which they were inverted three times, and centrifuged at 3390g (Microcentrifuge, Labnet, USA) for 5 min. The spot assay was repeated with the harvested sample to confirm the presence of bacteriophages, and once confirmed, bacteriophages were purified (section 6.2.3.5)

6.2.3.5 Purification of virulent bacteriophages and preparation of stocks

E. coli bacteriophages were purified from samples that confirmingly contained bacteriophages (section 6.2.3.4). Therefore, the respective supernatant from the enrichment assay (section 6.2.3.3) was serially diluted (10^{-2}, 10^{-4}, 10^{-6}) in SM buffer (100 mM NaCl, 10 mM $MgSO_4 \bullet 7H_2O$, 50 mM Tris-HCl, pH=7.4). Fifty µL of these serial dilutions were added together with 200 µL of *E. coli* MG1655 overnight culture to 3 mL of molten LB (supplemented with 10mM MgSo4, 0.2% Maltose and 10mM $CaCl_2$) top agar (0.6% agar). The tube was inverted gently three times, and poured onto prepared agar plates. These plates were incubated at 37°C for 48 hours under aerobic condition, and plates were checked for the presence of plaques the next day. Single plaques were harvested in SM buffer and processed as described above (section 6.2.3.4). The plaque assay was repeated, and again single plaques were harvested. This process was repeated three times to ensure that bacteriophage stocks contained single bacteriophages and not a mixture of several bacteriophages. To obtain a larger volume of bacteriophage stocks for transmission electron analysis (TEM, section 6.2.5) and host range testing (section 6.2.6), the harvested single bacteriophages were enriched in their host *E. coli* MG1655. Overnight cultures of *E. coli* MG1655 adjusted to OD_{600nm} of 0.1 (± 0.1) in 30mL of LB (supplemented with 10mM MgSo4, 0.2% Maltose and 10mM $CaCl_2$) were incubated aerobically at 37°C until an OD_{600nm} of 0.3 (± 0.1) was reached. Forty µL of purified bacteriophage sample was added and incubated under aerobic condition at 37°C overnight. The next day, 10% chloroform was added, the tube was inversed vigorously and placed at room temperature for 5 min, which was repeated twice. The *E. coli* cellular debris was pelleted by centrifugation at 4000g for 30 min at 4°C (Eppendorf centrifuge 5810R, Eppendorf AG, Germany), and supernatants were filtered (0.45 µm and 0.2 µM, Minisart® NML Syringe Filter, Sartorius Stedim Plastics GmbH, Germany). These pure bacteriophage stocks were stored at 4°C.

6.2.4 Induction of temperate bacteriophages from Lactobacillus isolates

Overnight cultures of probiotic (n=18, 6/18 from South African products, described in Chapter 3, and 12/18 from probiotics for vaginal health marketed in France [n=4 *L. crispatus* isolated from Jarro Dophilus® Women, Physioflor, Vacramal® and Probiovance® intime; n=1 *L. rhamnosus* from Lactogyn®, n=1 *L. reuteri* from Probiovance® intime; n=2 *L. acidophilus* from mediGYNE® and BactiGyn, n=2 *L. plantarum* from BactiGyn and Vacramal®; n=1 *L. salvarius* from BactiGyn; provided by Dr Remy Froissart, CNRS, France]) and vaginal *Lactobacillus* strains from South African women (n=39/57; including 10 *L. crispatus*, 7 *L. gasseri*, 11 *L. jensenii*, 5 *L. vaginalis,* and 6 *L. mucosae* strains, described in Chapter 5) were adjusted to an OD_{600nm} of 0.1 (±0.01) in 50 mL of MRS containing 10 mM $CaCl_2$ and incubated anaerobically at 37°C to reach an OD_{600nm} of 0.3 (±0.01). 1.5 mL of this culture was transferred to an Eppendorf tube as control (no Mitomycin C added). Then, 0.5 µg/mL of Mitomycin C (M0503, Sigma-Aldrich, USA) was added to the 50 mL tube. Again, 1.5 mL of this culture (now with Mitomycin C added) was transferred to another Eppendorf tube. Samples were incubated for 18 hours at 37°C, and the turbidity of cultures in Eppendorf tubes with and without Mitomycin were compared. If *Lactobacillus* cultures showed an indication for bacteriophage induction (defined as less growth in tubes with Mitomycin C added), 10% chloroform was added, the tube was inversed vigorously and placed at room temperature for 5 min, which was repeated twice. The suspension was then centrifuged for 30 min at 4000g (Eppendorf centrifuge 5810R, Eppendorf AG, Germany) at 4°C to pellet bacterial debris. The upper layer containing the induced bacteriophages was then removed and transferred to a new tube, filtered through a sterile 0.4 and 0.2 µM Minisart® NML Syringe Filter (Sartorius Stedim Plastics GmbH, Germany) and stored at 4°C until TEM imaging (section 6.2.5) and host range testing (section 6.2.6) was performed.

6.2.5 TEM analysis of virulent E. coli and induced Lactobacillus bacteriophages

Bacteriophages were pelleted in Eppendorf tubes by centrifuging at 20800g (Eppendorf centrifuge 5430R, Eppendorf AG, Germany) for 1 hour at 4°C. The supernatant removed, and the bacteriophages were resuspended in 100 µL of TEM buffer (0.1 M NH4-acetate, pH 7). If more than one tube of the same sample was centrifuged, the bacteriophages were pooled into a single Eppendorf tube, and filled

up with TEM buffer. The bacteriophages were pelleted again (1 hour, 20800g, 4°C), and the washing step was repeated twice. Finally, the bacteriophage pellet was resuspended in 50 µL of TEM buffer and kept at 4°C until TEM analysis.

To prepare the TEM grids (Square 200 mesh copper grids, Agar Scientific, UK) for negative staining with 2% uranyl acetate (AGR1260A, Agar Scientific, UK), 10 µL of bacteriophage sample was pipetted on top of a TEM grid and incubated for 10 min at room temperature. The grid was washed twice with 10 µL of H_2O, and stained with 10 µL of 2% uranyl acetate for 3 min. The grids were stored at room temperature in the dark, and a FEI Tecnai T20 (FEI Company, USA) was used to capture bacteriophage images (assisted by Dr Mohamad Jaffer from the Aaron Klug Centre for Imaging and Analysis, Electron Microscope Unit, UCT).

6.2.6 Host range testing of bacteriophages

The spot assay (section 6.2.3.4) was used to test host ranges of isolated, purified virulent *E. coli* bacteriophages (using pathogenic *E. coli* isolates as potential hosts, **Table 6.1**; kindly provided by Dr Bosco Kalule, Division of Medical Microbiology, UCT) and of induced temperate *Lactobacillus* bacteriophages (using 10/57 vaginal [representing each *Lactobacillus spp.*] and eight probiotic *Lactobacillus* strains as potential hosts, described in Chapter 3 and 5). In addition, the plaque assay (section 6.2.3.4) was also used to test the host ranges. Briefly, 200 µL of overnight cultures of *Lactobacillus* strains or *E.coli* MG1655 was added to 3 mL of molten MRS (supplemented with 10mM $CaCl_2$) or LB (supplemented with 10mM MgSo4, 0.2% Maltose and 10mM $CaCl_2$) top agar, in addition to 500 µL of bacteriophage sample. The tube was inverted gently three times, and poured onto prepared agar plates. These plates were incubated at 37°C for 48 hours under either anaerobic (*Lactobacillus* spp.) or aerobic conditions (*E. coli*). The next day, the plates were checked for the presence of spots and/or plaques.

Table 6.1 Overview of pathogenic *E.coli* isolates used for host range assay

Pathotype	Isolates (Source)
DAEC	777 (Mutton); 767/LR16 (River water); NY1.1/NY1.2/ NY3.2/NY28/NY58/ NY60/E33.1/E34.1 (Child); PK-STEC (Pork); 63.2 (Human)
STEC	NY29 (Child); 710 (River water); 29.4/29.5/602 (Human)
EPEC	789 (Beef); NY50 (Child); 15.1/15.2/271/279/284/286/291/344/344.1/600/722.1/722.2 (Human)
EaggEC	NY13.1/NY43 (Child); E101.1 (River water); 207/229/229.1/229.2/250/326/336/371.1/473/480/502/688/689/696/733 (Human)
EIEC	NY4 (Child)
Unknown	73/232.1 (Human)

6.2.7 Data analysis

Genome sequences were obtained from NCBI genome (https://www.ncbi.nlm.nih.gov/genome). Prophage sequences within genomes were identified using PHASTER (PHAge Search Tool Enhanced Release, http://phaster.ca/), and a completeness score was assigned to each identified prophage. To generate this score, three potential scenarios were considered, based on how much of the respective coding sequence were related to a known bacteriophage: (1) all; (2) >50%; or (3) <50% of the genes/proteins. For scenario 1, the region automatically had a maximal completeness score of 150. For scenario 2, the percentage of the total number of that bacteriophage organism in the reference database in the total number of proteins of the region is calculated and multiplied by 100; and the percentage of the length of that bacteriophage organism in the reference database in the length of the region was calculated and then multiplied by 50. For scenario 3, if any of the specific bacteriophage-related keywords (such as 'capsid', 'head', 'integrase', 'plate', 'tail', 'fiber', 'coat', 'transposase', 'portal', 'terminase', 'protease' or 'lysin') were present, the score was increased by 10 for each keyword found. If the size of the region was greater than 30 Kb, the score was increased by 10. If there were at least 40 proteins in the region, the score was increased by 10. If all of the phage-related proteins and hypothetical proteins constitute more than 70% of the total number of proteins in the region, the score was increased by 10. Comparing the total score of scenario 2 with the total score of scenario 3, the bigger one was chosen as the total score for that region. A prophage region was considered incomplete if its completeness score was <70, indeterminate if the score was between 70 and 90, and intact if the score was >90

(Arndt *et al*. 2016). The CRISPR finder program (http://crispr.i2bc.paris-saclay.fr/Server/) was used to identify CRISPR loci in genomes sequences, and CRISPRTarget (http://bioanalysis.otago.ac.nz/CRISPRTarget/crispr_analysis.html) was used to identify sequence similarities between spacers of confirmed CRISPR loci bacteriophages. To predict the most likely targets of the identified CRISPR loci, CRISPRTarget parameters used for the BLAST screen were as follows: a gap was penalised by -10, a gap extension was penalised by -2, a match rewarded by +1, and a mismatch penalised by -1. All matches that greater than a score of 20, defined by CRISPRTarget to eliminate self-matching results, were recorded. The Kruskal-Wallis one-way analysis of variance was used to compare variables of three or more groups, and adjusted p-values <0.05 were considered significant. GraphPad Prism6® (GraphPad Software, USA) was used for statistical analysis and to generate graphs.

6. 3 Results

6.3.1 Presence of prophage sequences in published Lactobacillus genomes

To investigate whether prophages were present in whole genome sequences of published probiotic *Lactobacillus* spp. (including 15 *L. rhamnosus*, 9 *L. reuteri*, 5 *L. acidophilus*, and 13 *L. helveticus* sequences; **Table 6.2**) and vaginal *Lactobacillus* spp. (including *L. crispatus* STI, *L. gasseri* ATCC 33323/4M13/DSM14869, *L. jensenii* SNUV360 and JV-V16, and *L. mucosae* LM1), PHASTER was used to identify and annotate prophage sequences (**Table 6.2**). Probiotic *Lactobacillus* sequences were included here, as it was of interest to evaluate whether these, which could be introduced in the FGT during probiotic treatment, also harbour temperate bacteriophages. Of these, 12/15 *L. rhamnosus,* 8/9 *L. reuteri*, 4/13 *L. helveticus* and 0/5 *L. acidophilus* probiotics strains and 3/7 vaginal isolates were determined to contain intact prophage regions (**Table 6.2**). The majority of the closest annotated bacteriophage hits mapped to previously described *Lactobacillus* bacteriophages (27/36 hits). The remaining bacteriophage hits (9/36), however, mapped to non-*Lactobacillus* bacteriophages (including 4 *Staphylococcus*, 1 *Enterococcus*, 1 *Escherichia*, 1 *Listeria*, 1 *Gordonia*, and 1 *Bacillus* bacteriophage), which suggested that bacteriophages from other bacterial genera might be able to integrate their genomes into *Lactobacillus* spp., or that these bacteriophages and *Lactobacillus* bacteriophages might share conserved sequences. Interestingly, *L. rhamnosus* strain BPL5, which was originally isolated from the vagina of a healthy women (Chenoll *et al*. 2016), contained one intact prophage region and its closest hit was the *Lactobacillus* bacteriophage PL-1. PL-1 was isolated in 1967 from an abnormally fermented batch of the probiotic drink Yakult (marketed since the 1930s in Japan and now world-wide; Dieterle *et al*. 2014), and its known host is *L. casei* (Stetter *et al*. 1978). Similarly, the probiotic strains *L. rhamnosus* WQ2 (isolated from traditional fermented milk, Hong Kong, 2012)[19], *L. rhamnosus* Pen (isolated from the human gastrointestinal tract, 2005, used to make the probiotic medicine Lakcid, Biomed-Lublin, Poland)[19], and *L. helveticus* MB2-1 (isolated from Sayram ropy fermented milk, China, 2008)[19] contained prophage regions closely related to the *Lactobacillus* bacteriophages ΦAT3, Lrm1 and Φadh, respectively. While the bacteriophage Lrm1 was described to be defective and no host strain has yet been identified (Durmaz *et al*.

[19] https://www.ncbi.nlm.nih.gov/biosample/, [15 October 2018]

2008), the know bacterial hosts of the bacteriophages ΦAT3 and Φadh were determined to be *L. casei* and *L. gasseri*, respectively[20].

Prophage regions were also identified in the genomes of vaginal *Lactobacillus* spp. (**Table 6.2**). The natural host of *Lactobacillus* bacteriophage KC5a (present as intact prophage region in *L. gasseri* strain ATCC® 33323) was confirmed to be *L. gasseri* (Kilic *et al*. 2001), and the host of *Lactobacillus* phage Lv1, present as prophage region in *L. jensenii* SNUV360, was confirmed to be *L. jensenii* (Martín *et al*. 2010; Martín *et al*. 2009). These findings suggest that vaginal *L. gasseri* and *L. jensenii* isolates may be susceptible to lysis should these *Lactobacillus* bacteriophages (such as Φadh, KC5a and Lv1) be spontaneously induced.

[20] Virus-host database, https://www.genome.jp/virushostdb/, [15 October 2018]

Table 6.1 Prophage sequences identified in the whole genomes of published probiotic and vaginal *Lactobacillus* strains

Isolate	Number prophage regions			Intact prophage regions				
	Intact[#]	Questionable[@]	Incomplete[&]	Length [Kb]	Keywords	# phage proteins	Most common bacteriophage hit	GC [%]
L. rhamnosus								
GG NC_013198.1	**2**	1	2	42.6	integrase, terminase, portal, capsid, tail, lysin	44	*Lactobacillus* phage Lrm1	45.24
				34.6	integrase, lysin, tail, head, protease, portal, terminase, recombinase, transposase	22	*Lactobacillus* phage Nu	40.7
Lc 705 NC_013199.1	**1**	2	0	41.6	integrase, terminase, portal, head, capsid, tail, lysin	46	*Lactobacillus* phage Lrm1	50.8
ATCC 8530 GCA_000233755.1	**0**	0	0	None				
LOCK 900 NC_021723.1	**1**	0	0	40.4	integrase, terminase, portal, head, capsid, tail, lysin	43	*Lactobacillus* phage Nu	45.23
LOCK 908 NC_021725.1	**1**	1	0	42.9	integrase, terminase, portal, head, capsid, tail, lysin	40	*Lactobacillus* phage Lrm1	43.98
HN001 NZ_CP001155.1	**0**	0	0	None				
BFE 5264 NZ_CP014201.1	**2**	2	4	18.9	head, capsid, tail, transposase, lysin	20	*Lactobacillus* phage Lrm1	46.06
				30.8	integrase, transposase	11	*Staphylococcus* phage SPbeta-like	43.76
Pen NZ_CP020464.1	**1**	0	0	54.9	lysin, tail, head, capsid, portal, terminase, integrase	49	*Lactobacillus* phage Lrm1	45.25
4B15 NZ_CP021426.1	**2**	1	4	34.6	terminase, portal, protease, head, tail, lysin, integrase	19	*Lactobacillus* phage ΦAT3	42.31
				23.8	terminase, portal, capsid, head, integrase	23	*Enterococcus* phage Ec-ZZ2	45.17
LR5 NZ_CP017063.1	**1**	0	2	41.7	transposase, portal, capsid, tail	52	*Lactobacillus* phage PLE3	44.32
DSM 14870 NZ_CP006804.1	**1**	1	1	33.2	integrase, lysin, tail, head, protease, portal, terminase	18	*Lactobacillus* phage Nu	41.79
SCT-10-10-60 NZ_CP019305.1	**1**	1	0	42.9	integrase, terminase, portal, capsid, head, tail	43	*Lactobacillus* phage PL-1	43.98
BPL5 NZ_LT220504.1	**1**	1	1	43.3	lysin, tail, head, capsid, portal, terminase, integrase	53	*Lactobacillus* phage PL-1	44.48
ASCC 290 NZ_CP014645.1	**0**	1	0	None				

WQ2 CP020016.1	**1**	2	1	33.6	integrase, lysin, tail, head, protease, portal, terminase	19	*Lactobacillus* phage ΦAT3	41.34
L. reuteri								
DSM 20016 NC_009513.1	**3**	1	3	60.1	terminase, portal, capsid, coat, tail, envelope, lysin, integrase	25	*Lactobacillus* phage LF1	38.62
				49.1	transposase, lysin, tail, head, protease, portal, terminase, integrase	39	*Lactobacillus* phage LF1	38.43
				36.8	transposase, lysin, integrase	9	*Staphylococcus* phage N315	38.66
JCM 1112 NC_010609.1	**1**	1	2	49.1	transposase, lysin, tail, head, protease, portal, terminase, integrase	33	*Lactobacillus* phage LF1	38.43
SD2112 NC_015697.1	**2**	1	2	58.2	transposase, terminase, portal, capsid, lysin, recombinase, integrase	26	*Eschericha* phage CFT073	38.52
				51.3	transposase, integrase, terminase, portal, head, capsid, tail	34	*Lactobacillus* phage LF1	39.17
I5007 NC_021494.1	**1**	0	0	43.5	integrase, head, terminase, portal, transposase	16	*Staphylococcus* phage ΦPV83	37.94
TD1 NC_021872.1	**1**	1	2	44.7	lysin, plate, tail, capsid, head, portal, terminase, integrase	44	*Listeria* phage B054	37.27
IRT NZ_CP011024.1	**1**	0	2	66.8	integrase, capsid, portal, coat, tail	38	*Gordonia* phage Bowser	38.63
ZLR003 NZ_CP014786.1	**1**	0	0	33.4	tail, transposase, head, capsid, portal, terminase, integrase	25	*Lactobacillus* phage LF1	38.76
I049 NZ_CP015408.2	**0**	1	6	None				
ATCC 53608 NZ_LN906634.1	**1**	0	0	11.5	transposase, tail	10	*Staphylococcus* phage ΦPV83	34.66
L. acidophilus								
NCFM NC_006814.3	**0**	0	1	None				
La-14 NC_021181.2	**0**	0	1	None				
FS14 NZ_CP010432.1	**0**	0	1	None				
ATCC 53544 NZ_CP022449.1	**0**	0	1	None				
LA1 NZ_CP017062.1	**0**	0	1	None				
L. helveticus								
CAUH18	**0**	0	1	None				

NZ_CP012381.1								
DPC 4571 NC_010080.1	**0**	0	0	None				
R0052 NC_018528.1	**0**	0	1	None				
H10 NC_017467.1	**0**	0	1	None				
CNRZ32 NC_021744.1	**1**	0	0	44.1	lysin, plate,tail, capsid, head, portal, terminase, integrase	45	*Lactobacillus* phage AQ113	36.11
H9 NZ_CP002427.1	**0**	0	2	None				
KLDS1.8701 NZ_CP009907.1	**0**	0	2	None				
MB2-1 NZ_CP011386.1	**1**	0	4	53.6	tail, head, protease, portal, integrase	39	*Lactobacillus* phage Φadh	35.74
D76 NZ_CP016827.1	**1**	0	0	2054.8	transposase, head, tail, terminase, lysin, capsid, protease, integrase, recombinase, plate, portal	530	*Bacillus* phage G	37.03
D75 NZ_CP020029.1	**0**	0	2	None				
FAM8627 NZ_CP015444.1	**0**	0	4	None				
FAM22155 NZ_CP015498.1	**0**	1	1	None				
FAM8105 NZ_CP015496.1	**2**	0	2	40.9	lysin, plate, tail, capsid, head, portal, terminase, integrase	48	*Lactobacillus* phage AQ113	35.10
				58.8	capsid, fiber, tail, terminase, transposase, integrase	38	*Lactobacillus* phage ΦJB	37.89
Vaginal *Lactobacillus spp.*								
***L. crispatus* ST1** NC_014106.1	**0**	0	1	None				
***L. gasseri* ATCC33323** NC_008530.1	**1**	0	2	97.7	integrase, terminase, portal, head, capsid, tail, lysin	115	*Lactobacillus* phage KC5a	36.04
***L. gasseri* 4M13** NZ_CP021427.1	**3**	0	2	54.4	lysin, tail, head, protease, integrase	51	*Lactobacillus* phage Φadh	34.72
				45.9	lysin, tail, capsid, terminase, integrase, transposase	37	*Lactobacillus* phage Φg1e	36.17
				29.0	lysin, capsid, fiber, tail, head, protease, portal, terminase	26	*Lactobacillus* phage JCL1032	37.03
***L. gasseri* DSM 14869**	**0**	0	3	None				

NZ_CP006803.1								
***L. jensenii* SNUV360** NZ_CP018809.1	**1**	0	1	50.3	integrase, lysin, capsid, fiber, tail, head, protease, portal, terminase, transposase	49	*Lactobacillus* phage Lv1	35.37
***L. jensenii* JV-V16** NZ_CM000953.1	**0**	0	1	None				
***L. mucosae* LM1** NZ_CP011013.1	**0**	1	2	None				

[#]PHASTER score >90; [@]PHASTER score 70 -90, [&]PHASTER score < 70

6.3.2 Detection of CRISPR loci in published Lactobacillus genomes

CRISPR loci give an indication whether *Lactobacillus* strains have previously survived bacteriophage or plasmid attacks (Barrangou *et al*. 2007). Thus, the presence of CRISPR loci in publically available whole genome sequences from 63 vaginal *Lactobacillus* isolates was investigated, in a total of 13 *L. crispatus* (**Table 6.3**), one *L. vaginalis,* 12 *L. gasseri* (**Table 6.4**), 14 *L. jensenii* (**Table 6.5**), 8 *L. mucosae* (**Table 6.6**), and 15 *L. iners* (**Table 6.7**) sequences that were available on NCBI, using the CRISPR finder software[17]. Grissa *et al*. (2007) has previously shown that candidate CRISPR loci have at least three defining motifs and at least two identical directed repeats. Using the criteria defined by Grissa *et al*. (2007), confirmed CRISPR loci were frequently present in the publically available *Lactobacillus* genomes, including in 12/13 *L. crispatus,* 13/14 *L. jensenii,* 9/15 *L. iners,* 4/8 *L. mucosae*, and 3/12 *L. gasseri* genomes. There was a significant difference in the frequency of confirmed CRISPR loci between *Lactobacillus* spp. (p=0.0010), with *L. crispatus* (adjusted p=0.0043) and *L. jensenii* (adjusted p=0.0030) strains harbouring CRISPR loci more commonly than *L. gasseri* strains, suggesting that these species might be attacked more frequently by bacteriophages than *L. gasseri*. There was no difference in CRISPR locus frequency between of vaginal and non-vaginal isolates (Tables 6.3-6.7). The one *L. vaginalis* sequence that was included did not contain any CRISPR locus (data not shown).

Of the *Lactobacillus* strains containing confirmed CRISPR loci, there was no major difference in the median number of CRISPR loci per strain: *L. crispatus, L. mucosae, L. gasseri* and *L. jensenii* strains had a median of 2 CRISPR loci (range 1 – 3), and *L. iners* strains had a median of 1 CRISPR loci (range 1-2). However, the length of CRISPR loci (p=0.0018) and number of spacers (p=0.0020) differed between *Lactobacillus* spp., and *L. crispatus* strains tended to have the shortest CRISPR loci (median length of 267 basepairs, IQR 267-486) and the least number of spacers (median 4; IQR 4-6), followed by *L. iners* (median length 457 basepairs, with 9 spacers), *L. gasseri* (median length 662 basepairs, with 10 spacers), *L. jensenii* (median length 927 basepairs; adjusted p=0.0017 vs. *L. crispatus*, with 14 spacers; adjusted p=0.0033 vs. *L. crispatus*), and *L. mucosae* (median length 1308 basepairs; adjusted p=0.0767 vs. *L. crispatus*, with 20 spacers; adjusted p=0.0522 vs. *L.*

[17] http://crispr.i2bc.paris-saclay.fr/Server/

crispatus), indicating that CRISPR loci are common in *L. crispatus* genomes but generally shorter than those of other *Lactobacillus* spp. included here.

To identify possible sequence similarities between these CRISPR loci and sequenced bacteriophages, the spacers of confirmed CRISPR loci were blasted against the Genbank bacteriophage database using CRISPRTarget[18]. Sequence similarities with bacteriophage sequences were only identified for a minority of the spacers (Tables 6.3-6.7). For those that aligned to published bacteriophage genomes, spacer sequences were similar to bacteriophages targeting *Clostridum*, *Enterobacteria* and *Vibrio* spp. Few spacers showed similarities with induced *Lactobacillus* bacteriophages, including: ΦAQ113 from the host *L. helveticus*; KC5a, Φjlb1and Φadh from the host *L. gasseri*; Lj965 from the host *L. johnsonii*; Lv-1 from a vaginal *L. jensenii* strain; ΦJB and ΦLdb from the host *L. delbrueckii*; and ΦAT3 from the host *L. casei*. Interestingly, sequence similarities to the *Lactobacillus* prophage Lv-1, that was induced from a vaginal *L. jensenii* isolate (Martín *et al*. 2010), was found in the CRISPR loci of all nine of *L. jensenii* isolates with confirmed CRISPR loci used in this part of the study (**Table 6.5**).

Table 6.3 CRISPR loci identified in publically available *L. crispatus* genomes

***L. crispatus* strain**	**NCBI accession number**	**Origin**	**Number of confirmed CRISPR loci**	**#**	**CRISPR length [bp]**	**Directed repeat length [bp]**	**Total Number Spacers**
JV-VOI	NZ_ACKR00000000.1	Vaginal	2	1	299	36	4
				2	267	23	4
MV-1A-US	NZ_ACOG00000000.2	Vaginal	2	1	267	23	4
				2	233	36	3
125-2-CHN	NZ_ACPV00000000.1	Vaginal	1	1[a]	233	36	3
MV-3A-US	NZ_ACQC00000000.1	Vaginal	1	1	299	36	4
SJ-3C-US	NZ_ADDT00000000.1	Vaginal	2	1	267	23	4
				2	486	36	6
214-1	NZ_ADGR00000000.1	Vaginal	2	1	267	23	4
				2	233	25	3
CTV-05	GL531736.1	Vaginal	2	1	420	36	5
				2	267	23	4
FB049-03	NZ_AGZF00000000.1	Urogenital	2	1	267	23	4
				2	486	36	6
FB077-07	NZ_AGZG00000000.1	Urogenital	2	1	486	36	6
				2	267	23	4
2029	AVFH00000000.2	Vaginal	2	1	486	36	6

[18] http://bioanalysis.otago.ac.nz/CRISPRTarget/crispr_analysis.html

				2	267	23	4
EM-LC1	NZ_AXLM00000000.1	Fecal	0	-	-	-	-
DSM20584	BBBS01000001.1	Eye	2	1	267	23	4
				2	455	28	7
ST1	NC_014106.1	Chicken crop	3	1[b]	1003	28	16
				2[c]	883	29	14
				3	453	29	7

[a]Sequence similarities in spacers to Clostridioides phage LIBA2945, specific for *C. difficile*
[b]Sequence similarities in spacers to *Lactobacillus phage ΦAQ113* specific for *L. helveticus* 1045; and Mexican rudivirus 1 specific to *Sulfolobales* spp.
[c]Sequence similarities in spacers to Clostridium phages ΦCTC2A, ΦCT19406A, ΦCD506, ΦCD481-1, ΦCDHM19, ΦCDHM14, ΦCDHM11, ΦCDHM13, ΦCD119 specific to *Clostridium* spp.; and *Staphylococcus* phages Φ879 and Φ575, specific for *Staphylococcus* spp.

Table 6.4 CRISPR loci identified in publically available *L. gasseri* genomes

***L. gasseri* strain**	**NCBI accession number**	**Origin**	**Number of confirmed CRISPR loci**	**#**	**CRISPR length [bp]**	**Directed repeat length [bp]**	**Total Number Spacers**
497_LGAS	NZ_JVEV00000000.1	Human	2	1[a]	629	36	9
				2[a]	233	36	3
JV-V03	NZ_ACGO00000000.2	Vaginal	1	1[b]	1289	36	19
K7	NZ_ASRG00000000.2	Human feces	0	-	-	-	-
224-1	ADFT00000000.1	Vaginal	0	-	-	-	-
SV-16A-US	NZ_ADDY00000000.1	Vaginal	0	-	-	-	-
MV-22	ABWH00000000.2	Vaginal	0	-	-	-	-
SJ-9E-US	NZ_ADDU00000000.1	Vaginal	0	-	-	-	-
ATCC33323	NC_008530.1	GIT	0	-	-	-	-
2016	NZ_AUUE00000000.1	Vaginal	0	-	-	-	-
CECT 5714	NZ_AKFQ00000000.1	Breastmilk	0	-	-	-	-
202-4	NZ_ACOZ00000000.1	Vaginal	0	-	-	-	-
130918	PNGR00000000.1	Unknown	1	1[c]	695	36	2

[a]Sequence similarities in spacers to *L. johnsonii* prophage Lj965, specific for *L. johnsonii*
[b]Sequence similarities in spacers to *Lactobacillus* phages *KC5a, Φjlb1 and Φadh*; specific for *L. gasseri*
[c]Sequence similarities in spacers to *Lactobacillus* phages KC5a and Φjlb1; specific for *L. gasseri*

Table 6.5 CRISPR loci identified in publically available *L. jensenii* genomes

***L. jensenii* strain**	**NCBI accession number**	**Origin**	**Number of confirmed CRISPR loci**	**#**	**CRISPR length [bp]**	**Directed repeat length [bp]**	**Total Number Spacers**
IM18-1	NZ_AZNN00000000.1	Unknown	1	1	629	36	9
IM18-3	NZ_AZNM00000000.1	Unknown	1	1	629	36	9
SJ-7A-US	NZ_ACQD00000000.1	Vaginal	1	1[a,b,c]	761	36	11
1153	NZ_ABWG00000000.2	Vaginal	1	1	1157	36	17
269-3	NZ_ACOY00000000.1	Vaginal	2	1	234	36	3
				2	299	36	4
MD IIE-70(2)	NZ_AVCU00000000.1	Vaginal	1	1[a]	894	36	13
DSM 20557	NZ_AYYU00000000.1	Vaginal	0	-	-	-	-
JV-V16	NZ_CM000953.1	Vaginal	1	1[a]	497	36	7

IM1	NZ_AZRD00000000.1	Unknown	1	1[a]	959	36	14
IM11	NZ_AZNO00000000.1	Unknown	1	1[a]	1686	36	25
115-3-CHN	NZ_ACQN00000000.1	Vaginal	1	1[a,b]	2147	36	32
27-2-CHN	NZ_ACOF00000000.2	Vaginal	1	1[a,b,d]	2147	36	32
IM59	NZ_AZRC00000000.1	Unknown	1	1[a,d,g-j]	2017	36	30
IM3	NZ_AZRE00000000.1	Unknown	1	1[a,d,k]	2742	36	41

[a]Sequence similarities in spacers to *Lactobacillus* phage Lv-1; specific for vaginal *L. jensenii* strain,
[b]Sequence similarities in spacers to *Lactobacillus* phage ΦJB; specific for *L. delbrueckii*
[c]Sequence similarities in spacers to *Listeria* phage LP-101, specific for *Listeria monocytogenes*
[d]Sequence similarities in spacers to *Lactobacillus* phage ΦLdB, specific for *L. delbrueckii*
[e]Sequence similarities in spacers to *Arthrobacter phages* Gorgeous, SorJuana, and Anansi, specific for *Arthrobacter* spp.
[f]Sequence similarities in spacers to *Moraxella phage Mcat16,* specific for *Moraxella catarrhalis*
[g]Sequence similarities in spacers to *Listeria phage WIL-3,* specific for *Listeria* spp.
[h]Sequence similarities in spacers to *Dickeya phages ΦDP23.1 and ΦDP10.3,* specific for *Pectobacterium* spp. and *Dickeya* spp.
[i]Sequence similarities in spacers to *Vibrio* phages pVa 1-8, Strym, Pel, Her, VaK, Cla, Len, P3, P2, H20, H8, H2, specific for *V. anguillarum*
[j]Sequence similarities in spacers to *Sulfolobus tengchongensis* spindle-shaped virus STSV1, specific for *S. tengchongensis*
[k]Sequence similarities in spacers to *Synechococcus* phage S-RSM4, specific for *Synechococcus* sp. WH 7803

Table 6.6 CRISPR loci identified in publically available *L. mucosae* genomes

***L. mucosae* strain**	**NCBI accession number**	**Origin**	**Number of confirmed CRISPR loci**	**#**	**CRISPR length [bp]**	**Directed repeat length [bp]**	**Total Number Spacers**
LM1	NZ_CP011013.1	Pig intestine	3	1	181	26	3
				2	821	29	13
				3[a]	2474	36	37
CRL573	NZ_JROC00000000.1	Human feces	1	1	429	34	6
DPC 6426	NZ_JSWI00000000.1	Bovine	0	-	-	-	-
Marseille	NZ_CVQW00000000.1	Human feces	1	1	1308	28	21
DSM 13345	NZ_AZEQ00000000.1	Pig intestine	2	1	1555	29	25
				2	1424	34	20
WCC8	NZ_FNIH00000000.1	Unknown	0	-	-	-	-
KHPC15	NZ_FNZY00000000.1	Unknown	0	-	-	-	-
KHPX11	NZ_FOSI00000000.1	Unknown	0	-	-	-	-

[a]Sequence similarities in spacers to *Lactobacillus* phage ΦAT3, specific for *L. casei* ATCC 393

Table 6.7 CRISPR loci identified in publically available *L. iners* genomes

***L. iners* strain**	**NCBI accession number**	**Origin**	**Number of confirmed CRISPR loci**	**#**	**CRISPR length [bp]**	**Directed repeat length [bp]**	**Total Number Spacers**
SPIN 1401G	NZ_AEXP00000000.1	Vaginal	1	1	822	29	13
UPII 60-B	NZ_AEXK00000000.1	Vaginal	0	-	-	-	-
LactinV 03V1-b	NZ_AEHP00000000.1	Vaginal	0	-	-	-	-
LactinV 09V1-c	NZ_AEHO00000000.1	Vaginal	1	1	365	36	5
LactinV 11V1-d	NZ_AEHN00000000.1	Vaginal	1	1	298	36	4
LactinV 01V1-a	NZ_AEHQ00000000.1	Vaginal	1	1[a]	1002	28	16
SPIN 2503V10-D	NZ_AEHR00000000.1	Vaginal	1	1	233	36	3

UPII 143-D	NZ_AEXJ00000000.1	Vaginal	0	-	-	-	-
LEAF 2052A-d	NZ_AEKI00000000.1	Vaginal	2	1	457	28	7
				2[b]	825	29	13
AB-1	NZ_ADHG00000000.1	Vaginal	0	-	-	-	-
DSM 13335	NZ_AZET00000000.1	Urogenital	0	-	-	-	-
ATCC 55195	NZ_AEPX00000000.1	Vaginal	0	-	-	-	-
LEAF 2053A-b	NZ_AEPX00000000.1	Vaginal	1	1	639	28	10
LEAF 2062A-h1	NZ_AEKJ00000000.1	Vaginal	0	-	-	-	-
LEAF 3008A-a	NZ_AEKK00000000.1	Vaginal	1	1	89	24	9

[a]Sequence similarities in spacers to Enterobacteria phages vB_EcoM_VR5, JS10, JS98, MX01, Bp7, WG01, QL01, specific for *E. coli*
[b]Sequence similarities in spacers to *Erwinia* phage vB_EamM_Stratt, specific for *Erwinia* spp.

6.3.3 Induction of temperate bacteriophages from vaginal Lactobacillus isolates

To investigate whether any of the vaginal and probiotic *Lactobacillus* isolates purified in this study (Chapter 3 and 5) contained temperate bacteriophages, bacteriophages were induced using Mitomycin C. For this part of the study, a subset of 39/57 of the vaginal *Lactobacillus* strains (including 10 *L. crispatus*, 7 *L. gasseri*, 11 *L. jensenii*, 5 *L. vaginalis,* and 6 *L. mucosae*) and 18 probiotic *Lactobacillus* isolates from probiotics for vaginal health marketed in South Africa and France (including 4 *L. crispatus,* 5 *L. rhamnosus*, 2 *L. reuteri*, 3 *L. acidophilus*, 2 *L. plantarum*, and each one *L. salivarius* and *L. bulgaricus* strain) were included. Initial experiments compared growth in the presence and absence of Mitomycin C in Eppendorf tubes (**Figure 6.1**A), during which 11/18 (61.1%) of the probiotic isolates and 28/39 (71.8%) of the vaginal *Lactobacillus* strains showed visibly reduced growth in the presence of Mitomycin C, indicating possible induction of temperate bacteriophages. Subsequently, for isolates that showed visible differences (n=11 probiotic and n=28 vaginal *Lactobacillus* strains), their growth in the presence and absence of Mitomycin C was monitored over 21 hours (**Figure 6.1**B) and the presence of bacteriophages in the supernatant fraction of these *Lactobacillus* cultures was further evaluated by TEM (**Figure 6.2**). There was no difference in bacteriophage induction frequency between *Lactobacillus* spp., or between probiotic and vaginal isolates (**Figure 6.1**). Eleven *Lactobacillus* isolates (7/11 probiotic and 4/28 vaginal *Lactobacillus* strains) showed >50% growth decrease in the presence of Mitomycin C (such as JDW4 and PF1 in **Figure 6.1**B and C), and all of these isolates contained bacteriophage particles in the supernatant, as determined by TEM (**Figure 6.2**). However, strains that did not show such a strong growth decline (such as G1 in **Figure 6.1**B) also had bacteriophage particles present in the supernatant.

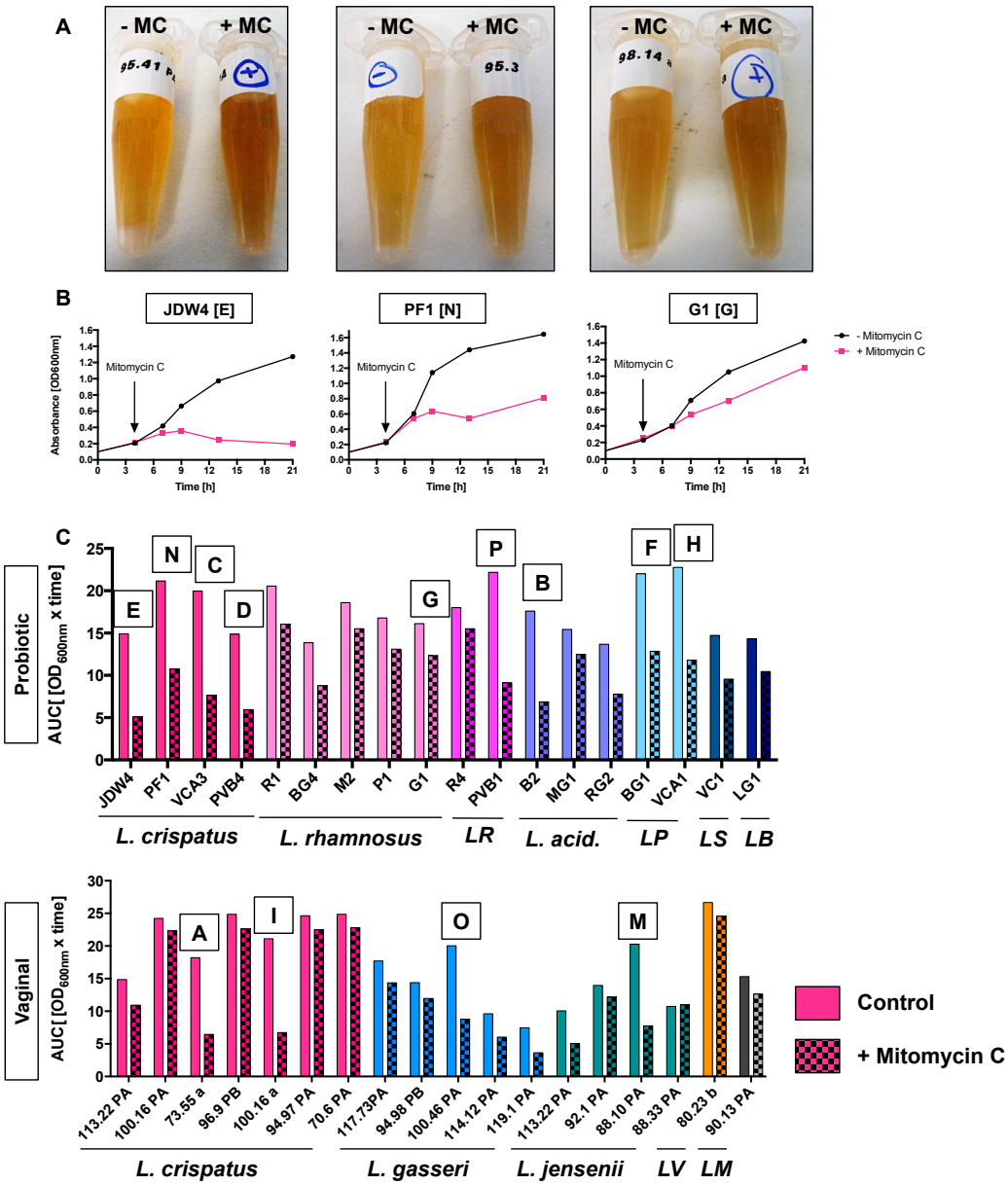


Figure 6.1. Induction of temperate bacteriophages from probiotic and vaginal *Lactobacillus* isolates. *Lactobacillus* strains were adjusted to OD_{600nm} of 0.1 (±0.01) and incubated until they reached and OD OD_{600nm} of 0.3 (±0.01). Mitomycin (0.5 µg/mL) was added, and (A) the growth compared in the presence and absence of Mitomycin C in Eppendor tubes. For isolates showing less growth in the presence of Mitomycin C, the absorbance (OD_{600nm}) of cultures with and without Mitomycin C was measured over time (three exemplary growth cures shown in B), and the area under the curve calculated (C). The single letters (in brackets in B, and in box in C) indicate which bacteriophage morphology belonged to which isolate in **Figure 6.2**. LR= *L. reuteri*, LP= *L. plantarum*, LS= *L. salivarius*, LB= *L. bulgaricus*, LV= *L. vaginalis*, LM= *L. mucosae*, MC= Mitomycin C.

Of the 39 *Lactobacillus* strains screened (n=28 vaginal, n=11 probiotic), the majority (21/39) contained capsids or podo-like particles, without tails or with very short tails that were difficult to visualize by TEM. Figure **Figure 6.2**A-C shows three representative TEM pictures of the bacteriophages that were isolated, possibly belonging to the *Podoviridae* family (based on having a capsid with a diameter 60 nm

and a short tail). Two samples of the probiotic *L. crispatus* isolates (JDW4 and PVB4) contained bacteriophages that appeared to belong to the *Myoviridae* family, based on having icosahedral capsids, with a diameter of 60 nm, and a long, thicker tail, with a diameter 16–20 nm (**Figure 6.2**D-E). Furthermore, 11/39 induced *Lactobacillus* supernatant samples [from five probiotic isolates (BG1=F, G1=G, PVB1=P, PF1=N, VCA1=H) and six vaginal strains (*L. crispatus* 100.16 a=I and 107.7 PB=K, *L. gasseri* 100.46 PA=O, *L. jensenii* 88.10 PA=M, *L. vaginalis*=J, and *L. mucosae*=L)] contained bacteriophages had head-tail structures characteristic for the *Siphoviridae* family, with icosahedral capsids that had a diameter of 60 nm, and long, thin filamentous tails with short terminal and subterminal fibres (**Figure 6.2**F-P).

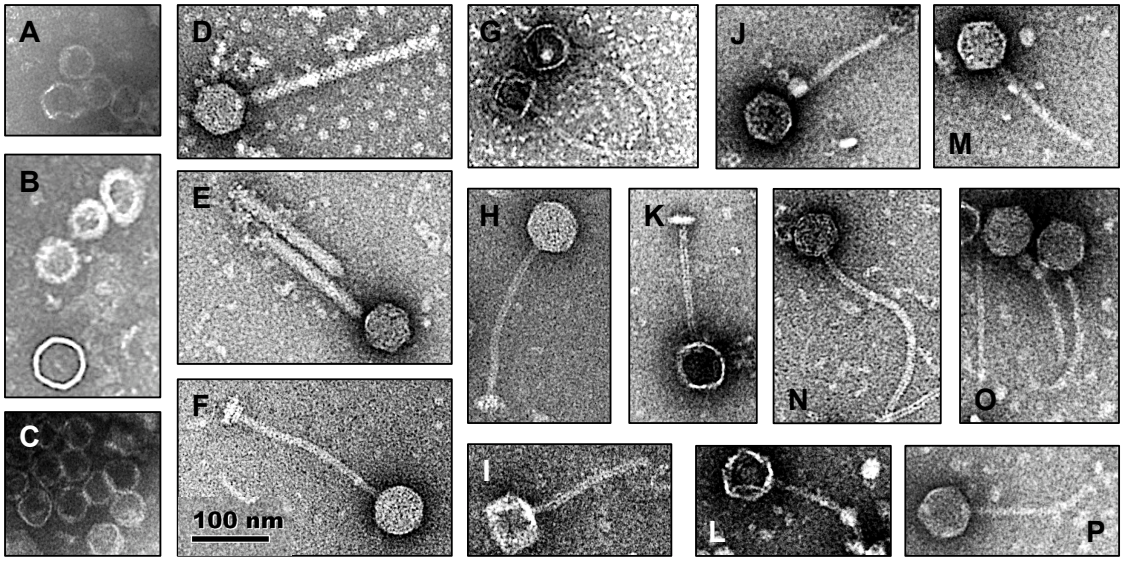


Figure 6.2. Electron microscopy of induced *Lactobacillus* bacteriophages. After Mitomycin C induction (0.5 μg/mL) of bacteriophages, the samples were concentrated and washed to remove bacterial debris, and visualised by electron microscopy (FEI Tecnai T20). The majority of samples (26/39) contained capsids, or podo-like particles (A-C), two samples contained bacteriophages that might belong to the Family *Myoviridae* (D-E), and eleven bacteriophages belonged to the group of *Siphoviridae* (F-P).

6.3.3.1 Host range testing of temperate bacteriophages from Lactobacillus strains

The host range of 10/13 of the induced *Myo*- and *Siphoviridae*, whose presence had been confirmed by TEM, was tested against 18 *Lactobacillus* strains, including each two vaginal isolates of *L. crispatus, L. gasseri*, *L. jensenii*, *L. vaginalis*, *L. mucosae;* and each two probiotic isolates of *L. crispatus*, *L. rhamnosus*, *L. acidophilus*, and *L. reuteri,* using spot and plaque assays. The remaining 3/13 induced bacteriophages was not screened as the sample volume was too little for comprehensive host range testing. However, none of these temperate bacteriophages induced from either vaginal or probiotic *Lactobacillus* strains appeared to be virulent against any of the 18 heterologous *Lactobacillus* strains tested (data not shown).

6.3.4 Screening for virulent bacteriophages against Lactobacillus in vaginal fluid

The *in silico* analysis of genomes of published *Lactobacillus* spp. commonly found in the FGT showed that most of them contain at least one CRISPR locus with several spacers (section 6.3.2), suggesting that vaginal *Lactobacillus* strains commonly undergo bacteriophage attacks. This implies that bacteriophages targeting vaginal *Lactobacillus* strains are commonly present in the lower FGT. Thus, a total of 440 vaginal samples that included five different FGT sample types (lateral vaginal wall ESwab™ in Amies medium, and enriched in MRS, SB, and TS, and endocervical swab in PBS) from 44 BV- [median Nugent Score 0 (IQR 0-1) of which 22/44 were also STI- (for *C. trachomatis, N. gonorrhoe, T. vaginalis, M. genitalum*, HSV-2)], 7 women with an intermediate vaginal microbiota (median Nugent score 4 [IQR4-4], 5/7 who were STI-), and 37 BV+ women (median Nugent Score 9 [IQR 8-10]; of which 12/37 were STI negative) were screened for the presence of virulent *Lactobacillus* bacteriophages, using five single vaginal host strains (including *L. crispatus* 73.55 a, *L. gasseri* 94.98 PB, *L. jensenii* 72.1 PA, *L. vaginalis* 81.17 a, and *L. mucosae* 80.23 a). However, despite testing a total of 440 samples, performing ten rounds of enrichments, and using five *Lactobacillus* host strains belonging to different species, no virulent bacteriophages could be detected.

6.3.5 Presence of virulent bacteriophages targeting E. coli in vaginal fluid

Bacteriophages targeting *E. coli* isolated from various environmental samples have commonly been described in the literature (Shousha *et al*. 2015; Lee & Park 2015; Chibani-Chennoufi *et al*. 2004). Since no virulent bacteriophages against

Lactobacillus or any other common bacterial species from the lower FGT have previously been described, the presence of lytic bacteriophages in cervicovaginal fluid from the same women described above were screened against the highly bacteriophage-sensitive *E. coli* strain MG1655 (Allen *et al*. 2017), using only the lateral vaginal wall ESwab™ in Amies medium. Of the 88 samples screened, 9/88 (10.2%) contained virulent *E. coli* bacteriophages that were lytic against *E. coli* MG1655. These bacteriophages were only present at low concentrations in cervicovaginal fluid in this cohort, as multiple rounds of enrichment were required for the bacteriophages to be detected. The first three bacteriophages appeared after the second round of enrichment on bacterial lawns (**Figure 6.3**A), while the other four bacteriophages were only visible after five to seven enrichment rounds. After isolating and enriching single bacteriophages targeting *E. coli* MG1655, their presence was confirmed by TEM. All nine samples contained bacteriophages with heads with an icosaedric symmetry (diameter 60 nm) and long tails (length 200 nm), and thus possibly all belong to the family of *Siphoviridae* (**Figure 6.3**B).

FGT secretions that contained lytic bacteriophages against *E. coli* MG1655 were predominantly from BV+ women (5/9), although 4/9 originated from women were BV-, and 1/9 woman had an intermediate Nugent score. Furthermore, four women had ≥1 STIs (2/4 were infected with *C. trachomatis*, 1/4 was infected with *T. vaginalis*, and 1/4 had concurrent infections with *N. gonorrhoe, T.vaginalis,* and *M. genitalium*). Five of the nine women had yeast infections, suggesting overall that *E. coli* bacteriophages might be more common in the FGT of women with BV, yeast and/or STIs.

Next, the susceptibility of a library of 53 pathogenic *E. coli* isolates [including Shiga-toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EaggEC), enteroinvasive (EIEC), and diffusely adherent *E. coli* (DAEC); **Table 6.1**)] to the nine vaginal *E. coli* bacteriophages was tested. Despite being lytic against *E. coli* MG1655, none of the 53 *E. coli* isolates appeared to be susceptible to any of the bacteriophages (data not shown).

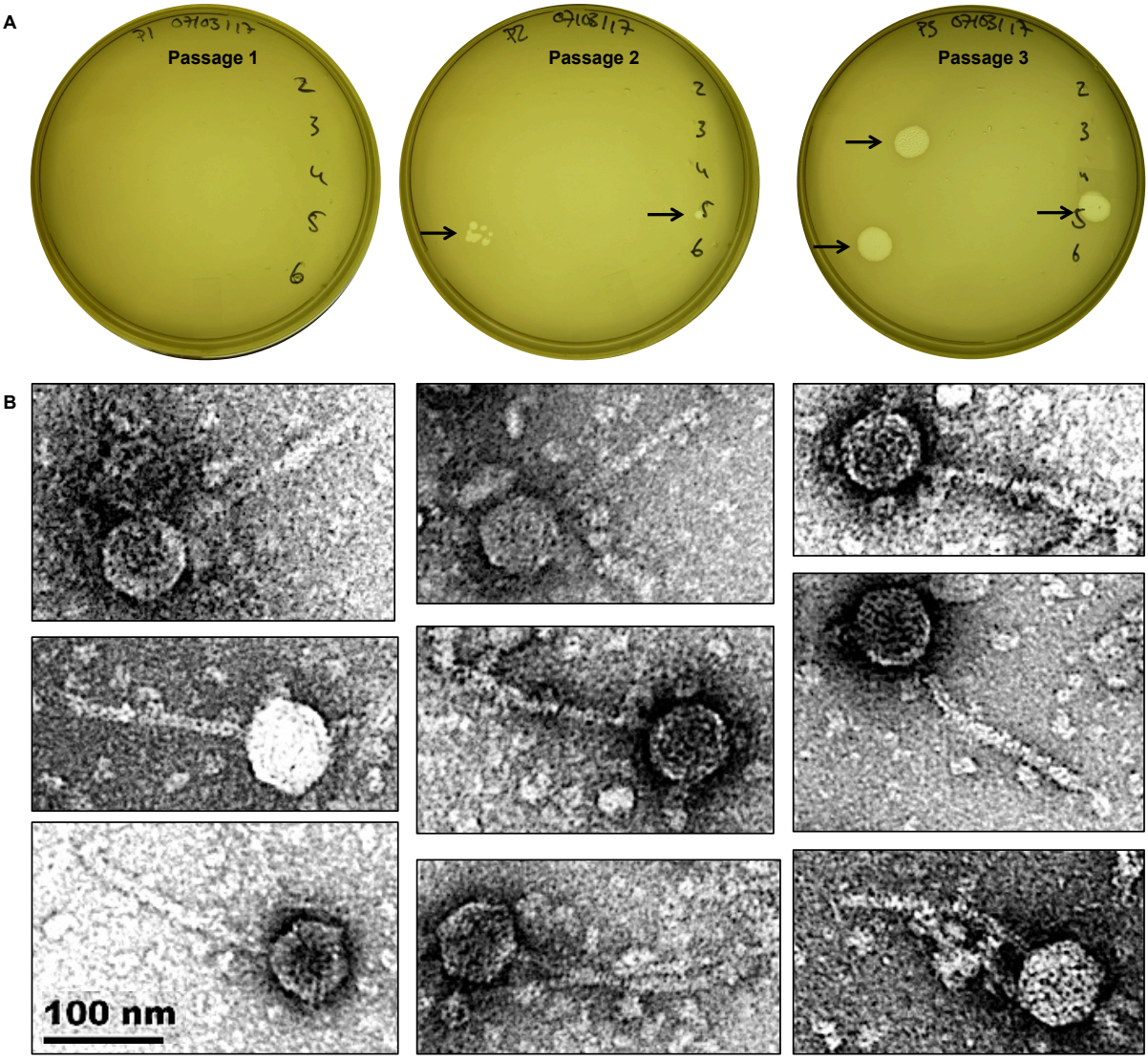


Figure 6.3. Isolation of virulent *E. coli* bacyteriophages from vaginal secretions. (A) A serial tranfer assay was used to enrich bacteriophages *E. coli* MG1655 from cervicovaginal secretions. After each round of amplification, the presence of phages was tested using the spot assay. While after the first round of enrichment (passage 1) no plaques, indicative for the presence of bacteriophages, were observed, single plaques were observed in two samples after the second round (passage 2), and three spots were visible after the third passage (passage 3). Arrows indicate presence of plaques and spots. After ten rounds of enrichment, nine samples showed spots. (B) The morphology of *E. coli* bacteriophages isolated from vaginal secretions was determined by TEM, and visualised using a FEI Tecnai T20. Bacteriophages from all nine samples containg bacteriophages possibly belong to the group of *Siphoviridae*.

6.4. Discussion

More than 20 years ago it was hypothesized that vaginal *Lactobacillus* spp. are infected by bacteriophages (Pavlova *et al*. 1997; Blackwell 1999), which causes the depletion of *Lactobacillus* spp. characteristic of BV (Ravel *et al*. 2011; Zhou *et al*. 2007). To date, however, no virulent *Lactobacillus* bacteriophages have been isolated from the FGT (Martín *et al*. 2009). In contrast, temperate *Lactobacillus* bacteriophages are commonly present in many vaginal *Lactobacillus* strains (Lo *et al*. 2005; Raya *et al*. 1989; Yoon & Chang 2011; Martín *et al*. 2010; Pavlova *et al*. 1997; Kilic *et al*. 2001). Thus, the aim of this Chapter was to determine whether lytic or temperate bacteriophages might be involved in the pathogenesis of BV, by (i) determining how frequent prophage sequences or CRISPR loci are present in published *Lactobacillus* genome sequences, (ii) inducing temperate bacteriophages from vaginal *Lactobacillus* isolates, and (iii) isolating virulent bacteriophages from vaginal secretions from South African women with and without BV.

Firstly, the *in silico* analyses using published *Lactobacillus* whole genome sequences revealed that the majority of the probiotic sequences available (including *L. rhamnosus* and *L. reuteri*) showed evidence of prophage sequences (indicative of temperate bacteriophages), as well as about half of the vaginal *Lactobacillus* strains. The sequences of those intact prophage regions aligned with sequences of 13 different *Lactobacillus* bacteriophages, as well as bacteriophage targeting other bacterial species. Of these 13 *Lactobacillus* bacteriophages, only three (Lc-Nu, PL-1 and ΦAQ113) were virulent bacteriophages, with each only having a single host strain that has been identified (Tuohimaa *et al*. 2006; Watanabe *et al*. 1970; Zago *et al*. 2013), indicating a very narrow host range. The remaining ten *Lactobacillus* bacteriophages were prophages that were induced from their host strains either spontaneously or by chemical induction. Two of these bacteriophages (Φadh and JCL1032) had a very narrow host range (Baugher *et al*. 2014; Forsman 1993), while KC5a was able to cause lytic infection in various vaginal *Lactobacillus* strains, indicative of a broad host range (Kilic *et al*. 2001). For the remaining seven bacteriophages that were induced, no propagating bacterial host could be identified (Durmaz *et al*. 2008; Lo *et al*. 2005; Dieterle *et al*. 2016; Yoon & Chang 2011; Martín *et al*. 2009; Guo *et al*. 2016; Kakikawa *et al*. 1996). Overall, Kilic *et al.* (2001) was

the only study that previously described the existence of a temperate bacteriophage that was spontaneously released and able to infect other vaginal *Lactobacillus* strains, while despite extensive testing and screening, the majority of other studies did not identify a single propagating host after bacteriophage induction. Thus, the contribution of prophage induction to the development of BV is questionable, if host ranges of induced bacteriophages are as restricted *in vivo* as *in vitro*. However, it is still interesting to speculate that the spontaneous induction of prophages, resulting in host cell lysis, could contribute to the depletion of *Lactobacillus* spp. during the onset of BV, even when not infecting other vaginal *Lactobacillus* strains.

Additional evidence of the presence of *Lactobacillus* bacteriophages in the FGT has been provided by the presence of CRISPR loci in the genomes of vaginal *Lactobacillus* spp., indicating that these strains previously survived plasmid or bacteriophage attacks. Analyses presented in this Chapter showed that CRISPR loci were present in about 80% of the published genomes of *Lactobacillus* strains commonly found in the FGT, and that their abundance varied by *Lactobacillus* spp. Pleckaityte *et al*. (2012) reported that CRISPR/cas systems were identified in about half of the genomes of *G. vaginalis* strains isolated from the FGT, which may suggest that some *Lactobacillus* spp. might be more frequently attacked by bacteriophages and/or plasmids than FGT pathobionts, and the presence of CRISPR loci might occur in response to bacteriophage pressure within the *Lactobacillus*-dominated vaginal microbiome. Further, CRISPR loci were more common in *L. crispatus* and *L. jensenii* compared to other *Lactobacillus* spp., suggesting that *L. crispatus* and *L. jensenii* strains might be attacked more frequently by bacteriophages and/or plasmids. However, it was not possible to determine the time frame over which this occurred (months, years, decades, etc.), thus conclusive conclusions are difficult to make. Some spacers within these CRISPR loci aligned with sequences of previously published genomes of *Lactobacillus* bacteriophages. However, the majority of spacer sequences aligned to sequences of bacteriophages other than those targeting *Lactobacillus* spp. This may suggest that these bacterial strains have survived attacks by different bacteriophage species, as one bacteriophage can simultaneously inhibit different bacterial species (Park *et al*. 2012). However, it also needs to be considered that spacer sequences are very short (<50 basepairs), and might therefore non-specifically align to conserved regions in bacteriophage genomes, which might

explain the high number of spacers aligning with the genomes of *Clostridum*, *Enterobacteria* and *Vibrio* bacteriophages. In addition, much fewer *Lactobacillus* bacteriophages have been sequenced than *Clostridium* and *Enterobacteria* bacteriophages, which may skew databases towards species other than *Lactobacillus* bacteriophages.

Interestingly, sequence similarities to the *Lactobacillus* prophage Lv-1, which was induced from a vaginal *L. jensenii* isolate (Martín *et al*. 2010), was found in the CRISPR loci of all nine of *L. jensenii* isolates used in this Chapter. It has, however, been suggested that the prophage Lv-1 is defective (as the capsid-tail junction of the Lv1 virions is fragile and therefore most virions become disrupted) and unable to generate fertile progeny (Martín *et al*. 2010), thus questioning whether those *L. jensenii* strains were indeed attacked by the bacteriophage Lv-1, or if the spacers align rather to a conserved region that is found in the genomes of *L. jensenii* isolates.

The genomes of the *Lactobacillus* spp. might also contain functional prophages that can be expressed in the presence of an environmental stimulus, thereby causing their depletion. To address the question as to whether and how frequently functional temperate bacteriophages were present in both probiotic and vaginal strains from South African women (from Chapter 3 and 5), Mitomycin C was used to induce bacteriophages. While temperate bacteriophages were common in both clinical and probiotic *Lactobacillus* strains, they did not infect any of the vaginal or probiotic *Lactobacillus* host strains tested. This suggested that these bacteriophages, although commonly harboured in *Lactobacillus* genomes, might not necessarily infect vaginal *Lactobacillus* spp. when being induced. This is supported by a number of other studies, which found that temperate bacteriophages can easily be induced using various chemical agents but very few have had their host ranges identified and these have been very narrow (discussed above, Durmaz *et al*. 2008; Lo *et al*. 2005; Dieterle *et al*. 2016; Yoon & Chang 2011; Martín *et al*. 2009; Guo *et al*. 2016; Kakikawa *et al*. 1996). Further, the high frequency of temperate bacteriophages in the *Lactobacillus* strains used in this study might have complicated the search for a susceptible host strain, due to superinfection immunity (expressed by a resident prophage to inhibit a

successful secondary bacteriophage infection) commonly present in lysogenic bacterial strains (Riipinen *et al*. 2007).

It also needs to be taken into consideration that host range determination depends on the overall approach used to evaluate this (reviewed by Ross *et al*. 2016; Hyman & Abedon 2010). For instance, plaque assays are used to determine whether a bacteriophage can productively infect the bacteria but not every host will allow the formation of plaques, even if the host does allow for a productive infection (reviewed by Hyman & Abedon 2010). Further, Holmfeldt *et al*. (2007) showed that the sensitivity of bacterial strains belonging to the same species to bacteriophage infection within one ecological niche is highly strain-specific, and that bacterial communities contain both sensitive and resistant bacterial strains. This suggests that the bacteriophage-host interaction and the development of bacteriophage-resistant strains within an ecological niche are highly complex. This also means that bacteriophage-resistant bacteria are constantly produced, and might gain dominance over bacteriophage-sensitive strains when the selective pressure from infectious bacteriophages is getting too strong to avoid large shift in bacterial communities. It therefore might be possible that the vaginal *Lactobacillus* strains used as hosts in this study were resistant clones.

Despite testing >400 vaginal samples from both BV+ and BV- women for the presence of virulent bacteriophages infecting vaginal *Lactobacillus* spp., none could be isolated in this study despite a number of enrichment cycles. Similarly, Martín *et al*. (2009) did not isolate any bacteriophages from FGT samples, despite using a two-rounds enrichment procedure of vaginal exudates against a panel of 45 *Lactobacillus* strains isolated from the FGTs of healthy women as hosts. This suggests either that virulent *Lactobacillus* bacteriophages are simply not present in the FGT, or that their frequency and abundance were very low at the time of sampling. Ultracentrifugation with a larger sample volume or concentration by dialysis could be useful in future to increase the concentration of bacteriophages present in FGT samples. The concentration of *Lactobacillus* bacteriophages present in the vaginal environment might also vary over time, as previously described in the Lotka-Volterra equations. This model described the dynamics of two species (here bacteriophages and vaginal *Lactobacillus* spp.) interacting in a biological system in which one acts as the predator

and the other as prey. Oscillations in the population size of both predator (bacteriophages) and prey (*Lactobacillus* spp.) occur, with the peak of the predator's oscillation lagging slightly behind the peak of the prey (Zhu & Yin 2009; Maslov & Sneppen 2017; Gavin *et al*. 2006). In the setting of the vaginal environment, this model predicts that the population size of bacteriophages may be highly dependent on the presence of a permissive host. If the permissive host is not present in the FGT, the concentration of bacteriophages will also decrease drastically. Longitudinal intensive sampling might therefore be more appropriate to detect bacteriophages in the FGT and allow better description of the dynamics of bacteriophage and bacterial populations over time. Lastly, the approach used for bacteriophage isolation may also not have been sensitive enough to identify lysis, as not every bacterial host forms plaques, even when productively infected (discussed above; reviewed by Hyman & Abedon 2010).

Further, as for majority of induced *Lactobacillus* bacteriophages described in the literature no or few host *Lactobacillus* strains have been identified (Durmaz *et al*. 2008, Lo *et al*. 2005, Dieterle *et al*. 2016, Kakikawa *et al*. 1996), *Lactobacillus* bacteriophages generally might have a very narrow host range, which is an important hurdle to consider in this attempt to isolate virulent *Lactobacillus* bacteriophages from FGT secretions. Recently, however, there have been a limited number of polyvalent bacteriophages described that are capable of infecting a number of different bacterial species and even genera (Park *et al*. 2012; Malki *et al*. 2015; Parra & Robeson 2016; Ackermann *et al*. 1978). The approach used in this Chapter made use of a single bacterial strain as host to isolate bacteriophages, which may have selected for bacteriophages with narrow host ranges. Ross *et al*. (2016) recently used multiple bacterial hosts during the isolation process, and found that bacteriophages with a wider host range were isolated, suggesting that further studies like this should use a mixture of several bacterial hosts. In a natural setting, when bacteriophages encounter a broad variety of potential hosts, the finding by Ross *et al*. (2016) indicates that their host range might be wider than observed in a laboratory setting, and that one bacteriophage might influence the growth of several bacterial species and genera within one ecological niche. The impact of bacteriophages on the bacterial communities will depend on the ratio of bacteria to bacteriophages. If the ratio of bacterial host to bacteriophage is equal, the impact of the bacteriophage infection on

bacterial communities would likely be severe (Meaden & Koskella 2013). In ecological niches where the ratio of host bacteria to bacteriophage is higher (eg. 100:1), the impact of bacteriophages on the bacterial community would most likely not be as noticeable. It is therefore important to factor the concentration of bacteriophage to bacterial host in a particular ecological niche into account to determine the potential impact they may have in such a niche.

Although no bacteriophages of *Lactobacillus* were isolated from FGT in this study, several characteristics of such bacteriophages would be interesting to evaluate, to determine their biological relevance in the vaginal niche should a bacteriophage be isolated in future studies, including: their phenotype, their complete genome sequence, and life history traits such as host-range. Obtaining longitudinal intensive (even daily) samples from women prior to and on the day of BV onset would allow to determine the population-wide prevalence and within host abundance of specific bacteriophage species (by qPCR). This would give critical insight into whether changes in bacteriophage communities are an underlying biological factor contributing to rapid alteration in the composition of vaginal bacterial communities that ultimately lead to the onset of BV.

Although *E. coli* bacteriophages have been shown to be abundant in several ecological environments (Shousha *et al*. 2015; Lee & Park 2015; Chibani-Chennoufi *et al*. 2004), this study is the first to report functional virulent *E. coli* bacteriophages isolated directly from cervicovaginal samples, to my knowledge. Further investigations are needed to characterize these isolated bacteriophages, including the determination of their full genome sequences. In addition, the origin of these bacteriophages should be determined to clarify whether these bacteriophages originated from the FGT, or if they were introduced by cross-migration from faeces. The *E. coli* bacteriophages were, however, present in a minority (10%) of FGT samples tested, and primarily appeared only after several rounds of enrichments, suggesting that they were not present at high concentrations in the initial sample. As previous studies have shown that virulent *E. coli* bacteriophages are frequently isolated from human gut samples (34 % of healthy humans had coliphages in their stool; Dalmasso *et al*. 2016; Chibani-Chennoufi *et al*. 2004), virulent *Lactobacillus*

have been primarily been isolated from dairy products, fermented vegetables and meat but seldom from human samples (reviewed by Villion & Moineau 2009), which further suggests that the abundance of *Lactobacillus* bacteriophages might be very low in FGT secretions, which contributes to how challenging they were to detect experimentally. While bacteriophages against the highly permissive *E. coli* MG1655 were detected in FGT samples, none of the 53 pathogenic *E.coli* isolates (belonging to EPEC, STEC, EaggEC, EIEC and DAEC) were permissive hosts. Shiga toxin 1 and 2 genes (contained in pathogenic STEC isolates) are not considered to be bacterial genes, but embedded within the genomes of integrated lambdoid prophages (Kruger & Lucchesi 2015; Wagner *et al*. 2002), which might reduce the susceptibility of those pathogenic isolates to other virulent bacteriophages (Riipinen *et al*. 2007).

Conclusion

Results in this Chapter showed that the majority of vaginal *Lactobacillus* strains from South African women harboured inducible temperate bacteriophages, and it is argued that spontaneous induction of these bacteriophages could contribute to the onset of BV, although this was not demonstrated here. However, virulent bacteriophages against vaginal *Lactobacillus* spp. could not be isolated from a wide range of FGT samples collected from South African women with a wide spectrum of vaginal health (BV-, intermediate and BV+). Studies evaluating the abundance and composition of bacteriophage communities in the FGT are few but urgently needed. Furthermore, relationships between *in vivo* changes in the abundance and/or composition of bacteriophage communities in the FGT and BV onset have not been established. Longitudinal studies assessing dynamic changes in both bacterial and bacteriophage communities in the FGT of women prior to the onset of BV, during BV episodes, and after BV cure are needed, likely to be important to resolve some of these questions.

7. Discussion

Vaginal health is important for women's overall health, for both reproductive and psychological wellbeing, since complaints of vaginal discharge have been linked to poor mental health, including depression and anxiety (Patel *et al*. 2008). BV is the most common vaginal condition in women of reproductive age (reviewed by Kenyon *et al*. 2013) that is typically evident by clinical changes, such as a greyish-white, malodorous discharge (Taha *et al*. 1998), increased genital inflammation (Mlisana *et al*. 2012; Mitchell & Marrazzo 2014; Campos *et al*. 2012; Hemalatha *et al*. 2012; Alcaide *et al*. 2017), and increased risk of acquiring and transmitting HIV (Cohen *et al*. 2012; Myer *et al*. 2005; Van de Wijgert *et al*. 2008; Anahtar *et al*. 2016; Gosmann *et al.* 2017) in addition to other STIs (such as *C. trachomatis, T. vaginalis, N. gonorrhoea,* HPV and HSV-2; Guo *et al*. 2012; Brotman *et al*. 2010; Nagot *et al*. 2007). In addition, BV has also been associated with PID and infertility in women wishing to conceive, or premature delivery, miscarriage, and intrauterine foetal death in pregnancy (Haggerty *et al*. 2016; Nelson *et al*. 2014; Clark *et al*. 1994).

Considering that the rates of BV, STIs and HIV are disproportionally high in women from sub-Saharan Africa compared to other more developed regions (Kenyon *et al*. 2013; Johnson *et al*. 2005; Barnabas *et al*. 2017; UNAIDS 2016), it is critical to develop more efficient and durable treatment options for BV. SOC treatment of BV with antibiotics like metronidazole or clindamycin is effective for curing BV in some women in the short-term but does not prevent recurrence in the long term (Barrons & Tassone 2008). In view of this, the overall aim of this thesis was to evaluate locally available probiotic products for the treatment of BV in South African women, and to compare these commercial *Lactobacillus*-containing products with vaginal *Lactobacillus* spp. derived from the FGTs of healthy BV- women. To achieve this, commercial probiotic formulations for vaginal health that are currently available in South Africa were reviewed, properties of these probiotic *Lactobacillus* strains were compared to vaginal *Lactobacillus* isolates, and the use of a currently available OTC probiotic for vaginal health as adjunct to antibiotics for BV treatment in South African women was explored in a pilot clinical trial, to test the regulatory landscape in South Africa overseen by SAHPRA.

The first results Chapter focused on surveying probiotic products available OTC (without a prescription needed) in two major cities of South Africa (Cape Town and Durban), from which only four probiotic products that were explicitly marketed for vaginal health (including Provacare® Probiotic Vaginal Care [manufactured in Canada], UltraFlora® Women's [manufactured in US], and Reuterina^{TM} Femme and Vagiforte® PLUS [manufactured in South Africa) were identified from over one hundred products. However, none of these contained *Lactobacillus* spp. that would commonly be found in the lower FGT of women, and the cost of one treatment course would make these products unaffordable to the majority of South Africans who are possibly at the greatest need. Detailed laboratory analysis of these vaginal probiotic products in Chapter 3 suggested that they mainly contained the bacterial species and doses as claimed by the local manufacturers.

Chapter 3 also characterised the commercially derived bacterial strains in terms of their performance as vaginal probiotics *in vitro* and their antibiotic sensitivity profiles. The majority of probiotic *Lactobacillus* strains grew best at pH 6.0, although they were all able to lower culture pH to 4.5, at which they demonstrated slower growth kinetics. Furthermore, they all showed some degree of adhesion to ectocervical epithelial cells, which was considered important to their ability to stably colonize the lower FGT, and produced various amounts and ratios of both L- and D-Lactic acid locally. None of the probiotic strains produced much H_2O_2 (<10 μM) under anaerobic conditions that would typically be found in the lower FGT, and they inhibited clinical *G. vaginalis* and *P. bivia* stains with variable efficacy. Overall, this data suggested that the commercially available probiotic strains have characteristics that align with their usefulness as probiotics. However, growth of some of the probiotic *Lactobacillus* strains was inhibited at low culture pHs. Ability to tolerate acidic conditions typical in vaginal health is considered important for probiotics intended to colonize the lower FGT (Succi *et al*. 2005; Hassanzadazar *et al*. 2012; Jin *et al*. 1998). In agreement with other researchers (Tachedjian *et al*. 2018), the low levels of H_2O_2 produced by probiotic isolates under anaerobic conditions, found in the lower FGT, suggest that H_2O_2 does not play a major role in pathogen inhibition by *Lactobacillus* spp. Although resistance to metronidazole was generally high across *Lactobacillus* strains evaluated in this study, the antibiotic resistance profiling revealed that none of the strains contained in South African products was resistant to clindamycin. This is

important to note as some international guidelines for BV treatment (such as the US CDC treatment guidelines) include the use of clindamycin, and thus probiotics from South Africa would not be useful in adjunctive antibiotic treatment of BV if administered concurrently with clindamycin. However, the South African treatment guidelines[1] recommend SOC treatment with metronidazole, therefore this finding might not be relevant in a South African setting. While the sensitivity of probiotic isolates to penicillin and amoxicillin varied between *Lactobacillus* strains, all commercial strains were highly sensitive to rifampicin, which is administered for six months during TB treatment (Nahid *et al*. 2016), and has shown to have a high tissue penetrations (Mindermann *et al*. 1998). As the prevalence of TB is high in South Africa (Ismail *et al*. 2018), the effect of long-term administration of rifampicin on the colonisation with probiotic (and commensal) *Lactobacillus* strains needs to be investigated.

In Chapter 4, one of the most promising probiotic products marketed for vaginal health that is currently available in South Africa - Vagiforte® PLUS Combo Pack (which contained *L. rhamnosus, L. acidophilus, B. bifidum* and *B. longum*) - was tested in a single blind, randomized pilot trial in adult South African women diagnosed with BV. This pilot trial taught several valuable lessons: (1) it initiated conversations with the regulatory authorities in South Africa, such as SAHPRA, thus laying the path for future clinical trials administering probiotics; (2) it demonstrated that symptoms of vaginal discharge were an incredibly poor predictor of BV, since only <50% of the women screened had Nugent confirmed BV; (3) it emphasized the need for BV and STI screening, enrolment and treatment allocation to take place on the same day, as vaginal microbiota can fluctuate weekly and even daily (Ravel *et al*. 2013; Lambert *et al*. 2013; Noyes *et al*. 2018; Vodstrcil *et al*. 2017; Srinivasan *et al*. 2010; Gajer *et al*. 2012), and quite a few of the women self-resolved BV between screening and enrolment; (4) testing for concurrent STIs was necessary as genital inflammation, which is influenced by the presence of STIs (Mlisana *et al*. 2012; Masson *et al*. 2014), was one of the secondary outcomes, although STI acquisition in the pilot trial was high, and should be considered in power calculations; (5) future trials should rather use oral metronidazole instead of vaginal gel, as the oral

[1] Sexually Transmitted Infections: Management Guidelines 2015, DOH, South Africa.

formulation is more commonly used in South Africa, due to the price difference between the formulations (ZAR30 vs. ZAR280, respectively, for one treatment course); and related to this, (6) power calculations for future trials need to take into account the trial design, as the effect size is likely to be smaller when probiotics are administered alone (no metronidazole) compared to adjunctive to antibiotics. The use of specific and sensitive rapid tests would allow easy BV and STI screening, and will be considered in future trial design. As rapid tests for BV tend to have low sensitivity (eg. BVBlue® test based on detection of sialidase; Madhivanan *et al*. 2014), performing Gram stains and Nugent scoring in real-time could be considered to reduce time between screening and enrolment. It would therefore be recommended that BV (by Nugent scoring) and STI testing (by GeneXpert®) are done at the clinic or in house at the laboratory. Although it would be desirable to conduct FGT sampling at the same phase of the menstrual cycle at each study visit, to minimize potential bias in microbiota changes associated with hormones, this would make the logistics of an already complicated visit schedule more difficult and increase trial design complexity and cost. Further, the pilot trial administered probiotics for 15 days as adjunct to a single course of vaginal metronidazole gel. The adjunctive study design is likely to influence effect size compared to probiotics alone (no antibiotics administered) that would necessitate a larger study in future to prove superiority to metronidazole alone. Nevertheless, it is out of question that metronidazole not be administered, as this is SOC in South Africa for BV treatment. A previous study suggested that probiotics helped some participants to resolve BV if they were administered for more than two months (Homayouni *et al*. 2014), suggesting that the probiotic (and placebo) should be administered for at least two months after an oral metronidazole course (500 mg, twice daily, for 7 days).

Interim analyses from this pilot trial suggested no improvement in BV cure rates with administration of adjunctive probiotics compared to antibiotics alone. Although disappointing, there are several likely explanations: (1) This pilot study was clearly underpowered to demonstrate superior BV cure compared to metronidazole alone; as such the number of women included in the PP analysis was very small due to the high number of participants who self-resolved their BV between screening and enrolment; (2) the rate of STI acquisition was high within the study, which might have influenced BV status; (3) choosing topical metronidazole over oral metronidazole might have led

to higher BV cure rates in the SOC arm, thus making it more difficult to detect any superiority in those using an adjunctive probiotic; (4) the first follow-up visits might have been scheduled at a time point that was too long after treatment completion (thus some participants might have had recurrent episodes of BV already when BV cure was measured); (5) the treatment duration with the probiotic might have been too short; and (6) the bacterial species (*L. rhamnosus, L. acidophilus, B. bifidum* and *B. longum*) contained in the administered product might not have been suitable for vaginal colonization. Vagiforte® PLUS not only contained *Lactobacillus* spp. that are not commonly found in the FGT of South African women (Lennard *et al*. 2017), but also contained *Bifidobacterium* spp. that have previously associated with BV in pregnant women (Rosenstein *et al*. 1996). In agreement with this later possibility, women in this interim analysis who showed increased vaginal colonization with *Bifidobacterium* spp. or *L. rhamnosus* tended to also be colonized with yeast (like *C. albicans* and *C. dubliniensis*) and other BV-associated bacteria (like *P. bivia*), and were also less likely to be colonized with *L. crispatus*. Overall, this suggests that a more suitable probiotic to test the effect on BV cure should contain *Lactobacillus* spp. commonly colonizing the lower FGT, preferably derived from healthy South African women.

Taking all of these lessons from the pilot trial and key factors into consideration, it is estimated that any future trial should aim to enrol no less than 130 women (n=65 per treatment group), which would enable detecting the difference in expected BV cure proportions between metronidazole alone and metronidazole with probiotics (0.88 versus 0.66 in the intervention and SOC arm, respectively) with 80% power, with the alpha level set at 0.05, taking into consideration that 20% of women may acquire an STI during follow up. In this pilot study, no women were lost to follow up so far. However, assuming that ~10% of participants might opt out over the course of the study, 144 woman (n=72 per treatment arm) should be enrolled.

Because it is desirable that future probiotic formulations for vaginal health should include *Lactobacillus* spp. commonly found in the lower FGT, Chapter 5 described the isolation and characterization of vaginal *Lactobacillus* strains from healthy BV-/STI- young South African women. The characteristics that were investigated included the growth of vaginal *Lactobacillus* isolates under various pH conditions,

their ability to lower pH, adherence to cervical cells (Ca Ski), production of L-lactic and D-lactic acid and H_2O_2, their antibiotic susceptibility profiles, ability to inhibit growth of pathobionts *G. vaginalis, P. bivia,* and GBS, and their effect on inflammatory markers and cervical cell viability *in vitro*. These respective characteristics appeared to be highly strain-specific, although *L. crispatus* strains appeared to perform better than the other vaginal species. This is in agreement with *in vivo* studies (Ravel *et al*. 2011; Gajer *et al*. 2012; Lennard *et al*. 2017) that have shown that a vaginal microbiota that is dominated by *L. crispatus* is associated with genital health. The vaginal *L. crispatus* strains grew significantly better *in vitro* than the other vaginal *Lactobacillus* isolates and slightly better than commercially available probiotic *Lactobacillus* isolates at both low and neutral pHs. Further, some vaginal *Lactobacillus* strains lowered the pH to below 4.0, in contrast to the commercial probiotic strains that lowered the pH to ~ 4.2. The vaginal *Lactobacillus* strains also produced more L- and D-Lactic acid than the probiotic strains (with the exception of *L. acidophilus*), while vaginal and probiotic strains had a similar inhibitory effect on the growth of FGT pathogens. The majority of tested vaginal *Lactobacillus* strains did not induce cell toxicity or an inflammatory response in co-culture with ectocervial cells, suggesting that they are likely to be safe for vaginal administration. Overall, several of the vaginal *Lactobacillus* strains had more desirable probiotic profiles than the commercially available strains currently marketed for vaginal health in South Africa, which was confirmed in a weighted scoring system developed as part of this study. Using this scoring system, two *L. crispatus,* one *L. gasseri*, one *L. vaginalis* and two *L. jensenii* strains, all from BV-/STI- women, were among the highest scoring vaginal *Lactobacillus* strains, while all commercially available probiotics ranked in the lower half of the isolates screened. These results suggest that several of the vaginal *Lactobacillus* isolates from healthy South African women were better suited as probiotics for vaginal health than the currently available products in South Africa, not just based on their FGT origin, but also based on their better performance in *in vitro* assays.

Following selection of the best performing vaginal *Lactobacillus* strains, an important consideration is what a probiotic for vaginal health relevant to South African women should look like? Due to the high variability of the vaginal microbiota between different women (Fettweis *et al*. 2014; Ravel *et al*. 2011; Gajer *et al*. 2012; Lennard *et*

al. 2017), is it likely that no single bacterial strain would be able to successfully colonize the FGT of all women. A previous study, using the probiotic *L. crispatus* strain CVT-05, reported that this probiotic isolate colonized best in women who were not endogenously colonized with *L. crispatus*, and that recent sexual activity, either with and without condom use negatively influenced colonization with the probiotic (Antonio *et al*. 2009), suggesting that the identity of the commensal microbiota and sexual practices may influence the efficacy of probiotics. Thus, co-formulating several probiotic bacterial species and/or strains into a product might increase the changes of colonization.

Production costs should be of special concern in resource-limiting settings like South Africa, to ensure that the final product will be accessible to those in most of need. Another question that needs to be addressed is whether the probiotic should be administered orally or vaginally, and at which dose. A review of several trials administering probiotics for vaginal health opted to administer these orally, with a presumption that these would be ano-genital transferred, or vaginally but at a higher dose $>10^8$ CFU for more than two months (Homayouni *et al*. 2014). While oral administration would possibly be easier to control, preference should be given to vaginal administration, as the oral route relies on a passive transfer from the rectum to the genital tract. On the other hand, willingness to administer probiotics vaginally needs to be high and women need to be comfortable doing this. Studies investigating vaginal application of other drugs in African women (contraceptive pill and contraceptive vaginal ring) showed that the acceptability was high, and it appeared even that women preferred vaginal over oral administration (Kestelyn *et al*. 2018; Ziaei *et al*. 2002). It is also worth noting that vaginal practices are common in South Africa (Scorgie *et al*. 2009; Scorgie *et al*. 2011), suggesting that women should feel comfortable to administer a probiotic vaginally. A vaginal product can be delivered in several different ways: spray, tablet, gel, cream, capsules or a vaginal ring. The vaginal delivery systems should ideally be easy to use, discreet, painless, cost-effective, widely available, and safe for continuous administration. It should also allow self-administration, with minimal interference with other body functions and daily life (Neves & Bahia 2006; Alexander *et al*. 2004). In the case of a vaginal probiotic, a sufficient amount of the probiotic bacteria must be delivered locally to be efficacious, and previous studies have shown that the concentration of *Lactobacillus*

spp. is $\geq 10^7$ CFU/mL in vaginal secretion of healthy women (Boskey *et al*. 1999). Using a spray, cream or gel to apply a vaginal probiotic would ensure greater vaginal distribution, in addition to encouraging vaginal retention of probiotic organisms to ensure efficacy. The volume of probiotic gel or product administered has been described as important factors in gel distribution and retention (Pretorius *et al*. 2002). In addition, sexual intercourse could increase the spread of the gel, but also increased leakage from the vagina (Pretorius *et al*. 2002). These previous studies suggest that a delivery method like capsules or tablets, for which distribution and retention is not dependent on applicator use and human behaviour, might be more suitable. Vaginal capsule administration was shown to result in an immediate release of all probiotic bacteria, and considering that approximately 10^7 - 10^8 CFUs are naturally eliminated in vaginal secretions daily, it is suggested that daily administration of the probiotic product is required (Dausset *et al*. 2018; Mitchell *et al*. 2011), which might result in poor compliance over long administration periods. In light of these considerations, it is possible that slow release muco-adhesive tablets would provide the best delivery system for vaginal probiotics. Dausset *et al.* (2018) recently described the use of such a delivery system that was administered twice a week, and was shown to be safe, well tolerated and efficacious in their ability to colonisation the vagina.

Once a new or improved vaginal probiotic is manufactured, another major consideration would be how it should be tested. Firstly, a Phase I trial with a small sample size should be conducted to investigate the safety and tolerance/acceptance of the product, but also whether the administered strains colonisation the FGT. Once the safe administration and successful colonisation of the probiotic strains is confirmed, their efficacy on BV cure and treatment durability needs to be determined in a randomised, placebo-controlled, double-blind trial with an adequate sample size, using standardized methodology. The primary end point should be BV cure, while secondary end points could include BV recurrence, changes in the vaginal microbiome, decrease of vaginal pH and genital inflammation over six months.

In Chapter 6, the likely contribution of bacteriophages to the onset of BV was evaluated. Prophages and CRISPR loci were found to be common in the *in silico* analyses using published *Lactobacillus* genomes that were conducted in this dissertation, and temperate bacteriophages were commonly induced from vaginal

Lactobacillus strains isolated from South African woman. However, no virulent bacteriophages targeting vaginal *Lactobacillus* spp. were isolated from vaginal secretions from women with and without BV. Since others (Martín *et al*. 2009) did not succeed in isolating virulent *Lactobacillus* bacteriophages from FGT secretions, other experimental approaches should be considered to evaluate whether bacteriophages are implicated in the etiology of BV, as hypothesized in this study. As such, analysing the relative abundance and composition of the vaginal virome, including bacteriophage communities, using culture-independent techniques would give more insight into the possible phenomenon of "vaginal bacterial phaginosis" (Blackwell 1999). Ideally, this should be done using intensively collected genital samples: prior to, on the day of BV onset, and during BV episodes with daily sampling.

Overall, this PhD thesis evaluated the use of vaginal probiotics for BV treatment in South African women who are at high risk of BV, STIs and HIV. While probiotics for vaginal health have great potential in the Sub-Saharan setting, the products that are currently being marketed in South Africa are not well suited, and should be replaced by probiotics containing FGT commensals that have beneficial properties, and that are marketed at an adequate price. Large randomised, controlled trials are still needed to evaluate the effect of a well-designed probiotic on BV cure and recurrence in South African women.

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APPENDICES

Appendix I. Summary of clinical trials that have tested the ability of probiotics to treat BV

Trial design	Intervention	Outcomes	Adverse Events	Ref
Placebo-controlled n = 60 BV+	Intra-vaginal capsule *L. acidophilus*, H_2O_2-producing 10^8-10^9 CFU Twice daily for 6 days	Immediately after treatment completion, 16/28 women treated with probiotics had normal vaginal wet smear results, in comparison to 0/29 treated with placebo.	Not reported.	(Hallen et al. 1992)
Placebo-controlled n = 32 BV+ or BV intermediate	Vaginal tablets (Gynoflor) *L. acidophilus*, H_2O_2-producing > 10^7 CFU/tablet 0.03 mg estriol (CAS 50-27-1) Once – twice daily for 6 days	The cure rate 2 and 4 weeks after the start of therapy was 77% and 88% in the intervention group and 25% and 22% in the placebo group.	Not reported.	(Parent et al. 1996)
Placebo-controlled n = 46 History of recurrent BV or Candida +	150 mL of oral yoghurt *L. acidophilus* 1.0 x 10^8 CFU/ mL Once daily for 2 months	60% reduction of BV at 1 month for probiotic vs. 25% for non-probiotic yoghurt.	Not reported.	(Shalev et al. 1996)
One group n=10 History of recurrent BV, candida or UTIs	3 ml skim milk *L. rhamnosus* GR-1 & *L. fermentum* RC-14 >10^9 CFU in total Twice daily for 14 days	In 6/10 participants with asymptomatic or intermediate microbiota, BV was resolved within 1 week of therapy.	Patients noted no adverse events.	(Reid et al. 2001)
Placebo-controlled n = 125 BV+	Oral metronidazole (500 mg) Twice daily from days 1 to 7 Oral capsules *L. rhamnosus* GR-1 & *L.reuteri*	88% cure rate in the antibiotic/probiotic group compared to 40% in the antibiotic/placebo group ($p < 0.001$) at 30-day follow-up	No adverse effects but persistent headache and increased appetite in 2/125 (1.6%) participants	(Anukam et al. 2006)

Probiotics as adjunct to antibiotics	RC-14 1×10^9 CFU/strain/dose unit Twice daily from days 1 to 30			
Placebo-controlled n = 59 Asymptomatic healthy women	Oral capsules *L. rhamnosus* GR-1 & *L.reuteri* RC-14 1×10^9 CFU/strain/dose unit Daily for 2 months	Prevalence of asymptomatic BV decreased from 27.6% to 3.5% in probiotic group while no change was observed in control group after 2 months.	Not reported.	(Reid et al. 2004)
Placebo-controlled n = 64 Asymptomatic healthy women	Oral capsules *L. rhamnosus* GR-1 & *L.fermentum* RC-14 Daily for 60 days	Restoration of asymptomatic BV to normal microbiota in 37% women during lactobacilli treatment compared to 13% on placebo (p = 0.02)	Patients receiving probiotics reported no adverse events. 2/64 developed a yeast infection, in placebo group only.	(Reid et al. 2003)
Placebo-controlled n = 255 BV + **Probiotics as adjunct to antibiotics**	Vaginal Clindamycin (100 mg) Once daily for 3 days Tampons *L. gasseri*, *L. rhamnosus* & *L. fermentum* 10^8 CFU / per tampon During one menstruation cycle	No improvement in the cure rate after treatment with *Lactobacillus*-containing tampons compared to placebo tampons (55% vs. 63%)	No significant difference in severity, nature and frequency of adverse events between treatment and placebo group. Most common adverse event was clinical candida infection with an incidence of 14.2% and 13.5% in the probiotic group and placebo group, respectively. No serious AE were reported.	(Eriksson et al. 2005)
Placebo-controlled n = 360 BV, Candida, *T. vaginalis* or fluor vaginalis +	SOC therapy with metronidazole or fluconazole Vaginal tablets (Gynoflor) *L. acidophilus*, H_2O_2-producing $> 10^7$ CFU/tablet 0.03 mg estriol & 600 mg lactose	The Normal Flora Index (Numbers of *Lactobacillus* and pathogenic spp., leucocytes and vaginal pH) increased significantly in probiotic group 3-7 days (p=0.002) and 4-6 weeks (p=0.006) after end of therapy compared to controls.	Adverse events in 1/240 (diarrhoea) participant in probiotic vs. 2/120 (diarrhoea, nausea) in control group.	(Ozkinay et al. 2005)

Probiotics as adjunct to antibiotics	Daily for 6 or 12 days			
Probiotics vs. SOC n = 40 BV +	Vaginal capsules *L. rhamnosus* GR-1 & *L. reuteri* RC-14 1×10^9 CFU/strain/dose unit Once daily for 5 days OR: 0.75% metronidazole vaginal gel Twice daily 5 days	Follow-up at day 6, 15 and 30 showed cure of BV in significantly more women in the probiotic group (16, 17 and 18/20) compared to metronidazole (9, 9 and 11/20; p = 0.016 at day 6, p = 0.002 at day 15, p = 0.056 at day 30)	2/17 participants in probiotics and 11/18 in metronidazole group reported side effects.	(Anukam et al. 2006)
Placebo-controlled n = 72 BV intermediate	Oral capsules *L. rhamnosus* GR-1 & *L. reuteri* RC-14 2.5×10^9 CFU/strain/dose unit Once daily for 14 days	21/35 probiotics (60%) in the intervention group and 6/37 probiotics (16%) in the control group showed a reduction in the Nugent score by at least two grades (p = 0.0001) at the end of the study.	Not reported.	(Petricevic et al. 2008)
Adjunctive probiotics vs. SOC n = 190 BV + **Probiotics as adjunct to antibiotics**	Oral Clindamycin (300mg) twice daily for 7 days Vaginal capsule (Gynophilus) *L. casei rhamnosus* Lcr35 $> 1\times10^9$ CFU/ dose unit Once daily for 7 days	69/83 women (83%) in the intervention and 31/88 women (35%) in the control group showed a reduction of Nugent score by at least 5 grades 4 weeks after the last administration of the medication.	Not reported.	(Petricevic & Witt 2008)
Adjunctive probiotics vs. SOC	Oral metronidazole (500 mg) Twice daily for seven days	At days 30, 90 and 180 cure rates were 81%, 71% and 67% in metronidazole only group and 88%, 88% and 83% in the probiotic	Not reported.	(Marcone et al. 2008)

n = 84 BV + **Probiotics as adjunct to antibiotics**	Vaginal tablet *L. rhamnosus* $>4\times10^4$ CFU /dose Once a week for two months	group.		
Placebo-controlled n = 100 BV + **Probiotics as adjunct to antibiotics**	2% vaginal clindamycin cream once daily for 7 days Vaginal capsules (EcoVag®) *L. gasseri* EB01 & *L. rhamnosus* PB01 1×10^9 CFU/ dose unit Once daily for 10 days during 3 menstrual cycles.	The one-month cure rate was 64% in the probiotics and 78% in the placebo group ($p > 0.05$). At the end of the study, 64.9% (24/37) of the probiotic treated women were still BV-negative compared to 46.2% (18/39) of the placebo treated women.	14/37 patients in the probiotics and 12/39 in the placebo group reported AEs (headache, menorrhagia, haemorrhoids, influenza, bronchitis, whiplash, asthma, urinary tract infection). Five patients in the probiotic and four patients in the placebo group were treated with antimycotic drugs for symptomatic candida infection. No difference between the groups in type or frequency of AE.	(Larsson et al. 2008)
Placebo-controlled n = 39 BV +	Vaginal tablets *L. brevis* CD2, *L. salivarius* subsp. *salicinius* FV2, *L. plantarum* FV9 $>1\times10^9$ CFU/dose Once daily for 7 days	After completion of therapy, all patients in the probiotic group (n = 18) were free of BV (83% normal and 17% intermediate), compared to only 2/16 (12% intermediate) in placebo group ($p < 0.001$). Two weeks after completion of therapy, treatment was successful in 61% of probiotic as compared to 19% in the placebo group ($p < 0.05$).	Patients noted no adverse events.	(Mastromarino et al. 2009)
Placebo-controlled n = 64 BV +	Single dose of tinidazole (2g) Oral capsules *L. rhamnosus* GR-1 & *L. reuteri* RC-14	At the end of treatment (day 28) the probiotic group had a significantly higher cure rate of BV (87.5%) than the placebo group (50%, $p=0.001$).	4 participants (12.5%) in the placebo and one (3.1%) in the probiotic group developed vulvo-vaginal candidiasis.	(Martinez et al. 2009)

Probiotics as adjunct to antibiotics	1×10^9 CFU/strain/dose unit 2 capsules once daily for 4 weeks			
Adjunctive probiotics vs. SOC n = 49 BV + **Probiotics as adjunct to antibiotics**	Oral metronidazole (500 mg) twice daily for 7 days Vaginal tablet *L. rhamnosus* $>4\times10^4$ CFU /dose Once weekly for 6 months	Metronidazole only: 91%, 83%, 74%, 74%, 69% vs. 96%, 96%, 96%, 91%, 91% in the probiotic group at day 30, 90, 180, 270, 360 with normal vaginal flora,	Not reported	(Marcone et al. 2010)
Placebo-controlled n = 95 BV or Candida + **Probiotics as adjunct to antibiotics**	Clindamycin or clotrimazole Vaginal capsules *L. gasseri* LN40, *L. fermentum* LN99, *L. casei subsp. rhamnosus* LN113 & *P. acidilactici* LN23 10^8 -10^{10} CFU/dose Twice daily for five days	93% of women receiving probiotics were cured 2–3 days after administration (placebo: 83%), and 78% after one menstruation (placebo: 71%) The intervention group experienced less malodorous discharge 2-3 days after administration ($p = 0.03$) and after the second menstruation ($p = 0.04$) compared with placebo.	Two (3.2%) women in the intervention group and two women (5.3%) in the placebo group with BV were diagnosed with vulvo-vaginal candidiasis infection during the study. Vulvo-vaginal pruritus was reported by one (1.6%) woman in the intervention group, and by five (13.2%) women in the placebo group. One woman (1.6%) in the intervention group experienced a small amount of vaginal bleeding, while one (2.6%) woman had a swollen and red vulva after use of the placebo capsules. One (2.6%) woman in the placebo group reported headache. No serious AEs were reported.	(Ehrstrom et al. 2010)
Placebo-controlled	Vaginal capsule (Probaclac Vaginal)	Probiotic prophylaxis resulted in lower recurrence rates for BV (15.8% [9/57	Aside from vaginal discharge and malodour, no adverse events were reported	(Ya et al. 2010)

n = 120 History of recurrent BV	*L. rhamnosus, L acidophilus & S. thermophiles* $8x10^9$ CFU / dose Daily for 7 days on, 7 days off, and 7 days on	women] vs. 45.0% [27/60 women]; p = 0.001) and *G. vaginalis* incidence through 2 months (3.5% [2/57 women vs. 18.3% [11/60 women]; p = 0.02). Between the 2- and 11-month follow-up periods, women who received probiotics reported a lower incidence of BV and *G vaginalis*.	in either group.	
Placebo-controlled n = 12 Healthy women **Phase I trial to test safety, tolerability and acceptability**	Vaginal applicator *L. crispatus* CTV-05 (LACTIN-V) 5×10^8, 1×10^9 or 2×10^9 CFU/dose Once daily for 5 days	LACTIN-V appeared to be safe and acceptable in healthy volunteers.	45 adverse events occurred, of which 31 (69%) were genitourinary AEs (vaginal discharge in 5 subjects (42%), abdominal pain in 4 subjects (33%), metrorrhagia in 4 participants (33%), vulvovaginitis in 4 participants (33%), vaginal candidiasis in 3 participants (25%), and vaginal odor in 3 participants (25%) that appeared evenly distributed between the three treatment blocks and between LACTIN-V and placebo arms. 41 (91%) AEs were mild (grade 1) in severity. All 4 moderate AEs (grade 2) were unrelated to product use. No grade 3 or 4 AEs or serious AEs occurred.	(Hemmerling et al. 2010)
Placebo-controlled n = 24 BV+ **Phase 2a trial to assess colonization efficiency,**	Vaginal applicator *L. crispatus* CTV-05 (LACTIN-V) 2×10^9 CFU/dose Once daily for 5 days, followed by once weekly for 2 weeks.	61% of the 18 women randomized to the LACTIN-V group were colonized with *L. crispatus* CTV-05 at day 10 or 28. Among LACTIN-V users with complete adherence to the study regimen, 78% were colonized at day 10 or 28.	Of the 120 AEs that occurred, 108 (90%) and 12 (10%) were of mild and moderate severity, respectively. AEs were evenly distributed between the LACTIN-V and placebo group. Of the total AEs, 93 (78%) were genitourinary in origin. The most common genitourinary AEs included vaginal discharge (46%), abdominal pain (46%), dysuria (21%), pollakiuria (21%),	(Hemmerling et al. 2010)

safety, tolerability, and acceptability			vaginal odour (21%), and genital pruritus (17%). No grade 3 or 4 AEs or serious AEs occurred and no deep epithelial disruption was seen during colposcopic evaluation.	
Probiotics vs. standard of care n = 310 BV +	Yoghurt (100g) *L. bulgaris*, *S. thermophilus*, probiotic *Lactobacillus* & *B. lactis* 1 x 10^7 CFU / mL Twice daily for 7 days OR: Oral clindamycin (300mg) Twice daily for 7 days	Ten patients (6.7%) in the probiotic and 9 (6%) in the clindamycin group had symptom recurrence ($p>0.05$). 132 patients (88%) in the probiotic and 105 (70%) in the clindamycin group had pH decrease ($p<0.0001$). The clinical response to clindamycin was 252/300 (84%) after completion of treatment in comparison with 241/300 (80%) of cases in yoghourt group, which showed no symptoms of BV through Amsel criteria.	Not reported	(Hantoushzadeh et al. 2012)
Adjunctive probiotics vs. SOC n = 40 BV +	Oral Ofloxacin/Ornidazole (200–500mg) Once daily for 5 days PLUS: Vaginal peccaries (co-kimaxazol) Once daily for 3 days Oral capsules *B. coagulans* Unique IS-2 1 x 10^9 CFU/capsule Twice daily for 90 days	At the end of the treatment 80% of probiotic group subjects showed significant positive response as revealed by reduction of BV symptoms compared to the control group (45%).	Participants did not report adverse events.	(Sudha et al. 2012)
Placebo-controlled n = 450 BV+	Oral metronidazole (400mg) Twice daily for 7 days PLUS: Vaginal cream	One-month BV recurrence was 3.6% (5/140), 6.8% (9/133) and 9.6% (13/135) in the metronidazole -clindamycin, metronidazole-probiotics and metronidazole -Placebo	One third of participants reported vaginal itching/soreness and 8% reported nausea. The only adverse symptom that was significantly different between treatments	(Bradshaw et al. 2012)

	Clindamycin (2%) Once daily for 7 days OR: Vagina peccary *L. acidophilus* KS400 > 1 x 10^7 CFU/capsule 0.03mg oestriol Once daily for 12 days.	groups, respectively. Cumulative 6-month BV recurrence was 28.2%; (95%CI 24.0–32.7%) with no difference between groups.	was increased vaginal discharge, which was reported by more participants in the probiotic group (p=0.006). No serious AEs occurred.	
Placebo-controlled n = 544 BV+	Oral capsules (Lactogyn) *L. rhamnosus* GR-1 & *L. reuteri* RC-14 > 1 x 10^9 CFU / capsule Twice daily for 6 weeks	At 6 weeks, balanced vaginal microbiota were reported in 40/149 (26.9%) in the placebo compared to 243/395 (61.5%, p < 0.001) in probiotic group. After 12 weeks normal vaginal microbiota were still present in 51.1% of the probiotic but only in 20.8% of placebo group (p < 0.001).	Not reported	(Vujic et al. 2013)
Probiotics vs. SOC n = 55 BV+	Vaginal suppositories *L. delbrueckii* subsp. *lactis* DM8909 > 1 x 10^9 CFU/dose Once daily for 10 days OR: Vaginal metronidazole (500 mg) once daily for 7 days	Follow-up visits at days 5 and 30 showed a greater BV cure rate in the probiotics-treated subjects (88.0 and 96 %, respectively) than the metronidazole-treated subjects (83.3 and 70 %, respectively [p = 0.625 at day 5 and p = 0.013 at day 30])	Not reported	(Ling et al. 2013)
Placebo-controlled n = 34 BV+	Vaginal tablets *L. fermentum* LF15 & *L. plantarum* LP01 4x10^8 CFU/strain/dose Once daily for 7 nights, followed by 1 tablet every 3	In probiotic group the Nugent score was significantly reduced below 7 after 28 days in 22/24 (91.7%, P<0.001). 8 women (33.3%) recorded a Nugent score between 4 and 6, whereas 14 (58.3%) showed a score <4. At the end of the second month, only 4 women	Participants did not report AEs.	(Vicariotto et al. 2014)

	nights for 3 weeks and 1 tablet per week long-term	registered a Nugent score >7, definable as BV (16.7%, P=0.065 compared with d28). In the placebo group, no significant differences were recorded at any time.		
Placebo-controlled n = 80 History of recurrent BV	Polyherbal microbicide (250 mg, BASANT) Once daily for 7 days OR: Vaginal capsules *L. fermentum* (TRF#36), *L. gasseri* (TRF#8) & *L. salivarius* (TRF#30) $3x10^7$ CFU/dose Once daily for 7 days OR: combination of both	Polyherbal microbicide regressed BV in 14/20, probiotics in 13/20, and the combination of the two in 19/ 20 women and placebo in 1/ 20 women.	Polyherbal microbicide capsules were well received and lack of any side effects or adverse events. AEs not reported for probiotic capsules	(Kriplano et al. 2014)
Placebo-controlled n = 34 BV+ or aerobic vaginitis+ **Probiotics as adjunct to antibiotics**	Oral metronidazole (500 mg) Twice daily for 7 days Oral capsules (prOVag®) *L. gasseri* 57C, *L. fermentum* 57A & *L. plantarum* 57B $>1 \times 10^8$ CFU/dose Twice daily for 10 days; afterwards once daily for 10 days perimenstrually.	prOVag lengthened the time to clinical relapse of BV or aerobic vaginitus symptoms up to 51% ($p < 0.05$) compared with placebo	There were 431 AEs in 160 participants; prOVag was used by 77 of these participants. There were no significant differences between the total numbers of adverse events reported in either of the study groups. No serious AEs related to the use of the probiotic product occurred.	(Heczko et al. 2015)
One group n = 10 BV +	Partner treatment with oral clindamycin Vaginal clindamycin cream (2	The cure rate after month 6 was 50% with four women cured and four with a relapse of BV.	One man treated with clindamycin had a skin rash and stopped taking the antibiotic after six days.	(Pendharkar et al. 2015)

	%) Once daily for 7 days PLUS: Oral clindamycin (300 mg) Once daily for 7 days Afterwards: Vaginal capsules (EcoVag®) *L. rhamnosus* DSM14870 & *L. gasseri* DSM14869 1 x 10^8 CFU/strain/dose Once daily for 5 days After next menstruation: Vaginal metronidazole (0.75 %) Once daily for 5 days PLUS: EcoVag® once daily for 5 days			
One group n = 30 BV + or Candia + 	Partner treatment with oral clindamycin Clindamycin or fluconazole as SOC Afterwards: Vaginal capsules (EcoVag®) *L. rhamnosus* DSM14870 & *L. gasseri* DSM14869 1 x 10^8 CFU/strain/dose Once daily for 10 days, and after the second menstruation once	The BV cure rate after month 6 and 12 was 67% with 3 relapses and 6 cured women in the probiotic group. All nine women infected with Candida and treated with fluconazole and EcoVag® were cured at the 6-month follow up, and 89% at the 12 month visit.	Two women stopped taking the last 10 and 2 EcoVag® capsules.	(Pendharkar et al. 2015)

	every week for the next four months			
Placebo-controlled n = 160 BV Intermediate	Vaginal capsule (inVag®) *L. fermentum* 57A, *L. plantarum* 57B & *L. gasseri* 57C >1 x 10^9 CFU/strain/dose Once daily for 7 days	Administration of inVag® contributed to a significant decrease in vaginal pH ($p<0.05$) and Nugent score ($p<0.05$), and a significant increase in the abundance of *Lactobacillus* ($p<0.05$).	In the probiotic group, 79 women reported AEs, and in the placebo group 67 women reported AEs. A total of 146 AEs were reported in both treatment groups, the most common being genitourinary tract events (60% of all reported AEs). The most common symptoms reported in probiotic group were vaginal discharge (16.46% of all symptoms observed), pruritus (12.66%), and hypogastric pain (7.59%), while in the placebo group it was vaginal discharge (19.40%), pruritus (11.94%), and burning sensation (10.45%). In both groups, a total of 71 AEs were unrelated to the application of either probiotic or placebo, and 75 adverse events were related to the application, but in most of these cases these were classified as "mild severity" and "unlikely related". Analysis of the AEs confirmed that no serious AE was related to the use of the probiotic.	(Tomusiak et al. 2015)
Placebo-controlled n = 250 BV + **Probiotics as adjunct to**	Oral metronidazole (500 mg) Twice daily for 7 days Vaginal tablets (NORMOGIN®) L. rhamnosus BMX 54 > 1 x 10^4 CFU/dose Once a day for 10 days, twice a	After 2 months, 90.4% in the probiotic and 79.2 % in the placebo group were BV negative ($p = 0.014$). After 6 months, 74.6% in the probiotic and 25.4 % in the placebo group were BV negative ($p = 0.014$).	No AEs were reported by participants.	(Recine et al. 2016)

antibiotics	week for 15 days, and once every 5 days for 7 months as maintenance therapy			
One group n = 103 BV +	Vaginal suppository (SymbioVag®) *L. acidophilus* & *L. gasseri* > 1 x 10^9 CFU/strain/gram Prebiotic inulin Once daily for 10 days	Vaginal pH was reduced in >80% of participants. The concentration of *Lactobacillus* spp. increased significantly. A decrease of *G. vaginalis* and A. *vaginae* was observed in < 60% of the participants. 80-90% of the participants noted an improvement in BV symptoms.	14 AEs were documented; all of them were considered as "not severe". Three of these (pain and burning, itching and burning, vaginal pain) were considered as "possible" and two (both vulvovaginal burning) as "probable" being related to the application of the study product.	(Schwiertz et al. 2015)
Probiotics vs. SOC n = 60 BV + **Probiotics as adjunct to antibiotics**	Oral metronidazole (400 mg) Three times daily for 7 days Oral capsule Strain data not available Dose data not available Twice daily for 30 days	Efficacy of combined probiotic and antibiotic therapy with antibiotic therapy alone in treatment of BV was 83.33% vs. 36.67%, respectively.	Not reported	(Arooj et al. 2017)
Placebo-controlled n = 36 BV+ **Probiotics as adjunct to antibiotics**	Oral metronidazole (500 mg) Twice daily for 7 days Oral yoghurt (125 g) *L. crispatus* LbV88, *L. gasseri* LbV150N, *L. jensenii* LbV116 & *L. rhamnosus* LbV96 1 x 10^7 CFU/strain/mL Twice daily for 4 weeks	After 4 weeks of intervention 0/17 had BV in the probiotic vs. 6/ 17 in the control group (p = 0.018). Amsel score decreased by 4 in probiotic and by 2 in the control group. The Nugent score decreased during the intervention period by 5.5 in the probiotic compared to 3.0 in the control group (p = 0.158).	In 11 women of the probiotic group compared to 12 women of the placebo group AEs were reported. The number of adverse events was 20 (3 antibiotic-associated diarrhoea, 7 gastroenteritis, 6 pain, 4 other) in the probiotic group compared to 27 (3 respiratory tract infection, 6 gastroenteritis, 11 pain, 7 other) in the placebo group. One participant withdrew the consent due to an AE.	(Laue et al. 2018)

Probiotics vs. SOC n = 100 BV + **Probiotics as adjunct to antibiotics**	Oral metronidazole (400 mg) Twice daily for 7 days Oral capsules *L. rhamnosus* Dose data not available Twice daily for 6 weeks	In the probiotic group the mean Nugent score before treatment was 8.02±0.699, which decreased to 3.87±0.86 after 6 weeks. In the control group the mean Nugent score before treatment was 8.26±0.78, which decreased to 4.84±0.85 after 6 weeks. Eleven participants in the probiotic and three in the control group had BV recurrence within the study time period.	No AE was recorded during the study period.	(Grewal et al. 2018)

Rows shaded green were those trials that suggested a positive outcome in women receiving probiotics; rows shaded orange were those trials that suggested no difference between probiotic and placebo or antibiotic alone arm; rows shaded blue are trial assessing safety, acceptability and colonization of vaginal probiotics (not efficacy), rows that are clear are those trials with equivocal outcomes that were difficult to classify as either a positive or negative outcome

Appendix II. Probiotics available in South Africa and their bacterial content

* More than one strain of one bacterial species

Vaginal

Immune system (IS)

Claims in several categories

GI / IS

Babies/Infants

Gastro-intestinal (GI)

	L. acidophilus	*L. rhamnosus*	*B. longum ssp. longum*	*B. animalis ssp. lactis*	*B. bifidum*	*L. plantarum*	*S. thermophilus*	*L. casei*	*L. reuteri*	*B.longum ssp. infantis*	*S. boulardii*	*L. paracasei*	*L. salivarius*	*L. sporogenes*	*Lactococcus lactis*	*L. helveticus*	*B. breve*	*L. Delbrucki ssp. bulgaricus*	*L. Delbrucki ssp. lactis*	*P. shermanii*	*S. cerevisiae*	*L. Delbrucki*	*L. diacetylactis*	*L. fermentum*	*E. mundtii*	*B. animalis ssp. animalis*	*B. lateosporus*	*B. subtilus*	*L. brevis*	**strains in total**	
Vibrant Health Green Vibrance® Version 15.0																													12	*
Vibrant Health Green Vibrance® Version 14.1																													12	*
Vibrant Health Maximum Vibrance®																													12	*
Nature Fresh PROBIOTIC-15 spray																													11	
Nature Fresh PROBIOTIC-15 liquid																													11	
The Real Thing PRO-Probiotic																													10	
Biogen PRE/PROBIOTIC 9 strain																													9	
ProbiFlora™ Adult Intensive Rescue 9 strain																													9	*
Healthy Living XTensiFlora9																													9	
Clicks 9 strain flora																													9	
Clicks virale eeze plus	No information available																												9	
Vivomixx™ VSL Probiotic																													8	
BIOremFERMENT Culture of Probiotic Bacteria																													7	
Probio Herbal Probiotics																													7	
UltraFlora® Spectrum																													7	*
Creche Guard Probiotic Oral Spray																													6	
CPharm Pro-B6 Suspension																													6	
Solgar® Advanced 40+ Acidophilus®																													5	
Viridian Synbiotic Daily																													5	
Activa Health ActivoVite™ PRO 4																													4	
Solgar® Advanced Multi-Billion Dophilus®																													4	
Solgar® Advanced Multi-Billion Dophilus® - Lactose Free																													4	
Nativa Digestive Complex																													4	
GastroChoice™ IBS																													4	
GastroChoice™ Probiotic																													4	
Holistix Probiotic																													4	
Bioflora Intestiflora® Spray																													4	
CPharm Pro-B5 Capsules®																													4	
ProbiFlora™ Adult Classic Bowel Support																													4	
ProbiFlora™ Adult Colon Ease																													4	
Biogen Probimax 4 Strains																													4	
Solal™ Probiotic Complex™																													4	
Solal™ Probiotic Maximum Potency																													4	
QuatroFlora™																													4	
sFera™ Biotic																													4	
Nativa Spasmopep™ Digest																													4	
Symbio-D®																													4	
Symbio-D® Junior																													4	
Bioflora Combiforte® Spray																													4	
ProbiFlora™ Adult Intensive Rescue 4 strain	No information available																												4	
ProbiFlora™ Infant - Drops																													3	
Bioflora Combiforte® Capsules																													3	
Crèche Guard Probiotic Skin Spray																													3	
Bioflora Intestiflora®																													3	
Bioflora Kiddie-forte®																													3	
ProbiFlora™ Infant																													3	
ProbiFlora™ Junior																													3	
Viralmed ProBio Baby Bum Spray																													3	
Trubiotic The Precision Biotic																													3	
UltraFlora® Acute Care																													3	
Bioflora Vagiforte® Plus																													3	
Viridian Tri-blend Acidophilus Complex																													3	
Sandoz Linex Forte																													2	
Solgar® Advanced Acidophilus Plus®																													2	
Adcock Ingram Bestum																													2	
Bioflora Bifidoflora®																													2	
Crèche Guard Baby Probiotic & Multivitamin Spray																													2	
Ampiro® Daily Flora																													2	
Entiro™ Junior																													2	
Entiro™ The complete probiotic																													2	
HydraChoice™																													2	
Lifestyle L-Reuteri probiotic																													2	
Pro-B Pro-B2 Drops®																													2	
ProbiFlora™ Adult Restore																													2	
ProbiFlora™ Adult Everyday Flora Balance																													2	
ProbiFlora™ Rx Intestinal Flora Care																													2	
Reuterina™ Femme																													2	
UltraFlora® Baby																													2	
Ultraflora® Balance																													2	
Ultraflora® Children's																													2	
Ultraflora® IB																													2	
UltraFlora® Immune Booster																													2	
UltraFlora® Synergy																													2	
UltraFlora® Women's																													2	
ViralGuard™ Fizzi Chews																													2	
ViralGuard™ Tablets																													2	
Viralmed ProBio																													2	
Bioflora Infantiforte® Spray																													1	
Biobalance® immo-gut																													1	
Ampiro ®Colon Ecology																													1	
Solgar® Cran Flora®																													1	
easiHEALTH COLON™																													1	
Enteron® LP299V®																													1	
Flora Balance																													1	
Aspen Flora-Balance™ L.R.																													1	
Healthy Living Florin 250																													1	
Zentiva Florawin																													1	
Adcock Ingram Inteflora 250®																													1	
Adcock Ingram Lp299v																													1	
Probi-Pin probiotic																													1	
Provacare® Probiotic Vaginal Care																													1	
Reuterina™ Acute																													1	
Reuterina™ Daily																													1	
Reuterina™ Drops																													1	
Reuterina™ Junior																													1	
Reuterina™ Vit. D																													1	
Clicks Saccharomyces boulardii supplement 250mg																													1	
Select Digest																													1	
Solal® 24-IN-1™																													1	
Cpharm MN-7																													1	
Ultima Probiotics Plus																													1	
Cipla Lactovita																													1	
UltraFlora® Intensive Care																													1	
Activovite supreme Pro4	No information available																												1	
Number of products containing species	54	38	36	34	27	20	13	11	11	11	10	10	9	9	7	7	6	6	4	3	3	2	2	2	2	1	1	1		

Appendix III. Package insert of MetroGel™

MetroGel™ V

Vaginal Gel

Scheduling status

S2

Composition

Metronidazole 37.5 mg/5 g

Preservatives: Methyl hydroxybenzoate 4.0 mg/5 g

Propyl hydroxybenzoate 1.0 mg/5 g

Pharmacological classification

A20.2.6 Antimicrobial: medicines against protozoa.

Pharmacological action

Metronidazole is a synthetic antibacterial agent. The antimicrobial effects result from the disruption of DNA and the inhibition of nucleic acid synthesis. Metronidazole has been shown to have activity against the following pathogens: Gardnerella vaginalis, bacteroides species and Mycoplasma hominis.

After intra-vaginal administration of MetroGel V, serum concentrations of metronidazole are about 2% of the maximum serum concentrations reached with a 500 mg tablet taken orally.

Metronidazole is metabolised in the liver by side chain oxidation and glucuronide formation and a large portion of the absorbed dose is excreted as metabolites. Both unchanged drug and metabolites are excreted mainly in the urine with about 35 -65% of the absorbed dose recovered over 24 hours.

Indications

MetroGel V is indicated for the treatment of bacterial vaginosis.

Contra-indications

MetroGel V is contra-indicated in patients with a price history of hypersensitivity to metronidazole, other nitroimidazoles or parabens.

Pregnancy and lactation

MetroGel V is contra-indicated during pregnancy.

Metronidazole is secreted in milk at concentrations similar to those in serum.

Warnings

See Dosage and Directions for use.

Reactions seen with oral metronidazole may also occur with MetroGel V.

In patients with renal failure, there is no accumulation of metronidazole, however the hydroxy and acid metabolites are retained. Haemodialysis removes both metronidazole and the two metabolites.

Although patients with severe hepatic dysfunction metabolise metronidazole slowly leading to retention of metronidazole and its metabolites, a total treatment course of 187.5 mg is unlikely to lead to excessive serum levels.

Known or previously unrecognised candidiasis may present more prominent symptoms during therapy with MetroGel V and may require treatment with a candicidal agent.

Metronidazole is a nitroimidazole and should be used with care in patients with evidence of a history of blood dyscrasias. Leukopenia has been observed during oral metronidazole administration.

Metronidazole may interfere with certain types of determination of serum chemistry values, such as aspartate aminotransferase (AST, SGOT), alanine aminotransferase (ALT, SGPT), lactic dehydrogenase (LDH), triglycerides and glucose hexokinase. Values of zero may be observed.

Seizures and peripheral neuropathy have been reported. MetroGel V should be administrated with caution in patients with central nervous system diseases.

Dosage and directions for use

For vaginal administration.

Pierce the sealed end of the tube and screw the end of the applicator tightly onto the tube of gel. Squeeze the tube, filling the applicator with gel. Remove applicator from the tube and gently insert the applicator into the vagina as far as it will comfortably go. Push the plunger to release the gel. Dispose of the applicator as instructed.

One applicator full of MetroGel V (5 g) should be inserted into the vagina once daily, at bedtime, for 5 days. If the patient does not respond to initial therapy it is recommended that appropriate laboratory measures be used to rule out conditions other than bacterial vaginosis before repeating the treatment.

MetroGel V is not recommended for use during menses.

MetroGel V is not recommended for use in children since safety and effectiveness have not been established.

Known symptoms of overdosage and particulars of its treatment

There is no human experience of overdosage with Metrogel V. Treatment is symptomatic and supportive. Metronidazole is readily removed from the plasma by haemodialysis.

Identification

A colourless to straw-coloured, transparent, slightly hazy, single-phase gel.

Presentation

Aluminium tubes with polyethylene screw caps containing 40 g product. The product is packed with five, 5 g vaginal applicators.

Storage instructions

Store below 25°C

KEEP OUT OF REACH OF CHILDREN

Registration number

33/20.2.6/0243

Name and business address of applicant

iNova Pharmaceuticals (Pty) Ltd
15e Riley Road, Bedfordview

Date of publication of this package insert

23 October 2000

PI Ref. No: R030(b)/231000

BOTSWANA

Scheduling status: S2

Pharmacological classification: ATC: G01A F01-Anti-infectives and antiseptics excl. combinations with corticosteroids, imidazole derivatives

Registration number: BOT 0200490

ZIMBABWE

Scheduling status: Prescription Preparation (PP)

Pharmacological classification: 7.6 Anti-infective drugs: antiprotozoals

Registration numbers: 2000/14.17/3648

NAMIBIA

Scheduling status: NS1

Registratio. number: 10/20.2.6/0472

How to use the applicators

Your tube of MetroGel V vaginal gel is packed in a box which also contains five throw away vaginal applicators.

When filled with gel, each applicator contains one single dose of MetroGel V gel for application into the vagina.

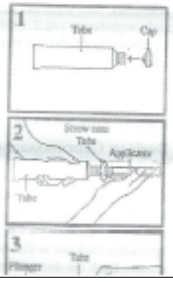
Hygiene

To ensure maximum hygiene, always

- wash your hands before opening the tube or touching the applicators
- use a fresh applicator for each dose
- immediately throw away the used applicator in the waste bin

Opening the tube

Before using MetroGel V vaginal gel for the first time, you will need to puncture the metal seal on the tube. To do this, simply unscrew the cap from the tube and pierce the seal using the pointed tip of the cap [1].



Filling the applicator

Remove an applicator from the wrapper. Unscrew the cap from the tube and screw the open end of the applicator onto the tube [2].

Squeeze the tube slowly from the bottom to fill the applicator with gel; the plunger will move as the applicator fills and will stop moving when the applicator contains the correct amount of gel [3].

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MetroGel V is not recommended for use during menses.

MetroGel V is not recommended for use in children since safety and effectiveness have not been established.

Side effects and special precautions

Adverse reactions are: vaginal candidiasis, vulvovaginal itching/burning/swelling, increased pelvic pressure and abnormal vaginal discharge. Abdominal cramping or pain, nausea, metallic taste in the mouth, diarrhoea, constipation, decreased appetite, decreased/increased white blood cells, headache, dizziness, and generalised rash/pruritis may occur.

Leukopenia was observed in some patients, but this was not clinically significant.

In addition, post-marketing experience revealed the following adverse events: vomiting, incoordination, insomnia, flushing, cystitis, incontinence and modification of taste.

Interaction with other medication

Oral metronidazole has been associated with a disulfiram-like reaction.

The possibility of a similar reaction occurring with MetroGel V cannot be excluded.

Lithium treatment should be tapered or withdrawn prior to administering metronidazole. Concomitant administration may lead to lithium retention and the possibility of renal damage.

Oral metronidazole has been reported to potentiate the anticoagulant effect of warfarin and other coumarin anticoagulants, resulting in a prolongation of prothrombin time. This possible drug interaction should be considered when MetroGel V is prescribed for patients on this type of anticoagulant therapy.

Storage instructions

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Registration number

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Name and business address of applicant

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Filling the applicator

Remove an applicator from the wrapper. Unscrew the cap from the tube and screw the open end of the applicator onto the tube [2].

Squeeze the tube slowly from the bottom to fill the applicator with gel; the plunger will move as the applicator fills and will stop moving when the applicator contains the correct amount of gel [3].

Unscrew the applicator and replace the cap on the tube.

Inserting the applicator

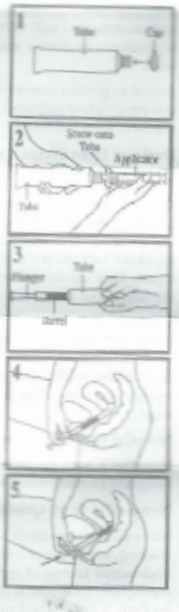
Hold the filled applicator by its barrel and gently insert it into your vagina as far as it will comfortably go [4].

You may find it easiest to do this whilst lying on your back with your knees bent. Alternatively, choose any position you find comfortable.

Applying the gel

Slowly press the plunger to deposit the gel into your vagina; continue pressing until the plunger stops (this will ensure you have released the correct amount of gel) [5].

Remove the applicator from the vagina and throw it in the waste bin immediately.



YOU MAY WANT TO READ THIS LEAFLET AGAIN. PLEASE DO NOT THROW IT AWAY UNTIL YOU HAVE FINISHED YOUR MEDICINE.

inova pharmaceuticals

iNova Pharmaceuticals (Pty) Ltd
15e Riley Road, Bedfordview

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Appendix IV. Package insert of Vagiforte® PLUS Combo Pack

VAGIFORTE® Plus Combo Pack PACKAGE INSERT

SCHEDULING STATUS: Nutritional supplement (Act 54/1972)

PROPRIETARY NAME *(AND DOSAGE FORM)*: VAGIFORTE® Plus Combo Pack comprises 15 oral capsules and vaginal spray with 30 dosages per bottle.

COMPOSITION: ***capsule:*** Each probiotic oral capsule contains 25.8 mg of a probiotic mixture consisting of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus GG*, *Bifidobacterium bifidum* and *Bifidobacterium longum* (in a lyophilized form), which yields in total not less than 2 billion ($2x10^9$) CFU (colony forming units) per capsule.

Vaginal spray: Each squirt of the probiotic spray has a volume of 250 µl (microliters) and contains 2 billion CFU (colony forming units) of a mixture of: *Lactobacillus acidophilus*, *Lactobacillus rhamnosus GG*, *Bifidobacterium bifidum* and *Bifidobacterium longum*.

PHARMACOLOGICAL ACTION: VAGIFORTE® Plus Combo oral capsules and vaginal spray contain a mixture of lyophilised probiotic bacteria namely, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus GG*, *Bifidobacterium bifidum* and *Bifidobacterium longum* which are natural inhabitants of the human vagina and intestinal tract. VAGIFORTE® Plus Combo Pack is administered to restore and maintain the normal vaginal flora (mainly lactic acid bacteria) frequently destroyed by broad-spectrum antibiotics, the use of disinfectants, soaps and deodorants. Lactic acid bacteria control the overgrowth of pathogenic microorganisms in the human digestive tract and prevent their potential transfer to the uro-genital tract. Administration of high doses of these bacteria helps to prevent the development of vaginosis (bacterial vaginal infections) and of vaginal thrush caused by *Candida albicans*, frequently associated with the broad-spectrum antibiotic treatment.

INDICATIONS: VAGIFORTE® Plus Combo Pack is indicated primarily for the treatment of vaginal thrush induced by antibiotic treatment or the excessive use of strong soaps, disinfectants and deodorants, for the restoration of the bacterial flora occurring naturally in the human vagina and for the prevention of transfer of *Candida albicans* (a natural intestinal inhabitant) to the vagina. Some forms of vaginosis also respond to the treatment with VAGIFORTE® Plus Combo Pack.

CONTRA-INDICATIONS: None known

DOSAGE AND DIRECTIONS FOR USE: Take one oral capsule per day for 5 days. Continue for the next 10 days with one oral capsule and two squirts of the VAGIFORTE® Spray per day onto vagina (one in the morning one in the evening, before bedtime). Continue using the vaginal spray until the content is finished. Shake bottle very well prior to application. The course can be repeated if necessary.

SHAKE BOTTLE VERY WELL PRIOR TO APPLICATION

SIDE-EFFECTS AND SPECIAL PRECAUTIONS: Large doses may cause loose stools in some individuals.

KNOWN SYMPTOMS OF OVERDOSAGE AND PARTICULARS OF ITS TREATMENT: Loose stools whereupon the administration of VAGIFORTE® Plus Combo Pack should be discontinued temporarily.

IDENTIFICATION: White capsules containing white to cream coloured, granular material and an amber spray bottle with metered spray insert.

PRESENTATION: 15 white capsules and an amber spray bottle with metered spray insert.

STORAGE CONDITIONS: Store in a cool, dry place below 25°C.

KEEP OUT OF REACH OF CHILDREN.

DATE OF PUBLICATION OF THIS PACKAGE INSERT: July 2014

NAME AND BUSINESS ADDRESS OF MANUFACTURER:

Creative Care Solutions Medical (Pty) Ltd Reg No: 2003/022808/07
Unit 8, Keymax Park P.O. Box 8142, Centurion, 0046
1006 Ergon Road, Lyttelton Ext 6 Tel: +27 (0) 12 644 1584
Centurion 0140, Gauteng; Fax: +27 (0) 12 644 1585

The Name of the Applicant: Bioflora® CC
45, Idol Road Tel/Fax: +27 (0) 12 348 2367
Lynnwood Glen, Pretoria E-mail: bioflora@lantic.net

Appendix V. Enrolmment questionnaire BV trial

Live biotherapeutics for treatment of bacterial vaginosis in HIV prevention in South African women – BV-trial1

Principal Investigator:
University of Cape Town, South Africa: Dr Shaun Barnabas

Co-Investigators:
University of Cape Town, South Africa: Dr Jo-Ann Passmore, Anna-Ursula Happel and Dr Heather Jaspan

Funder: Medicines Research Council

University of Cape Town:
Phone 021 650 7963

Introduction
Hello my name is _____. I am part of a research team from the University of Cape Town (UCT). In this study we would like to compare two different ways to treat bacterial vaginosis. When you have bacterial vaginosis, you have abnormal bacteria in your vagina. We also want to know about various factors in your life that may change your risk of getting infected with these abnormal bacteria. So, we are going to ask you questions about your sexual behaviour, substance use, education, social history and vaginal practices. This information will be kept confidential and your personal information will not be shared with anyone.
You have been invited as a possible participant because you are between 18 - 45 years old and have bacterial vaginosis. If you agree to participate in this study, please fill in the attached questionnaire. You will be asked to sign a consent form before you fill in this questionnaire, which you will get a copy of to keep. Please read the information on the consent form, and ask questions about anything you do not understand, before deciding whether you would like to participate or not.

INTERVIEW DETAILS

Participant Identification _____

Date of Interview (DD/MMM/YYYY) _____

Location of interview: _____

Interviewer Name: _____

Interview start time: (HH:MM AM/PM): _____

Interview stop time: (HH:MM AM/PM): _____

SECTION 1: DEMOGRAPHICS AND SOCIO-ECONOMIC STATUS

1.1: Demographics

What is your date of birth? _____/_____/_____DAY/MONTH/YEAR

What is your current marital status?
Select one.

- ❑ Married (legal or traditional) or living as married
- ❑ Single
- ❑ Separated / Divorced
- ❑ Widowed
- ❑ Other, please specify: _____
- ❑ Prefer not to answer

What do you consider to be your racial and/or ethnic background? (For example Zulu, Xhosa, Coloured, White, etc.) _____

SECTION 2: WOMEN'S REPRODUCTIVE HEALTH

2.1 Menstrual History

How old were you when you had your <u>first</u> menstrual period? *Probe best estimate.*

Age in years: _____

- ❑ Don't know
- ❑ Prefer not to answer
- ❑ I never had a menstrual period

When was the <u>last</u> day of your <u>last</u> menstrual period?
_____/_____/_____DAY/MONTH/YEAR

2.2 Vaginal Hygiene Practices

Which vaginal practices have you heard about? Please select all that apply.

- ❑ Douching
- ❑ Cleansing with water
- ❑ Cleansing with soap
- ❑ Cleansing with detergents
- ❑ Cleansing with salt solution
- ❑ Cleansing with lemon juice
- ❑ Use of traditional herbs
- ❑ Insertion of creams
- ❑ Wiping with cloths/paper/etc.
- ❑ Other: _____

	Have you ever…			**If yes, how often have done this in the last month** (weekly, daily, number of times)**?**	**Why did you use the vaginal practice?** Possible reasons see below. Please look at **list below** and **name all numbers that apply**.
	Yes	**No**	**Prefer not to answer**		
Washed with water inside your vagina?	❑	❑	❑		
Washed with soap inside your vagina?	❑	❑	❑		
Washed with something else besides water or soap inside your vagina, including detergents or antiseptics?	❑	❑	❑		
Used your fingers to wash inside your vagina?	❑	❑	❑		
Used anything else to wash inside your vagina, including cloths or sponges?	❑	❑	❑		
Put or kept traditional medicines or herbs inside your vagina?	❑	❑	❑		
Put or kept medicine from a doctor/nurse/pharmacy inside your vagina?	❑	❑	❑		
Put or kept paper, cloth, or cotton wool inside your vagina?	❑	❑	❑		
Used a tampon?	❑	❑	❑		
Put or kept anything else inside your vagina?	❑	❑	❑		
Used anything to dry or tighten your vagina for sex?	❑	❑	❑		
Practiced douching to clean your vagina?	❑	❑	❑		
Had sex during your period?	❑	❑	❑		

Why did you use these products?

1) To clean the vagina
2) To reduce discharge
3) To reduce smell
4) To reduce itching
5) To clean blood after period
6) To avoid pregnancy
7) To reduce risk of sexually transmitted infections (e.g. Syphilis, gonorrhoea, herpes simplex virus type 2, chlamydia, etc.)
8) To reduce risk of HIV
9) For my own sexual pleasure
10) For my partner's sexual pleasure

11) Other: Please state reason

Have you ever experienced any problems after using any of these products?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

If yes, what did you experience? Please select all that apply.

- ❑ Itching or Irritation
- ❑ Pain
- ❑ Vaginal infections
- ❑ Bleeding
- ❑ Discomfort
- ❑ Other: _____

What do you think about using products in your vagina? Please select all that apply.

- ❑ They are good for me
- ❑ They cause discharge or smell
- ❑ They increase my chances of getting an STI
- ❑ They increase my chances of getting or transmitting HIV
- ❑ My partner wants me to use them

2.3 Pregnancy History

Have you ever been pregnant? This includes all pregnancies, whether the outcome was a live birth, miscarriage, stillbirth, termination of pregnancy (abortion), or an ectopic/tubal pregnancy. *Select one.*

- ❑ Yes
- ❑ No
- ❑ Prefer not to answer

If yes, how many times have you been pregnant? _____
If yes, when was the last pregnancy? (Month/Year) _____/_____

2.4 Contraceptive use

In the past have you used any form of contraception, safer sex method, or any other means to prevent pregnancy, to avoid getting or giving sexually transmitted infections, or to regulate your periods?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

In the past, what forms of contraception, safer sex method, or any other means to prevent pregnancy, to avoid getting or giving sexually transmitted infections, or to regulate your period have you used? Please select all that apply.

- ❑ Oral contraceptive, also known as 'the pill'
- ❑ An injection/injectable, also known as 'Depo-Provera' or Nur-Isterate (circle correct one)
- ❑ NuvaRing, a vaginal ring that you insert once a month
- ❑ A contraceptive patch, also known as Ortho Evra and used once a week

- ❑ Implanon, also known as a "progestin implantable contraceptive"
- ❑ An Intrauterine Device, also known as an "IUD" or "Copper IUD"
- ❑ An Intrauterine System, also known as an "IUS" or "Mirena"
- ❑ A diaphragm, also known as a "cervical cap"
- ❑ The sponge
- ❑ Any vaginal creams, jellies, or foams
- ❑ Any emergency contraception, commonly known as "Escapelle", "the morning after pill", or "Norlevo"?
- ❑ Condoms
- ❑ The rhythm method, also known as "periodic abstinence"
- ❑ The withdrawal method
- ❑ Chosen to practice abstinence with male partners
- ❑ Any other method? (please specify) _____

Do you smoke?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

2.5 Vaginal discharge

Do you have vaginal discharge?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

If yes, is it a chronic/long-term problem or a new/recent problem?

- ❑ Long-term
- ❑ New

If yes, what is the colour of your discharge? _____

If yes, what is the consistency of your discharge?

- ❑ Thick/cheesy
- ❑ Creamy
- ❑ Thin

If yes, what is the smell of your discharge?

- ❑ Strong
- ❑ Mild
- ❑ None

SECTION 3: WOMEN'S SEXUAL HEALTH

The next section includes some personal questions about your sexual activities. Some of these questions are very personal and may make you feel uncomfortable. Since the survey is confidential, no one will know your answers. We would appreciate your participation in answering these questions as truthfully as possible. If you would like to complete this section yourself, you are welcome to do so. If you would like me to guide you through this whole section, that's okay too.

How old were you the first time you had vaginal sex?

Indicate age in years: _____

- ❑ Don't know
- ❑ Prefer not to answer

How many different people have you had sex with in your lifetime?

Number of different people: _____

- ❑ Don't know
- ❑ Prefer not to answer

During the past 6 months have you had vaginal sex?

- ❑ Yes **If yes, with how many partners**? ____
- ❑ Don't Know
- ❑ Prefer not to answer

During the past 6 months, have you had anal sex?

- ❑ Yes **If yes, with how many partners**? ____
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

During the past 6 months, have you had oral sex?

- ❑ Yes **If yes, with how many partners**? ____
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

How many of these relationships were "exclusive" or "mutually monogamous" (meaning while you were in the relationship, you and your partner only had vaginal or anal sex with each other, and had no other sexual partners outside the relationship)? _____

If you are using condoms with your partners, how often do you use them?

- ❑ Always, every time I've had sex
- ❑ Most of the time
- ❑ Occasionally
- ❑ Never
- ❑ Unsure
- ❑ Prefer not to answer

During the past 6 months, have you used condoms regularly?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

Did you use barrier protection (male or female condoms) with your last sexual act?

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

3.2 Risk factors

Have you ever been diagnosed with or been treated for a sexually transmitted infection?
Examples: Syphilis, gonorrhoea, non-gonococcal urethritis, herpes simplex virus type 2 (HSV-2), chlamydia, pelvic inflammatory disease (PID), trichomonas, mucopurulent cervicitis, epididymitis, procitis, lymphogranuloma vernereum, chancroid, hepatitis B.

- ❑ Yes
- ❑ No
- ❑ Don't Know
- ❑ Prefer not to answer

Have you or your partner had a STI in the past 6 months?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

How many sexually transmitted infections have you had in your lifetime?_____

Appendix VI. Follow-up questionnaire BV trial

Live biotherapeutics for treatment of bacterial vaginosis in HIV prevention in South African women – BV-trial1

Principal Investigator:
University of Cape Town, South Africa: Dr Shaun Barnabas

Co-Investigators:
University of Cape Town, South Africa: Dr Jo-Ann Passmore, Anna-Ursula Happel and Dr Heather Jaspan

Funder: Medicines Research Council

University of Cape Town:
Phone 021 650 7963

Introduction
Hello my name is _____. I am part of a research team from the University of Cape Town (UCT). You are taking part in a study comparing two different ways to treat bacterial vaginosis. When you have bacterial vaginosis, you have abnormal bacteria in your vagina. We would like to ask how you responded to the treatment. This information will be kept confidential and your personal information will not be shared with anyone.

INTERVIEW DETAILS

Participant Identification _____

Date of Interview (DD/MMM/YYYY) _____

Location of interview: _____

Interviewer Name: _____

Interview start time: (HH:MM AM/PM): _____

Interview stop time: (HH:MM AM/PM): _____

1.1 Use of METRONIDAZOLE GEL

Have you used the Metronidazole gel?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

If yes, did you complete the entire course?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

If no, how many days did you take it for? _____

If no, why did you stop treatment?

- ❑ Medication did not work
- ❑ Vaginal itching or irritation
- ❑ Vaginal pain
- ❑ Vaginal infections
- ❑ Vaginal bleeding
- ❑ Discomfort
- ❑ Other: _____

Have you ever experienced any problems after using the study product?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

If yes, what did you experience? Please select all that apply.

- ❑ Vaginal itching or irritation
- ❑ Vaginal pain
- ❑ Vaginal infections
- ❑ Vaginal bleeding

- ❑ Discomfort
- ❑ Other: _____

1.2 Use of STUDY PRODUCT

NOTE: PLEASE ONLY COMPLETE THIS SECTION IF YOU WERE GIVEN THE PROBIOTIC FOR VAGINAL HEALTH. IF YOU WERE ONLY GIVEN THE METRONIDAZOLE GEL, PLEASE GO TO SECTION 1.3

Have you used the study product?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

<u>If yes,</u> did you complete the entire course?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

<u>If no</u>, how many days did you take it for? _____

<u>If no,</u> why did you stop treatment?

- ❑ Medication did not work
- ❑ Vaginal itching or irritation
- ❑ Vaginal pain
- ❑ Vaginal infections
- ❑ Vaginal bleeding
- ❑ Discomfort
- ❑ Other: _____

Have you ever experienced any problems after using the study product?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

If yes, what did you experience? Please select all that apply.

- ❑ Vaginal itching or irritation
- ❑ Vaginal pain
- ❑ Vaginal infections
- ❑ Vaginal bleeding
- ❑ Discomfort
- ❑ Other: _____

What do you think about using products in your vagina? Please select all that apply.

- ❑ They are good for me
- ❑ They cause discharge or smell
- ❑ They increase my chances of getting an STI
- ❑ They increase my chances of getting or transmitting HIV
- ❑ My partner wants me to use them

1.3 Vaginal discharge

Has using the medication improved your discharge?

- ❑ Yes
- ❑ No, there has been no change.
- ❑ No, symptoms worsened.
- ❑ Prefer not to answer

<u>If yes,</u> how did the discharge improve?

- ❑ Less discharge
- ❑ Change in colour
- ❑ Change is smell
- ❑ Don't know
- ❑ Other _____

<u>If symptoms have become worse</u>, what were the changes?

- ❑ More discharge
- ❑ Change in colour
- ❑ Change is smell
- ❑ Don't know
- ❑ Prefer not to answer
- ❑ Other _____

Appendix VII. Final visit questionnaire BV trial

Live biotherapeutics for treatment of bacterial vaginosis in HIV prevention in South African women – BV-trial1

Principal Investigator:
University of Cape Town, South Africa: Dr Shaun Barnabas

Co-Investigators:
University of Cape Town, South Africa: Dr Jo-Ann Passmore, Anna-Ursula Happel and Dr Heather Jaspan

Funder: Medicines Research Council

University of Cape Town:
Phone 021 650 7963

Introduction
Hello my name is _____. I am part of a research team from the University of Cape Town (UCT). You just finished taking part in a study comparing two different ways to treat bacterial vaginosis. When you have bacterial vaginosis, you have abnormal bacteria in your vagina. If you were in the group that was given the study product please complete this questionnaire.
In our study, you used the adjunctive probiotic for the treatment of bacterial vaginosis, and we would like to ask how you felt about using this treatment. This information will be kept confidential and will not be shared with anyone.

INTERVIEW DETAILS

Participant Identification _____

Date of Interview (DD/MMM/YYYY) ____/____/_____

Location of interview: _____

Interviewer Name: _____

Interview start time: (HH:MM AM/PM): _____

Interview stop time: (HH:MM AM/PM): _____

1. Use of the study product

During the study, did you like using the study product?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

<u>If yes,</u> why did you like using it?

_____
_____

<u>If no</u>, why did you not like using it?

_____
_____

Would you use the study product again?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

<u>If yes</u>, why would you use it again?

- ❑ For prevention of vaginal discharge
- ❑ To treat vaginal discharge
- ❑ Prefer not to answer

Would you recommend this product to other woman?

- ❑ Yes
- ❑ No

- ❑ Don't know
- ❑ Prefer not to answer

Did you use the product as recommended (oral, vaginal application)?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

<u>If no</u>, why did you not use it as recommended?

_____

_____

Did you complete the entire course of the product?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

<u>If no</u>, why did you not complete the course?

_____

_____

2. Acceptability of the study product

How easy or difficult was the vaginal application of the product?

- ❑ Easy
- ❑ Medium
- ❑ Difficult
- ❑ Don't know
- ❑ Prefer not to answer

For the vaginal application of a product, which formulation would you prefer?

- ❑ Spray
- ❑ Capsule
- ❑ Tablet
- ❑ Gel
- ❑ Tampon

Did you prefer the oral or vaginal application?

- ❑ Oral
- ❑ Vaginal
- ❑ Both applications
- ❑ Don't know
- ❑ Prefer not to answer

Would you prefer a product with oral or vaginal application only?

- ❑ Oral only
- ❑ Vaginal only

- ❑ Oral and vaginal
- ❑ Don't know
- ❑ Prefer not to answer

How comfortable did you feel about others knowing that you use this product?

- ❑ Very comfortable
- ❑ Comfortable
- ❑ Neither comfortable nor uncomfortable
- ❑ Uncomfortable
- ❑ Very uncomfortable
- ❑ No-one knew I was using the product
- ❑ Don't know
- ❑ Prefer not to answer

Was it difficult to store the study product in your home?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

Would you buy the product?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

<u>If yes,</u> how much money would you be willing to spend?

- ❑ Up to R25
- ❑ Up to R50
- ❑ Up to R100
- ❑ Up to R200
- ❑ Up to R300
- ❑ More than R300

<u>If yes,</u> where would you want to buy the product?

- ❑ Pharmacy
- ❑ Health store
- ❑ Grocery shop
- ❑ Clinic
- ❑ Other _____

Who would you ask for advice regarding the use of probiotics?

- ❑ Doctor
- ❑ Nurse
- ❑ Pharmacist
- ❑ Friends
- ❑ Partner
- ❑ Internet
- ❑ Other _____

Do you believe that you received some benefit from using the product?

- ❑ Yes
- ❑ No
- ❑ Don't know

- ❑ Prefer not to answer

We are going to ask you some personal questions; you do not have to answer. You can choose to either answer these on your own or to have someone assist you to complete the questions.

Did you inform your partner(s) that you were using the product?

- ❑ Yes
- ❑ No
- ❑ Prefer not to answer

<u>If yes,</u> what was your partner(s) response to being informed that you were using the product?

- ❑ Positive
- ❑ Negative
- ❑ Indifferent
- ❑ Prefer not to answer

Did you have sexual intercourse while using the product?

- ❑ Yes
- ❑ No
- ❑ Prefer not to answer

<u>If yes,</u> did your partner notice during sexual intercourse that you were using the product?

- ❑ Yes
- ❑ No
- ❑ Don't know
- ❑ Prefer not to answer

Did the use of the product affect your sex life?

- ❑ Yes
- ❑ No
- ❑ Prefer not to answer

<u>If yes,</u> was the effect positive?

- ❑ Yes
- ❑ No
- ❑ Prefer not to answer

<u>If yes,</u> what was the positive effect?

_____
_____

<u>If no</u>, what was the negative effect?

_____
_____