# Investigate the effect of Bacterial Vaginosis-associated bacteria on the efficacy of Anti-HIV therapy

By

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# Table of Contents

## Declaration

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# List of abbreviations

| | |
|---|---|
| ABC | Abacavir |
| ART | Antiretroviral therapy |
| ARV | Antiretroviral |
| ATCC | American Type Culture Collection |
| BCA | Bicinchoninic acid |
| BLAST | Basic Local Alignment Search Tool |
| bp | Base pairs |
| BV | Bacterial vaginosis |
| BVAB | Bacterial vaginosis associated bacteria |
| Ca | Calcium |
| CCR5 | C-chemokine receptor type 5 |
| $CD4^+$ | Cluster of differentiation 4 |
| CDC | Centre for Disease Control and Prevention |
| CFU | Colony forming units |
| $CO_2$ | Carbon dioxide |
| CVL | Cervical lavage |
| CXCR4 | CXC chemokine receptor 4 |
| dATP | Deoxyadenosine triphosphate |
| dGTP | Deoxyguanosine triphosphate |
| $dH_2O$ | Distilled water |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DPV | Dapivirine |
| *E. Coli* | *Escherichia coli* |
| *Env* | Envelope |
| EtBr | Ethidium bromide |
| FBS | Fetal bovine serum |
| FGT | Female genital tract |
| *Gag* | Group-specific antigen |
| GV | *Gardnerella vaginalis* |
| $H_2$ | Hydrogen |

| | |
|---|---|
| $H_2O_2$ | Hydrogen peroxide |
| HEK293T | Human embryonic kidney 293T |
| HIV-1 | Human immunodeficiency virus type 1 |
| HPLC | High performance liquid chromatography |
| HRMS | High resolution mass spectrometry |
| $IC_{100}$ | Full maximal inhibitory concentration |
| $IC_{50}$ | Half maximal inhibitory concentration |
| ICAM-1 | Intracellular adhesion molecule-1 |
| IDA | Information dependent acquisition |
| IL-1α | Interleukin-1 alpha |
| IL-1β | Interleukin-1 beta |
| IL-8 | Interleukin-8 |
| IMC | Infectious molecular clone |
| IN | Integrase |
| kb | Kilobase |
| kHz | Kilohertz |
| L | Litres |
| LA | Luria agar |
| LB | Luria Bertani |
| LTR | Long terminal repeat |
| mA | Milli amperes |
| MVC | Maraviroc |
| MSM | Men who have sex with men |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MW | Molecular weight |
| $N_2$ | Nitrogen |
| NaCl | Sodium chloride |
| NCBI | National Centre for Biotechnology Information |
| ng | Nanogram |
| nM | Nanomolar |
| nm | Nanometres |
| NNRTI | Non-nucleoside reverse transcriptase inhibitor |
| NRTI | Nucleoside reverse transcriptase inhibitor |

| | |
|---|---|
| NYCIII | New York City III |
| ºC | Degree Celsius |
| OAT-1 | Organic anion transporter-1 |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PEI | Polyethyleneimine |
| *Pol* | Polymerase |
| PrEP | Pre-exposure prophylaxis |
| PSV | Pseudovirus |
| QTOF | Triple quadrupole time-of-flight instrument |
| RCF | Relative centrifugal force |
| RLU | Relative luminescence units |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| rRNA | Ribosomal RNA |
| RT | Reverse transcriptase |
| qPCR | Qualitative polymerase chain reaction |
| SDS | Sodium dodecyl sulphate |
| STI | Sexually transmitted infection |
| t0 | Time point 0 |
| t24 | Time point 24 |
| TAE | Tris acetate EDTA |
| TAF | Tenofovir alafenamide |
| $TCID_{50}$ | Median tissue culture infectious dose |
| TDF | Tenofovir disoproxil fumarate |
| TNF-α | Tumour necrosis factor-alpha |
| TNF-β | Tumour necrosis factor-beta |
| Tris | Tris(hydroxymethyl)aminomethane |
| TFV | Tenofovir |
| µg | Microgram |
| µL | Microlitre |
| µM | Micromolar |

| | |
|---|---|
| UV | Ultraviolet |
| V | Volts |
| VLY | Vaginolysin |
| WHO | World Health Organization |

## Abstract

Infection with human immunodeficiency virus type 1 (HIV-1) remains one of the leading causes of death worldwide with young women in sub-Saharan Africa the most affected. Although pre-exposure prophylaxis (PrEP) with antiretroviral (ARV) drugs such as tenofovir (TFV) and emtricitabine have been shown to protect men who have sex with men (MSM) from HIV infection, PrEP trials have not been as successful for women. Although this disparity is most likely multifactorial, studies have shown an association between decreased drug efficacy and increased diversity of the female genital tract (FGT) microbiome. The shift from a homogenous *Lactobacillus* spp. dominated population to one defined by bacterial vaginosis (BV) associated bacteria such as *Gardnerella* spp. not only increased the risk of HIV acquisition but also reduced the ability of ARVs to protect against infection. Additionally, *Lactobacillus crispatus* (*L. crispatus*) was used in this study because it has previously shown to suppress the growth of *Gardnerella vaginalis* (*G. vaginalis*) in a porcine vaginal mucosa model. Recently, it was confirmed that metabolism of TFV by *G. vaginalis* reduced the extracellular level of ARV available to inhibit infection. We hypothesised that *G. vaginalis* not only internalises/metabolises TFV but also other ARVs and that the presence of *L. crispatus* could negate this effect. The main objective of this study was to investigate if exposure of TFV, tenofovir disoproxil fumarate (TDF), maraviroc (MVC) and abacavir (ABC) to *G. vaginalis* reduced extracellular ARV drug concentrations to a level unable to inhibit HIV infection and to determine whether *L. crispatus* was able to counteract this effect.

*G. vaginalis* and *L. crispatus* were grown for 24 hours (t24) in the presence and absence of drugs and cells were lysed and the culture medium harvested before (t0) and after incubation. Using mass spectrometry, we found that *G. vaginalis* and *L. crispatus* took up not only TFV but also ABC and MVC. However, this uptake did not correspond to a significant decrease in extracellular ARV drugs. Extracellular and intracellular TFV, TDF, and MVC samples were added to the reporter cell line, TZM-bl, during infection with infectious molecular clones and pseudovirus infection. Intracellular TDF, TFV, and MVC at t24 had no impact on HIV infectivity, suggesting that the concentration of ARVs internalised by the bacteria was not sufficient to inhibit infection to detectable levels.

At t0, extracellular ARVs inhibited infection by approximately 40% whereas at t24, inhibition decreased to approximately 20%. Therefore, incubation with *G. vaginalis* consistently reduced drug efficacy 2-fold, irrespective of the ARV, although this change was not statistically significant. Although *L. crispatus* had a similar effect as *G. vaginalis* on TFV and TDF

efficacy, extracellular MVC present after incubation with *L. crispatus* inhibited infection by 50%, similar to t0. Therefore, *L. crispatus,* despite internalising MVC, had no effect on the drug's efficacy.

Residual extracellular TDF and TFV at t24 obtained from co-culture samples also lowered inhibition 2-fold, indicating that *L. crispatus* did not impact the effect of *G. vaginalis* culture had on drug efficacy. However, extracellular MVC present after co-culture, inhibited infection by 50%, similar to t0. Therefore, *L. crispatus,* was able to abrogate the effect of *G. vaginalis* on MVC efficacy. We showed that *G. vaginalis* not only internalised MVC, an entry inhibitor, but reduced its efficacy and this effect was lost in the presence of *L. crispatus*. This finding was contrary to what was observed for TFV and TDF, suggesting that the effect of *G. vaginalis* on ARV efficacy was dependent on the type of drug.

These findings confirm the importance of the FGT microbiome in modulating the efficacy of ARV PrEP. Therefore, PrEP formulations with TFV, TDF, and ABC might not be as protective for women with BV compared to those that are BV-negative. Therefore, it is recommended that individuals at high risk of acquiring HIV should be regularly screened for BV.

# Chapter I

## Literature review

### 1.1 Introduction

The prevalence of human immunodeficiency virus type 1 (HIV-1) is high among young women in South Africa. Despite widespread awareness and global effort to lower the numbers of newly acquired HIV infections, HIV remains a global public health issue. According to the World Health Organization (WHO), it is estimated that 38.4 million people currently live with HIV, and a total of 40.1 million people have succumbed to the disease. Many of the new cases of HIV infections occur in African countries, more specifically, sub-Saharan African countries [1, 2]. Young women have a much higher HIV prevalence than men the same age in some areas of South Africa. Prevalence is strikingly high in young pregnant women in rural areas, where it is difficult to gain access to prevention strategies or treatment [3]. In addition, non-inflammatory and non-ulcerative bacterial vaginosis (BV) affects up to 55% of women in sub-Saharan Africa and is associated with sexually transmitted infections (STIs) as well as pregnancy complications. BV has also been linked to increased risk of HIV infection, although some studies did not support this finding [4, 5]. A recent trial using Tenofovir (TFV) - containing microbicides indicated that application before and after exposure reduced HIV infection by 39%. However, when they determined whether this protection was influenced by genital tract microbiota, they found that those that carried BV-associated bacteria (BVAB) were less protected than those women whose microbiome was dominated by *Lactobacillus* spp. Lactobacilli have been associated with “healthy" female genital tracts (FGTs) and the loss of these bacteria is concomitant with the outgrowth of BVAB. The presence of BVAB lowered the concentration of TFV in the FGT potentially by uptake of the drug [6]. By understanding how the composition of the genital microbiome affects antiretroviral (ARV) efficacy, one might better understand the risk of HIV acquisition in people that use microbicides or pre-exposure prophylaxis (PrEP) as a preventative measure. Ultimately, this could inform whether screening for specific BVAB and/or BV treatment before the use of microbicides and PrEP could be beneficial in preventing HIV infection by ensuring the efficacy of ARVs.

### 1.2 HIV Replication

HIV-1 is an RNA virus that is comprised of three structural genes, *Env*, *Gag*, and *Pol*. Envelope (*Env*) gene encodes the gp160 polyprotein which is then cleaved into glycoproteins gp120 and

gp41. These glycoproteins interact with cell surface receptors which facilitate membrane fusion between the virus and host membrane. The group-specific antigen (*Gag*) gene encodes structural proteins, namely, the capsid protein (p24), Gag protein matrix (p17), nucleocapsid (p7), and p6. Finally, the polymerase (*Pol*) gene encodes enzymes that are essential for viral replication, the reverse transcriptase (RT) and integrase (IN) [7].

The first step in HIV-1 infection is attachment of gp120 to the $CD4^+$ receptor and co-receptors present on the host cell surface of helper T-cells. This leads to the fusion of the viral and host plasma membranes. Once the membranes are fused, the viral core and genome are released into the host cytoplasm. Following entry, the viral RNA is reverse transcribed by the RT into double-stranded DNA. This pro-viral DNA is transported into the nucleus and incorporated into the host genome by IN. Structural, accessory, and regulatory viral proteins are synthesized from the pro-viral DNA and assembled to create new viral particles. New virions containing all the necessary proteins bud off from the host cell plasma membrane. Finally, the virus undergoes further processing which leads to the maturation of the viral particle [7].

### 1.3. HIV treatment

There is a wide range of ARVs currently available to treat HIV infection. Antiretroviral therapy (ART), although not a cure, suppresses viral replication in infected individuals, ensuring prolonged life. To date, the WHO estimates that 73% of HIV infected people are receiving ART, leaving just over a fourth of the global infected population still needing treatment. However, in order for the treatment to be successful, it is imperative that the infected individuals adhere to treatment, and dosage [8]. HIV treatment is usually lifelong, and ARVs, when taken consistently and correctly, are used in combination help control HIV infection and prevent disease progression (AIDS). Proper adherence to ARV treatment supresses viral replication to undetectable levels, allowing individuals to live with HIV and reduces the risk of transmitting the virus to other people [8]. The ARV combination is tailored to the individual's specific needs and is often guided by factors such as side effects, drug resistance, and adherence to the treatment regime.

#### 1.3.1 Antiretroviral Pharmacokinetics

ARVs have different mechanisms of action and are classified within groups based on which step of the viral replication cycle they inhibit. However, even though they fall within the same

class of ARV, they have different uptake and processing mechanisms (Table 1.1). The non-nucleoside RT inhibitors (NNRTIs) such as nevirapine, dapivirine (DAP), and efavirenz (EFA) bind reversibly and non-competitively to RT and is not dependent on processing to an active form within the host cell. DAP moves freely across the plasma membrane and has a relatively short half-life [9]. It has been suggested that EFA is taken up by cells by both facilitated transport and passive diffusion although transporters have not been identified [10]. On the other hand, nucleoside RT inhibitors (NRTIs) such as abacavir (ABC), lamiduvine, tenofovir (TFV) and tenofovir disoproxil fumarate (TDF) inhibit the activity of RT by competing with nucleosides and preventing chain elongation. ABC is a small, lipophilic molecule that is internalised by non-facilitated diffusion. Once it crosses the lipid bilayer of the cell membrane of the host cell, it undergoes a series of phosphorylation steps and gets converted to carbovir triphosphate, an analogue of deoxyguanosine-5'-triposphate (dGTP), which lacks the 3'hydroxyl (3'-OH) group [11]. Thus, incorporation of carbovir triphosphate causes DNA chain termination.

TFV is a South African first line prevention ARV and is thus one of the most often used drug to treat HIV infection [12]. TFV is actively taken up by host cells and is phosphorylated to form TFV diphosphate which has a long half-life and competes with deoxyadenosine 5'-triphosphate (dATP) causing premature termination of DNA strand elongation [13]. It has been suggested that the transporters responsible for TFV uptake are organic anion transporters (OAT) 1 and 3. However, due to the absence of OAT-1 and -2 in vaginal cells and because uptake was reduced by an inhibitor of endocytic membrane transport, it is more likely that TFV is taken up by energy dependent endocytosis [14-16].

TDF, is a prodrug of TFV and after uptake, it is hydrolysed first to monophosphate TFV by carboxylesterases, then by phosphodiesterases to form TFV and finally converted to TFV diphosphate which also competes with dATP [14, 17]. However, contrary to TFV it is passively taken up by the cell because TDF has two fumarate molecules which mask the phosphate of TFV and allows it to cross the plasma membrane by passive diffusion [18]. If TDF is not metabolised to TFV-diphosphate it diffuses out of cells and is hydrolysed to TFV within the vaginal fluid. The mode of uptake could impact its activation as TDF has been shown to have 40-fold higher levels of active metabolite than TFV. Another study suggested that variation in transport mechanisms affected efficacy: TFV inhibited HIV-1 and herpes simplex virus (HSV) at 100-times higher concentrations than TDF[19, 20]. However, it has not consistently been

shown that TDF is more efficacious than TFV although this could be due to varying adherence and virological factors across clinical trials [4, 5, 21-23].

In addition to NRTIs there are also entry inhibitors, such as maraviroc (MVC), Tak779 and enfuvirtide (T20). Entry inhibitors block viral entry into the host cell and thus have the advantage of preventing infection unlike NNRTIs and NRTIs that prevent HIV replication after entry. MVC binds to the chemokine receptor, CCR5, and stops the viral particles from infecting human cells [24].

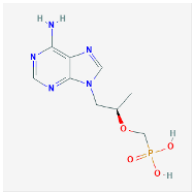
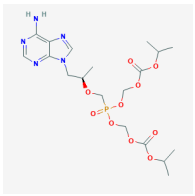
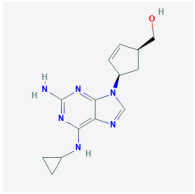
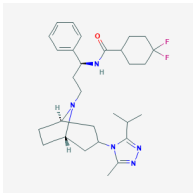
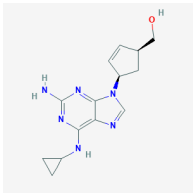
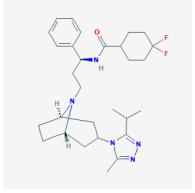
It is important to note that ARVs are not only used for treatment but are now also used to prevent HIV infection. This is known as pre-exposure prophylaxis (PrEP).

### 1.4 HIV prevention

In the absence of a licensed HIV vaccine, male and female condoms are the most easily accessible and reliable form of protection against HIV-1 infection [25]. As antiretroviral drugs are approved to be used as PrEP as a preventative measure to lower the risk of acquiring HIV-1, the Southern African HIV Clinicians Society published the first African guideline for PrEP in 2012. High risk individuals, such as discordant couples or sex workers, are now able to take Truvada® (emtricitabine and TDF) or Descovy® (emtricitabine and tenofovir alafenamide) pills, which are two safe and effective medications approved for use as PrEP by the Centre of Disease and Control (CDC). PrEP has demonstrated to be efficacious against HIV infection, although different studies have shown varying degrees of efficacy. The iPrEx and ANRS IPERGAY randomized studies showed that taking daily oral doses of 300mg Tenofovir/200mg Emtricitabine (TDF-FTC) conferred 44% and 42%, respectively, protection against HIV infection among men who have sex with men (MSM) [26]. Additionally, the TDF2 study carried out in Botswana demonstrated that HIV infection in sexually active heterosexual subjects prevented HIV infection when TDF-FTC prophylaxis was taken daily. Furthermore, the Partners PrEP study also showed 75% effectiveness against HIV infection in heterosexual serodiscordant couples [27]. However, both FEM-PrEP and the Vaginal and Oral Interventions to Control the Epidemic (VOICE) trials showed conflicting results. In these studies, PrEP with oral TDF-FTC did not reduce the incidence of HIV infection among African women [5, 22]. However, the results obtained from the VOICE study could be attributed to low adherence to the prescribed gel application [5]. To circumvent potential variation due to oral delivery, there

has been an increase in microbicide research to prevent HIV transmission in women [5, 6, 21]. However, microbicide trials have also yielded contradicting outcomes [28].

**Table 1.1:** Antiretroviral drugs showing the respective mode of action, structure, uptake mechanism, and function.

| ARV | Mode of action | Structure | Uptake mechanism | Function |
|---|---|---|---|---|
| TFV | Nucleoside RT inhibitor, adenosine analogue |  | Active transport | Chain terminator of DNA synthesis |
| TDF | Nucleoside RT inhibitor, adenosine analogue |  | Passive diffusion | Chain terminator of DNA synthesis |
| ABC | Nucleoside RT inhibitor, guanosine analogue |  | Passive diffusion | Chain terminator of DNA synthesis |
| MVC | Entry and fusion inhibitor |  | Not applicable | Blocks CCR5 chemokine receptor |
| DAP | Non-Nucleoside RT inhibitor |  | Passive diffusion | Binds to RT allosterically and inhibits RT |
| EFA | Non-Nucleoside RT inhibitor |  | Active and passive transport | Binds to RT allosterically and inhibits RT |

*TFV: Tenofovir, TDF: Tenofovir disoproxil fumarate, ABC: Abacavir, MVC: Maraviroc, DAP: Dapivirine, EFA: Efavirenz, CCR5: C-C chemokine receptor type 5, RT: Reverse transcriptase, DNA: Deoxyribonucleic acid.

### 1.5 PrEP: Microbicides

Microbicides are topical PrEP, and their role is to prevent sexually transmitted infections or HIV transmission by releasing ARVs within the FGT or rectal region. Different types of microbicides are currently being developed, but it is important to note that presently there is no effective microbicide on the market [29]. They exist in the form of gels, films, creams, vaginal rings, inserts, and suppositories [29]. Ensuring effective drug delivery to specific tissues and cells is essential to thwart HIV transmission. Various studies have played an important role in pinpointing the factors that could influence the effectiveness of these microbicides [30]. In 2010, the Centre for the AIDS Program of Research in South Africa (CAPRISA) 004 study demonstrated for the first time that a vaginal gel containing 1% TFV decreased the number of new HIV infections by 54% in women that adhered to the protocol [21]. Subsequently, a larger study called The Follow-on African Consortium for Tenofovir

**Table 1.2: PrEP Trials.** Summary of main findings.

| Study | Cohort | Intervention | Finding | Reference |
|---|---|---|---|---|
| CAPRISA 004 | South Africa | 1% TFV gel | Lowered the risk of HIV-1 infection by 54% | Abdool Karim Q., *et al*., 2010 |
| iPrEx | Brazil, Ecuador, Peru, South Africa, Thailand and United States | Oral TDF/FTC | Lowered the risk of HIV-1 infection by 27% | Grant R. M., *et al*., 2010 |
| TDF2 | Botswana | Oral TDF or TDF/FTC | Lowered the risk of HIV-1 infection by 44% | Thigpen M. C., *et al*., 2012 |
| FEM-PrEP | Kenya, South Africa and Tanzania | Oral TDF/FTC | Did not lower the risk of HIV-1 infection | Van Damme L., *et al*., 2012 |

| | | | | |
|---|---|---|---|---|
| Partners PrEP | Kenya and Uganda | Oral TDF or TDF/FTC | Lowered the risk of HIV-1 infection by 67% and 75%, respectively | Beaten J. M., *et al*., 2012 |
| VOICE | South Africa, Uganda and Zimbabwe | Oral TDF, TDF/FTC or 1% TFV gel | Did not lower the risk of HIV-1 infection, −49.0% with TDF, −4.4% with TDF-FTC and 14.5% with TFV gel | Marrazzo J. M., *et al*., 2015 |
| ANSR IPERGAY | Canada and France | Oral TDF/FTC | Lowered the risk of HIV-1 infection by 86% | Molina J. M., *et al*., 2015 |
| MTN-020-ASPIRE | Malawi, South Africa, Uganda and Zimbabwe | Dapivirine | Lowered the risk of HIV-1 infection by 27% | Beaten J. M, *et al*., 2016 |
| THE RING | South Africa and Uganda | Dapivirine | Lowered the risk of HIV-1 infection by 31% | Nel A., *et al*., 2016 |
| FACTS 001 | South Africa | 1% TFV gel | Did not lower the risk of HIV-1 infection | Delany-Moretlwe S., *et al*., 2018 |
| DREAM | South Africa and Uganda | Dapivirine | Lowered the risk of HIV-1 infection by 63% | Nel A., *et al*., 2021 |
| CHARM-03 | United States | Oral MVC and 1% MVC gel | Did not lower the risk of HIV-1 infection | McGowan I. M., *et al*., 2022 |

Studies (FACTS 001) was performed in order to validate the results obtained by the CAPRISA

004 study. In this study, the same 1% TFV vaginal gel was used, the trial was unable to replicate the results from the earlier study. This suggested that the topical gel was unable to mitigate the risk of women from acquiring HIV-1. In addition, the VOICE study carried out in South Africa, Uganda and Zimbabwe, which included both oral and microbicidal delivery systems of TDF, TDF-emtricitabine and 1% TFV vaginal gel, also showed contradicting results [5]. Additionally, the Microbicide Trial Network 020 – Randomised, double-blind, placebo-controlled study to Prevent Infection with a Ring for Extended Use (MTN-020-ASPIRE) demonstrated that a vaginal ring that contained DAP reduced the incidence of HIV infection by 27% among women in Malawi, South Africa, Uganda and Zimbabwe [31]. A similar study, The Ring study, showed that the DAP ring lowered the risk of becoming infected with HIV-1 by 31% [32]. In 2019, participants in the Ring study were enrolled into the open label DREAM study at five South African sites with the aim of determining safety, adherence and efficacy of the DAP ring. Compared to the placebo group of the Ring study, HIV incidence was reduced by 63% although the difference in timing of the Ring and DREAM studies was a possible caveat of the trial [33]. In 2021 South Africa approved the use of a DAP ring as PrEP.

It has been suggested that MVC might be a valuable addition to PrEP microbicide formulations given that its half-life in cervicovaginal fluid far exceeded that of its plasma concentrations after an oral dose [34]. A recent Phase 1 study (CHARM-03) in men showed that 1% MVC gel was not a potential candidate for PrEP [35].

Variability in clinical trial results have been widely attributed to drug adherence and inadequate drug concentrations in the FGT and virological characteristics [36]. In 2017 it was shown that the efficacy of the TFV-containing PrEP was much higher when the FGT microbiome was dominated by *Lactobacillus* spp [6]. Thus far, the association between FGT microbiome has been limited to clinical trials with TFV, and given the variation in pharmacokinetics of drugs, it is important to study whether the FGT microbiome differentially impacts the efficacy of ARVs from other classes if included in microbicide formulations.

### 1.6 Pharmacomicrobiomics

The human microbiome is a complex array of microbes that consists of bacteria, viruses, bacteriophages, fungi, and protozoa. It is estimated that the human microbiome consists of 10-100 trillion microorganism and the majority of these are present in the gastrointestinal tract

[37]. The impact of these microorganism on the host health has been widely studied. Changes in the microbiome have been associated with diabetes, cancers, and HIV-1 infection, and now ARV efficacy [6, 38-41].

Multiple mechanisms have been suggested as explanations for the relationship between FGT microflora and the efficacy of ART in the FGT. Microorganisms present in the FGT can affect drug pharmacodynamics and pharmacokinetics by decreasing ARV uptake by host cells, secreting enzymes that metabolise the drugs, producing effectors of the host cell proteome and production of metabolites that competitively inhibit its action [42]. It has been reported that *G. vaginalis* and other bacteria increase the pH of the FGT and produce adenine which inhibits TFV endocytosis [42]. On the other hand, DAP which is taken up by passive diffusion was rendered unavailable to the host by binding irreversibly to *Gardnerella* species [43]. These findings suggest that the mechanism by which the FGT microbiome affects ARV efficacy is highly complex.

## 1.7 Female genital tract microbiota

### 1.7.1 *Lactobacillus* spp. associated with "healthy" female genital tracts

The FGT microbiome plays an important role in reproductive health and confers protection against a number of pathogens that can result in disease, including STIs and HIV-1 [44, 45]. As previously reported, "healthy" FGTs are associated with a simple *Lactobacillus* dominant microbiota [46-50]. Lactobacilli are gram-positive rods that produce antimicrobial agents, namely hydrogen peroxide, acidolin, and lactacin B [51]. Multiple studies were able to successfully isolate *Lactobacillus* spp. from healthy women in North America, Japan, and South Africa [52, 53]. More particularly, a BV negative diagnosis is strongly associated with the presence of *Lactobacillus crispatus* [49, 54, 55]. A study showed that the FGT pH is linked to the composition of the microbiome [51]. Lower pH correlates to a higher *Lactobacillus* spp. count compared to high pH [56]. A "healthy" FGT has a low pH, normal vaginal discharge, negative whiff test, and lacks clue cells, which are vaginal epithelial cells that have fuzzy borders which indicate the presence of BV [49]. *Lactobacillus* spp. are believed to have a virucidal effect, against HIV, by producing hydrogen peroxide ($H_2O_2$) and lactic acid [57]. *In vitro* studies have shown that $H_2O_2$ and lactic acid inhibit the growth of *G. vaginalis*, *Prevotella bivia*, and *Mobiluncus* spp. Thus, suggesting that lowering of the pH in the vaginal tract confers

protection to the host by making the vagina inhospitable to pathogenic microorganisms [58-60]. In addition, the cervicovaginal microbiome of various asymptomatic women in North America was characterized into five main groups, namely *L. crispatus*, *Lactobacillus gasseri*, *Lactobacillus jensenii*, *Lactobacillus iners*, and a fifth polymicrobial bacterial community consisting of various strictly anaerobic bacteria. The fifth bacterial group consisted of *Atopobium*, *Dialister*, *Gardnerella*, *Megasphaera, Peptoniphilus,* and *Prevotella*, which are bacterial species highly associated with BV. Furthermore, Borgdorff *et al.* (2014) suggested that a microbial shift from a *Lactobacillus* predominant microbiota to a BVAB biome subsequently results in less protection against diseases in African women [50]. Interestingly, a bacterium-specific polymerase chain reaction (PCR) assay was also unable to detect *L. crispatus* in individuals with BV [46]. These data is supported by a study done in South Africa which showed that women with a highly diverse population of anaerobic bacteria in their cervicovaginal region, instead of a *Lactobacillus* dominant population, were at a higher risk of acquiring HIV-1[61]. These results highlight the need to understand the importance of beneficial *Lactobacillus* spp., such as *L. crispatus*, in the cervicovaginal microbiota.

### 1.7.2 Bacterial Vaginosis

BV is a common genital tract condition that affects a large proportion of women and has been associated with several health problems, such as pelvic inflammatory disease, preterm birth complications, acquisition and transmission of various STIs including HIV [62-64]. BV is not considered an STI, as it results from an overgrowth of facultative and highly anaerobic bacteria and a decrease in Lactobacilli and there remains controversy surrounding its classification as a non-inflammatory or inflammatory condition [65]. During BV, the vaginal microbiome shifts from being predominantly colonized by Lactobacilli spp. to a more diverse, anaerobe-dominated community [66]. This condition can be clinically diagnosed using the Amsel clinical criteria defined by the presence of a fishy odour after the addition of 10% potassium hydroxide, having an elevated vaginal pH (>4.5), thin discharge and the presence of clue cells (squamous epithelial cells covered by Gram negative rods), in which three out of four criteria must be met in order to make a positive diagnosis [67]. However, medical diagnosis can be challenging as the clinical symptoms described can be ambiguous and thus can be easily missed by the clinician performing the test. Thus, Nugent *et al.* (1991) developed an effective standardized test to evaluate the vaginal flora of women. The Nugent scoring system is based on Gram staining of vaginal smear samples. In brief, it assesses the amount of three bacterial

morphotypes present in vaginal fluid. The three morphotypes are *Lactobacillus* (large gram-positive rods), *Gardnerella/Bacteroides*, (small Gram-variable rods), and *Mobiluncus* (curved gram-variable rods). The scoring criteria (0 to 10) calculates the weighted sum of three bacterial morphotypes and it categorizes the vaginal flora as 'normal', intermediate, or bacterial vaginosis. A low score of less than 3 is consistent with a healthy *Lactobacillus*-dominant flora, a 4 – 6 score suggests the vaginal flora is altered and a score of 7 or higher is considered indicative of bacterial vaginosis [68].

Fluctuation in the FGT microbiome during menstruation, intercourse, smoking, or changes in response to high number of sexual partners, and intravaginal practices (douching, finger-cleansing, wiping, or inserting traditional substances) can alter the composition of the vaginal microbiome, increasing the risk of BV [47, 48, 64, 69, 70]. A cross sectional study done in Zambia revealed that women that practiced vaginal cleansing practices had a higher incidence of BV [70]. It is also important to note that women's menstrual cycle constantly affect the makeup of the vaginal microflora. Different types of microorganisms are present at different times in the menstrual cycle, which could result in the misdiagnosis of BV [47, 69]. For instance, *G. vaginalis* levels increased during the menstrual period of healthy women and a systematic review of various studies showed that hormonal contraception decreased the risk of BV [47, 71]. Additionally, studies have shown that menstruation and changes in oestrogen levels have an effect on bacterial diversity present in women during the course of their lives [48, 72-74]. All in all, greater focus should be placed on promoting an optimal FGT microbial community to maximise protection against pathogens.

### 1.7.3 Bacterial Vaginosis-Associated Bacteria (BVAB)

Several groups have shown that BV-positive women have greater bacterial diversity with a paucity of *Lactobacillus* spp. compared to healthy controls [46, 49]. Bacteria strongly associated with BV include *G. vaginalis*, *Prevotella* spp., *M. hominis*, *Atopobium*, *Megasphaera*, *Mobiluncus* sp., and *Clostridales* [46, 54]. Treatment for this condition includes the use of antibiotics, namely metronidazole and clindamycin, although a substantial amount of individuals experience relapse shortly after treatment is complete [75]. The aetiology of BV remains unknown although in 1955, incidence of BV was linked to the presence of the facultative, gram variable anaerobic bacteria, *G. vaginalis* [76]. However, *G. vaginalis* was also detected in individuals that were not clinically diagnosed with BV, suggesting that it might

not be the aetiological agent of BV. It has recently been shown that *Gardnerella* spp. genetic diversity is highly complex which has led to an alternative suggestion that not all *Gardnerella* spp. cause BV but that only some virulent strains are linked with BV [77].

Ahmed *et al*. (2012) and Vaneechoutte *et al*. (2019) identified four distinct species of *Gardnerella* namely: *G. vaginalis, Gardnerella piotii, Gardnerella leopoldii* and *Gardnerella swidsinskii* [78, 79]. Women positive for BV had FGT microbiota dominated by *G. vaginalis* and *G. piotii* and not *G. leopoldii and G. swidsinskii*, suggesting that only the former might be involved in FGT dysbiosis [80]. However, when each species was characterized according to their virulence potential, defined as sialidase activity, production of the toxin vaginolysin (VLY) and ability to form biofilm, *G. vaginalis, G. piotii* and *G. swidsinskii* had similar VLY expression and biofilm forming ability [79, 80]. However, only *G. vaginalis and G. piotii* strains had sialidase activity with sialidase activity associated with most *G. piotii* strains [79].

Sialidase releases sialic acid residues from glycoproteins of the mucus membrane, potentially disrupting the integrity of the protective mucus layer and facilitating the adherence of *Gardnerella* spp. and secondary colonizers to the vaginal epithelium [81-83]. Sialidases are also known to reduce the viscosity of FGT mucus by degrading mucin allowing pathogens to gain access to the epithelium more easily [84].

VLY is a pore-forming cytolysin that results in the lysis of human erythrocytes and vaginal epithelial cell facilitates *G. vaginalis* adherence to the vaginal wall, which promotes biofilm formation.[85-87] The role of VLY in BV is unclear, however it could play a similar role as sialidase and disrupt the epithelial barrier and/or impact the host immune response [88]. Both sialidase and VLY have been implicated in the formation of biofilm which forms a protective environment for BVAB, allowing it to survive within the FGT [89-91]. *Gardnerella* spp. are thought to be the primary coloniser of the FGT epithelium facilitating the attachment of *P. bivia, Mycoplasma hominis, Staphylococcus hominis, Breveibacterium mcbrellneri* and, *Enerococcus faecalis* leading to the pathology of BV by forming the biofilm [90, 91]. These virulence factors could also aid HIV infection although no clear relationship has been established.

### 1.7.4 Bacterial Vaginosis associated with increased HIV infection

There is increasingly more data available that suggests BV could increase susceptibility to HIV-1 infection. Multiple observational studies have shed light on the possible association

between BV and HIV-1 transmission, where HIV seropositive women had BV positive flora [92]. A longitudinal study performed in Malawi by Taha *et al.* (1998) suggested that high bacterial diversity in the cervicovaginal microbiome was associated with increased rate of HIV acquisition in pregnant and post-natal women [93]. A prospective cohort study performed in Kenya, looked at the relationship between BV, Lactobacilli and HIV-1 infection and reported that the presence of a non-optimal vaginal flora, lacking Lactobacilli, was linked to increased risk of HIV-1 infection [94]. Furthermore, a significant association was found between the concentration of specific vaginal bacteria (*Parvimonas* spp., *G. asaccharolytica*, *M. hominis*, *Snethia* spp., *Eggerthella* spp., and *Megasphaera* spp.) and risk of HIV-1 acquisition. Interestingly, *G. vaginalis* showed no significant association with HIV-1 infection even though past studies had indicated an association with HIV-1 transmission [95]. The relationship between HIV acquisition and BV is likely to be multifactorial. The displacement of protective *Lactobacillus* spp. by BVAB could result in increased FGT pH that favours the survival of HIV [93]. Intact mucosal epithelium is a formidable barrier against HIV infection and thus any disruption of this protective layer by BVAB sialidase, VLY and biofilm formation could increase HIV acquisition [96]. *Lactobacillus* spp., on the other hand, might be protective by directly countering the effect of these virulence factors. An *in vitro* study has shown that *L. crispatus* is able to decrease expression of VLY by *G. vaginalis*, thereby reducing cell cytotoxicity [97]. Currently, the mechanism remains unknown.

### 1.8 Inflammation of the FGT increases risk of HIV infection

Inflammation has been linked to increased risk of HIV infection in women, although the exact biological mechanism behind this phenomenon still remains to be elucidated. It is possible that BV indirectly increases HIV acquisition by inducing inflammatory responses in the FGT [98]. Women with FGT inflammation have been shown to have microbial proteins predominantly from BVAB such as *G. vaginalis*, *Prevotella* spp., *Megasphera* spp., and *A. vaginae* and that inflammation was linked to an increase in abundance of anaerobic bacteria, namely *G. vaginalis* and *P. bivia* [99].

BV is classified as a non-inflammatory disorder and yet the presence of VLY in the FGT resulted in an increase in interleukin-8 (IL-8) production, a neutrophil chemotactic, suggesting that VLY may play a role in FGT inflammation [86]. Both IL-1β and IL-8 are proinflammatory cytokines indicating that during inflammation, levels of both cytokines should be raised.

However, Cauci *et al*., (2003) showed that the FGT of women with and without BV had similar levels of IL-8 but that women with BV had significantly greater concentrations of the proinflammatory interleukin-1β (IL-1β) compared to healthy controls [100]. These findings could suggest that BV does not affect IL-1β levels but could inhibit IL-8 which prevents the influx of neutrophils, explaining why asymptomatic women show no sign of genital inflammation. Findings by Thurman *et al*., (2015) confirmed that cervicovaginal lavage of BV positive women had significantly higher levels of IL-1β, as well as tumour necrosis factor-alpha (TNF-α), and intercellular adhesion molecule-1 (ICAM-1) [101]. Masson *et al*., (2015) also reported that interleukin-1α (IL-1α), IL-1β, and tumour necrosis factor-β (TNF-β) were all significantly elevated and chemokines were downregulated in BV-positive women compared to healthy controls [102]. As increased levels of IL-8 were highly associated with increased anti-HIV-1 activity in cervicovaginal lavages, by BV lowering IL-8 levels, it would increase the chance of HIV infection. Therefore, BV could disrupt the "normal" inflammatory response but still maintain key features that facilitate HIV infection such as causing damage to the FGT mucosa, resulting in the influx of $CD4^+$ T cells, the targets for HIV-1 entry and infection [98, 103].

Alternatively, BVAB might not directly stimulate inflammation, but by mere displacement of *Lactobacillus* spp. disrupt "normal, healthy" anti-inflammatory responses. As opposed to laboratory strains, some clinical *Lactobacillus* spp. isolated from South African women were able to counter the inflammatory response elicited by *G. vaginalis*, suggesting that some species are better able to modulate FGT immune responses. In fact, it has been suggested that probiotics should comprise of clinical samples of *Lactobacillus* spp. such as *L. jensenii, L. crispatus, and L. mucosae*, because of their potential anti-inflammatory properties [104]. Recently, Manhanzva *et al*. (2020) supported this finding by comparing *Lactobacillus* spp. isolated from BV-positive and BV-negative women and found that isolates from BV positive women tended to be pro-inflammatory whereas those from BV-negative women, such as *L. jensenii* reduced *G. vaginalis* associated inflammation *in vitro*. The authors reported that FGT inflammation was due to *Lactobacillus* biological processes such as lactic acid production, and not their relative abundance, and suggested that probiotic pro-inflammatory characteristics should be evaluated [105]. The study also reported that certain lactobacilli isolates, from women that possessed a non-optimal microbiota, produced significantly lower levels of D-lactate [105]. These findings are important and could explain why the presence of lactobacilli species alone is not enough to not confer protection against HIV.

## 1.9 Mechanisms of how BVAB could decrease drug efficacy

### 1.9.1 BVAB internalises ARVs in the FGT

Multiple factors affect the ability of ARVs to control viral replication including viral load at the time of treatment commencement; adherence, presence of drug resistant mutations, and the FGT microbiome [8, 106]. The FGT microbiome might affect the efficacy of TFV-containing microbicides via several different mechanisms: lowering ARV uptake, the secretion of hydrolysing enzymes, production of inhibitors or activators of host cell processes that interfere with intracellular ARV processing and increasing the pH of the FGT which inhibits TFV endocytosis [42]. Furthermore, it has been shown that FGT inflammatory responses impact the efficacy of TFV, suggesting that the immune responses to BVAB could reduce ARV efficacy and not the bacteria itself [107].

*In vitro* studies have shown that a *G vaginalis* strain, lowered the concentration of extracellular TFV by uptake of the drug [6]. It was suggested that TFV is taken up by endocytosis [14]. However, bacteria are thought to take up small molecules or peptides passively, because most are considered unable to undergo endocytosis [14, 16, 108]. However, Cheu *et al.,* (2020) recently showed that inhibitors of endocytosis prevented the uptake of TFV by both Jurkat cells and *G. vaginalis*, suggesting a similar mechanism for both bacteria and eukaryotic cells [109]. The authors also showed that cervical lavages with high levels *Lactobacillus* spp. increased uptake of TFV and DAP by Jurkat cells compared to lavages with lower numbers of *Lactobacillus* spp. whereas *in vitro* co-culture with *G vaginalis* decreased uptake. Interestingly, these changes in uptake corresponded to differences in HIV infectivity with single culture of *G. vaginalis* and co-culture of *G. vaginalis* and *L. crispatus* resulting in an increase and decrease of HIV infected cells, respectively. This effect was not significant when tenofovir alafenamide (TAF) was used. Overall, the authors report that *G vaginalis* reduced uptake and metabolism of TFV and DAP resulting in higher infection rates and that *L. crispatus* was able to somewhat counter this effect [109].

Therefore, one of the mechanisms by which BVAB might lower the efficacy of ARVs is by reducing the levels of drug taken up by host cells, diminishing its inhibitory effect on HIV infection [6, 109]. The authors suggest that ARV efficacy is not only affected by the type of FGT microbial populations but also the characteristics of the ARV. It is thus important to determine whether other ARVs are similarly affected by FGT microbiota.

## 1.10 Aims and objectives

The aim of this study was to determine whether *G. vaginalis* and *L. crispatus* impact the ability of antiretroviral drugs to inhibit HIV infection *in vitro*.

### 1.10.1 Specific Aims:

1) Determine whether *G. vaginalis* culture reduces the extracellular levels of TFV, TDF, MVC, and ABC so that residual ARV is no longer able to inhibit HIV-1 infection.
2) Determine whether the co-culture of *L. crispatus* abrogates the effect of *G. vaginalis* on ARV efficacy.

# Chapter II

## Materials and Methods

### 2.1 Mammalian cell strains and culture conditions

#### *2.1.1 Cell lines, bacterial strains, and plasmids*

The adherent cell lines, Human Embryonic Kidney 293T, HEK293T, and TZM-bl cells were obtained from the AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institute of Health, from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc [110]. HEK293T cells were used to produce pseudovirus (PSV) and infectious molecular clones (IMCs). TZM-bl cells are modified HeLa cells expressing $CD4^+$, CCR5, and CXCR4 with a luciferase gene under the control of the HIV long terminal repeat (LTR) promoter. Plasmids, namely, the subtype B, X4 provirus, pNL4 was a gift from Professor Carolyn Williamson, University of Cape Town. The non-infectious construct, pSG3Δenv (ARP-11051), was obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH, Human Immunodeficiency Virus Type 1 (HIV-1) contributed by Dr. John C. Kappes, Dr. Xiaoyun Wu. The subtype C, R5 *env* clone used in the pseudovirus assay was generated in our lab [111].

#### *2.1.2 Culture conditions*

Mammalian cell lines were grown and maintained in complete Dulbecco's Modified Eagle's High Glucose Medium (DMEM) (Sigma-Aldrich, Burlington, Massachusetts, United States) with 10% (v/v) fetal bovine serum (Sigma-Aldrich, Burlington, Massachusetts, United States) and 1U/mL penicillin and 1µg/mL Streptomycin (Sigma-Aldrich, Burlington, Massachusetts, United States) at 37°C and 5% $CO_2$ in the Heracell™ VIOS 160i $CO_2$ incubator with Cell Locker™ System (Thermo Fisher Scientific™, Waltham, Massachusetts, United States). Cells were regularly checked under the Nikon TMS inverted microscope (Nikon, Japan) for confluency and for the presence of contamination.

### 2.2 Production of PSV and IMCs

PSV and IMCs are common tools to measure HIV infection *in vitro*. PSVs are non-infectious viral particles due to an inactive *env* gene in the provirus clone, pSG3Δenv [112]. Whereas

IMCs produced using the pNL4_3 provirus clone is infectious and can undergo multiple rounds of infection.

### *2.2.1 Propagation and transformation of plasmids*

Chemically competent *Escherichia coli* JM109 cells (Promega, Madison, Wisconsin, United States) were transformed with 100pg of plasmid DNA and incubated on ice for 25 minutes. After incubation at 42°C for 45 seconds, the cells were transferred to ice for 2 minutes before 1 mL Luria-Bertani (LB) broth (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl in distilled water) was added and incubated at 30°C, with shaking for 1 hour. The culture was centrifuged at 5000rpm for 5 minutes, and 900µl supernatant was removed before the cell pellet was resuspended in the remaining supernatant and spread onto Luria agar (LA) (LB with 1.5% *w/v* agar) plates containing 100µg/mL of carbenicillin disodium salt (Sigma-Aldrich, Burlington, Massachusetts, United States). Plates were incubated at 30°C with shaking overnight. Glycerol stocks were prepared by mixing 500µl of culture with autoclaved 500µl 50% (v/v) glycerol in a cryotube and flash frozen in liquid nitrogen. Stocks were stored at -80 °C.

### *2.2.2 Plasmid DNA extraction and purification*

Glycerol stocks of transformed *Escherichia coli* JM109 cells were inoculated into 5mL of LB broth containing 100µg/mL carbenicillin disodium salt and grown shaking at $30^{O}$C for 16 hours. Starter cultures were transferred to 50mL LB broth containing 100mg/mL carbenicillin disodium salt and grown overnight at $30^{O}$C shaking.

Plasmid DNA was extracted using the Plasmid DNA Purification kit (QIAGEN, Germany) according to the manufacturer's instructions. A NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, United States) was used to determine the plasmid DNA concentration and stored at -$20^{O}$C. The extracted pSG3Δenv plasmid DNA was subjected to an enzyme digest using PvuII (New England Biolab, Ipswich, Massachusetts, United States) enzyme. To confirm integrity of the plasmid DNA, digest products were mixed with 6x loading dye (New England Biolab, Ipswich, Massachusetts, United States). The products were then separated in a 1% (w/v) agarose gel made using 1x TAE buffer (40mM Tris, 1mM EDTA and 20mM (v/v) glacial acetic acid) that contained 0.16µg/mL ethidium bromide (EtBr). A 1kb DNA ladder (Thermo Fisher Scientific™,

Waltham, Massachusetts, United States) was used as a molecular weight marker. Products were electrophoresed in 1x TAE buffer for 40 minutes at 100V. Gel was visualized in the Gel Doc™ XR UV transilluminator (Bio-Rad Laboratories, Hercules, California, United States).

### *2.2.3 Transfection of HEK293T cells*

HEK293T cells were plated in a 6-well plate at final density of $2x10^6$ cells per well in 2mL of complete DMEM that was prepared as described in section 2.1.2. Cells were incubated overnight at 37ºC with 5% $CO_2$. DNA transfection was done when cells reached 40-60% confluency. Each transfection reaction was prepared in a 15mL falcon tube. Plasmid DNA was added, in a ratio 3:1 (PEI:DNA), [(PSV: 2.5µg Env plasmid + 5µg of pSG3Δenv + 22.5µL PEI) and (IMCs: 5µg of pNL4_3 + 22.5µL PEI)], to 400µL of serum-free medium. Reactions were vortexed for 15 seconds and incubated for 10 minutes at room temperature (20-25 °C) to allow for complex formation. The growth media was removed from the 6-well plate and replaced by complete DMEM. Transfection reactions were added in a dropwise manner. Plate was gently swirled to evenly distribute the PEI:DNA complexes. Cells were incubated for 6 hours at 37ºC with 5% $CO_2$. After incubation, media was removed, discarded and 2mL of fresh pre-warmed full DMEM was added to each well. Cells were incubated for 48 hours to allow for HIV PSV/IMC production. The HIV PSV/IMC particles released into the supernatant were harvested by filtering the growth media using 0.22µM filters. PSVs and IMCs were stored at -80ºC in aliquots supplemented with 20% FBS.

### *2.2.4 Median Tissue Culture Infectious Dose ($TCID_{50}$) assay*

TZM-bl cells were seeded in a 96-well plate at a final density of $1x10^4$ cells per well in 200ul of complete DMEM. Cells were incubated overnight at 37ºC with 5% $CO_2$. Media was removed and 100µl of fresh media containing 20.8µg/mL DEAE-Dextran (Sigma-Aldrich, Burlington, Massachusetts, United States) was added to each well. PSVs/IMCs were thawed at room temperature and serially diluted 4-fold 10 times, and 100µL of each viral dilution was added to each well in triplicate. The cells were incubated for 48 hours at 37ºC with 5% $CO_2$. Then, 150µL of media was removed from each well and a total of 50µL BrightGlo™ (Promega, Madison, Wisconsin, United States) lysis reagent was added and left for 2 minutes to allow for lysis. Finally, media was mixed thoroughly and 75µL from each well was transferred to an opaque 96-well plate. Luminescence was read using a Glomax® 96 Microplate Luminometer

(Promega, Madison, Wisconsin, United States). Viral quantitation was calculated using the Reed-Muench method [113].

## 2.3 Determination of half-maximal inhibitory concentration ($IC_{50}$) values

### *2.3.1 Antiretroviral drugs and $IC_{50}$ value determination*

Antiretroviral drugs, namely Maraviroc (Selzentry, ARP-11580), Tenofovir (ARP-10199), Tenofovir disoproxil fumarate (ARP-10198), and Abacavir (Ziagen, ARP-4680) were obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH. The $IC_{50}$ concentrations were determined using the TZM-bl luciferase reporter cell line. TZM-bl cells were seeded in a 96-well plate at a final density of $1x10^4$ cells per well in 200µl of complete DMEM. Cells were incubated overnight at 37°C with 5% $CO_2$. Media was removed and 100µl of fresh media containing DEAE-dextran was added to each well. PSVs/IMCs stocks were thawed at room temperature and diluted to 200 $TCID_{50}$/well. A total of 50µL of virus was added to each well in triplicate. Drugs were serially diluted and added as shown in Table 2.1. The cells were incubated for 48 hours at 37°C with 5% $CO_2$. Then, 150µL of media was removed from each well and a total of 50µL BrightGlo™ (Promega, Madison, Wisconsin, United States) lysis reagent was added and left for 2 minutes to allow for lysis. Finally, media was mixed thoroughly and 75µL from each well was transferred to an opaque 96-well plate. Luminescence was read using a Glomax® 96 Microplate Luminometer (Promega, Madison, Wisconsin, United States). Three biological repeats were done for each drug. The $IC_{50}$ and $IC_{100}$ values were calculated on GraphPad Prism statistical software (version 8, GraphPad Software, La Jolla, California, United States) and averaged. The $IC_{100}$ values determined were used to determine the effect of antiretroviral drugs on HIV infectivity in the presence of *G. vaginalis* culture medium and cell lysates.

**Table 2.1: Antiretroviral dilution used to determine $IC_{50}$ and $IC_{100}$ values.**

| Drug | Stock concentration (nM) | Dilution factor | Number of dilutions |
|---|---|---|---|
| Maraviroc | 4 | 1 in 5 | 8 |
| Tenofovir | 400 | 1 in 5 | 8 |
| Tenofovir disoproxil fumarate | 400 | 1 in 5 | 8 |
| Abacavir | 400 | 1 in 5 | 8 |

## 2.4 Bacterial strains and culture conditions

*Gardnerella vaginalis,* ATCC 14018 (group C), was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, United States). *Lactobacillus crispatus* clinical specimen was kindly donated to us by Dr. Brian Kullin. Bacteria were maintained on complete New York City III (NYCIII) agar (5% bacteriological agar, 0.25% (w/v) HEPES, 1.5% (w/v) protease peptone, 0.5% (w/v) NaCl, 0.4% yeast extract, 0.5% (w/v) glucose supplemented with heat inactivated 10% horse serum) plates according to the manufacturer's instructions. Agar plates and 15mL broth media were supplemented with 10% horse serum (Biowest, France) and incubated under anaerobic conditions (80% $N_2$, 10% $CO_2$, and 10% $H_2$) in a tightly sealed BD GasPak EZ Container (Thomas Scientific, Swedesboro, New Jersey, United States) at 37$^{o}$C for 48 hours. AnaeroPack® gas packs (Mitsubishi Gas Chemical America, New York, New York, United States) were used in order to generate an anaerobic environment suitable for growth. Frozen glycerol stocks were made from liquid bacterial cultures and stored at -80$^{o}$C in 60% (v/v) glycerol.

## 2.5 Analysis of 16S rRNA gene sequencing

### *2.5.1 Amplification of 16S rRNA gene*

*Gardnerella vaginalis* and *Lactobacillus crispatus* were grown on complete NYCIII agar plates supplemented with 10% horse serum (Biowest, France). A single colony was picked for each bacterial species and mixed into 200µL of sterile 1x PBS. Samples were treated with 2µl of Invitrogen™ Proteinase K solution (Thermo Fisher Scientific™, Waltham, Massachusetts, United States) and incubated at 50$^{O}$C for 1 hour. Proteinase K (Thermo Fisher Scientific™, Waltham, Massachusetts, United States) was inactivated by incubating the samples at 80$^{O}$C for 30 minutes. Samples were centrifuged at 4000rpm for 10 minutes at 4$^{O}$C. Supernatant containing the bacterial DNA was transferred to a clean and labelled Eppendorf tube. Amplification of the 16S rRNA gene was performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, California, United States). The 16S universal primer set, F27 and R5, is listed in Table 2.2 with the cycling conditions. The expected PCR product size was 1.5kb. The reaction set up is indicated in Table 2.3. All reactions were performed using KAPA HiFi HotStart ReadyMix PCR kit (KAPA biosystems, Cape Town, South Africa) according to the manufacturer's instructions.

**Table 2.2: List of primer sets used in this study.**

| Primer name | Sequence (5'-3') | PCR parameters | Target gene | Size (bp) | Reference |
|---|---|---|---|---|---|
| **Sequencing** | | | | | |
| F27 | AGA GTT TGA TCI TGG CTC AG | 2' 95°C, (15" 95°C, 15" 55°C, 1' 72°C), ×30 | 16S rRNA | 1500 | Weisburg W. G., *et al*., 1990 |
| 1492R | ACG GCT ACC TTG TTA CGA CTT | | | | |
| **qPCR** | | | | | |
| R-GV3 | AGC CTA GGT GGG CCA TTA CC | 10' 95°C, (45" 94°C, 1 60°C, 45'' 72°C), ×50 | *G. vaginalis* 16S rDNA | 167 | Designed |
| F-GV1 | TGA GTA ATG CGT GAC CAA CC | | | | |
| LcrisR | AGC TGA TCA TGC GAT CTG CTT | 10' 95°C, (15" 95°C, 45" 55°C, 45' 72°C), ×40 | *L. crispatus* 16S rDNA | 120 | Byun R., *et al*., 2004 |
| LcrisF | AGC GAG CGG AAC TAA CAG ATT TAC | | | | |

**Table 2.3: General PCR method used in this study.**

| Component | Final concentration | 1x reaction (µL) |
|---|---|---|
| 10 µM Forward primer | 0.2 µM | 2.5 |
| 10 µM Reverse primer | 0.2 µM | 2.5 |
| 10 mM dNTPs | 0.2 mM | 0.5 |
| 5X OneTaq® Standard reaction buffer* | 1X | 5 |
| OneTaq® HotStart Polymerase | 0.025 U/µL | 0.125 |
| DNA/Sample | 10 ng/µL | 3 |
| Nuclease-free water | n/a | Add to 25 µL |
| **Final volume** | | **25** |

### *2.5.2 Visualization of PCR amplicons by gel electrophoresis*

PCR products were mixed with 2µl of 6x loading dye (New England Biolabs, Ipswich, Massachusetts, United States) and loaded into a 1% (v/v) agarose gel made using 1x TAE buffer (40mM Tris, 1mM EDTA and 20mM (v/v) glacial acetic acid) that contained 0.16µg/mL ethidium bromide (EtBr). Amplicons were electrophoresed in 1x TAE buffer for 40 minutes at 100 V. A 1kb DNA ladder (Thermo Fisher Scientific™, Waltham, Massachusetts, United

States) was used as a molecular weight marker. Products were visualized using a Gel Doc™ XR UV transilluminator (Bio-Rad Laboratories, Hercules, California, United States).

### *2.5.3 Sequencing*

*G. vaginalis* and *L. crispatus* 16S sequencing was performed routinely to confirm the purity of our bacterial samples. The 16S PCR products were sent to be sequenced to the Central Analytical Facility (CAF) DNA Sequencing Unit at Stellenbosch University, Stellenbosch, South Africa. Sequencing results obtained from Stellenbosch were subjected to a BLAST search on the NCBI database to confirm identity of the bacteria being used in this study.

### *2.5.4 Gram staining*

In order to confirm the purity and/or presence of *G. vaginalis* and *L. crispatus* in samples, bacterial samples were gram stained with the bacterial gram staining kit (Sigma-Aldrich, Burlington, Massachusetts, United States) according to the manufacturer's instructions. Sterile loops were used to pick single colonies from culture plates and smeared onto a slide with 20µl of sterile 1x PBS. Slides were heat-fixed. The smear was covered in crystal violet for 1 minute, and gently rinsed with tap water. Next, iodine solution was added to the slide and washed with tap water. Decolourizer was added for 5 seconds, carefully rinsed with tap water and stained with safranin for 30 seconds. Finally, slides were dried in an incubator at 37°C and visualized under a light microscope under oil immersion at a magnification of 1000x.

## 2.6 Colony forming units per mL (CFU/mL) and bacterial growth curves

### *2.6.1 Determination of CFU/mL*

*G. vaginalis* and *L. crispatus* were grown individually in complete NYCIII broth supplemented with 10% horse serum (Biowest, Nuaillé, France) for 48 hours. Bacterial cultures were diluted to a final 0.2 $OD_{600nm}$. A serial dilution was performed for each strain and plated in duplicate on NYCIII agar plates. Plates were placed inside a BD GasPak EZ Container (Thomas Scientific, Swedesboro, New Jersey, United States) with an AnaeroPack® gas pack (Mitsubishi Gas Chemical America, New York, New York, United States) to generate anaerobic conditions (80% $N_2$, 10% $CO_2$, and 10% $H_2$) in a 37°C incubator for 48 hours. Colony forming units were

counted and the average CFU/mL was calculated in Microsoft® Excel®. Three biological repeats were performed for each bacteria species.

### *2.6.2 Growth curves*

To determine the growth curve of *G. vaginalis*, the ATCC 14018 strain was grown in NYCIII broth supplemented with 10% horse serum for 48 hours. Bacterial cultures were diluted to a final OD of 0.23 and 0.15 at a wavelength of 600nm. A total of 200µL of each bacterial culture was dispensed in triplicate into a 96-well clear flat-bottom plate. Plate was covered with a plastic film and placed inside the GloMax® Explorer microplate reader (Promega, Madison, Wisconsin, United States) at 37°C for 48 hours. Absorbance measurements were taken every hour for 48 hours. Three biological repeats were performed for each dilution. Similarly, for co-culture experiments, *G. vaginalis* and *L. crispatus* were grown individually in NYCIII broth supplemented with 10% horse serum for 48 hours, bacterial cultures were diluted to a final OD of 0.23 and 0.15 at a wavelength of 600nm and growth was monitored as described. Two biological repeats were performed for each species.

## 2.7 Sample preparation

### *2.7.1 Bacterial culture growth*

*G. vaginalis* and *L. crispatus* bacterial cultures were grown in 15mL of NYCIII broth as described in section 2.4 and passaged at least 3 times prior to starting the drug experiments. After 48 hours, the culture was diluted to a final 0.2 $OD_{600nm}$ and then further diluted to achieve an (1:20) initial inoculum of $5x10^5$ CFU/mL in a total of 15mL of NYCIII media. Each antiretroviral drug was added at their respective $IC_{100}$ concentrations as determined in section 2.3.1. Samples were grown in an anaerobic environment in an incubator at 37°C for 24 hours.

### *2.7.2 Processing of culture medium and cell lysate*

Samples were then centrifuged in the Heraeus Megafuge™ 16R centrifuge (Thermo Fisher Scientific™, Waltham, Massachusetts, United States) at 5000rpm at 4°C for 20 minuets. Growth media was filter sterilized through a 0.22µM filter and stored at -20°C. Bacterial pellets were washed twice with 1x PBS and resuspended in 300µL of nuclease-free water. Cell suspensions were lysed using a sonication wand for 20-30 seconds at a frequency of 20kHz

while kept on ice. After sonication, samples were left on ice for 5 minutes and then centrifuged at 5000rpm at 4ºC for 5 minutes. The supernatant was filter sterilized through a 0.22µM filter and transferred to a clean, labelled microcentrifuge tube. Growth media and cell lysate samples were aliquoted in duplicate and half of the samples were heat inactivated at 100ºC for 5 minutes. Samples were stored at -20ºC or transferred to a mass spectrometry vial for further analysis as described in section 2.10.

### *2.7.3 Sample quantification using Bicinchoninic acid (BCA) protein assay*

The protein concentration for each sample was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific™, Waltham, Massachusetts, United States) according to the manufacturer's protocol. BCA protein standard was serially diluted to generate a standard curve which was used to quantify the protein concentration of unknown samples. Absorbance was read at $OD_{595nm}$ using the BioTek microplate reader with Gen5™ Software (Agilent Technologies, California, United States). Data was analysed on Microsoft® Excel®.

## 2.8 Determine the impact of *G. vaginalis* culture medium and cell lysate on the efficacy of TFV, TDF, and MVC in HIV infectivity

The premise for the methodological approach is that when ARVs are incubated with *G. vaginalis*, their concentration decreases in the culture medium due to uptake/metabolism by the bacteria. When the culture medium is added to the infection assay, inhibition should decrease relative to drug not incubated with bacteria. Conversely, uptake of ARVs by *G. vaginalis* would lead to the inhibition of infection by cell lysates (Figure 2.1).

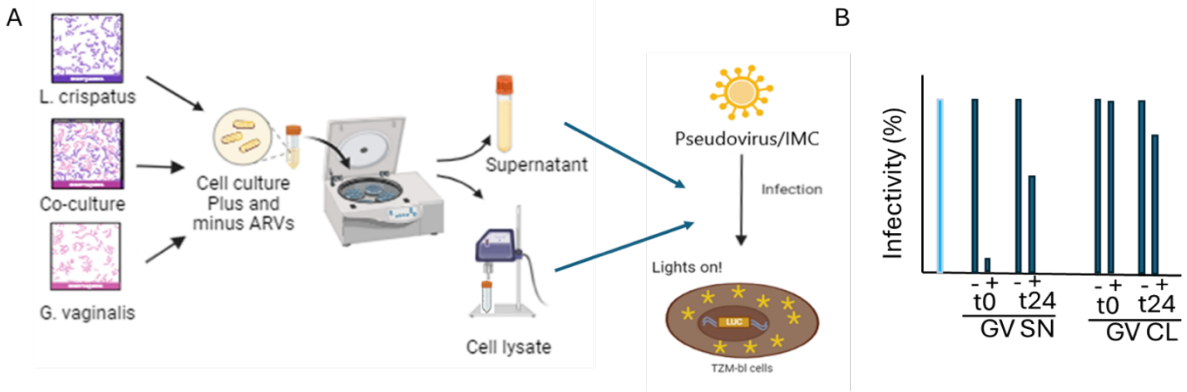



**Figure 2.1: Methodological approach to link bacterial uptake/metabolism of ARVs to HIV infection *in vitro* (A)** Equal titres of *L. crispatus* (LC) and *G. vaginalis* (GV) are grown singly and an as co-culture and incubated in the presence of antiretroviral drugs (ARVs).

Immediately after mixing (t0), a subset of samples was centrifuged, and the culture medium (supernatant) was collected and the bacterial cells lysed by sonication. The rest of the cultures were incubated for 24 hrs before centrifugation (t24). The supernatant was collected and the bacterial cells lysed by sonication. The supernatants and cell lysates from cultures at t0 and t24 were added to the infection assay. Infectivity was measured by relative light units and shown as infectivity relative to the positive control. **(B)** The positive control (blue bar) represents infection in the absence culture samples. We hypothesised that all bars representing samples with no ARVs added (-) will have the same relative infectivity as the positive control. At t0, the supernatant should have $IC_{100}$ concentration of ARVs and thus inhibit infection by approximately 100% whereas the cell lysate at this timepoint should have no effect. At t24 the bacteria should have taken up/metabolised the ARVs and thus infection should increase relative to t0 whereas the cell lysate at t24 should inhibit infection. Therefore, a decrease in efficacy was measured as the increase in infectivity between t0 and t24 in the presence of drug. The schematic indicates the expected effect of *G. vaginalis* on infection. The predicted effect of *L. crispatus* on infection is not shown on the hypothetical graph.

### *2.8.1 Infectivity assay*

TZM-bl cells were seeded in two sets of 96-well plates at a final density of $1x10^4$ cells per well in 200µl of complete DMEM. Cells were incubated overnight at 37°C with 5% $CO_2$. Media was removed from one set of plates and 100µl of fresh media containing 20.8µg/mL DEAE-Dextran (Sigma-Aldrich, Burlington, Massachusetts, United States) was added to each well. PSVs/IMCs stocks were thawed at room temperature, diluted and 200 $TCID_{50}$ units were added per well. Total protein was calculated for each culture medium and lysate samples and 10µg of total protein was added to each well. Samples were added to each well in triplicate. Plates were incubated for 48 hours at 37°C with 5% $CO_2$. Then, 150µL of media was removed from each well and a total of 50µL BrightGlo™ (Promega, Madison, Wisconsin, United States) lysis reagent was added and left for 2 minutes to allow for lysis. Finally, the cell lysate was mixed through pipetting action and 75µL was transferred to an opaque 96-well plate. Luminescence was read using a Glomax® 96 Microplate Luminometer (Promega, Madison, Wisconsin, United States). Three biological repeats were done for each drug. The $IC_{50}$ values were calculated and averaged on GraphPad Prism statistical software (version 8, GraphPad Software, La Jolla, California, United States).

### *2.8.2 Determining cell viability by MTT assay*

An MTT assay was included in all infectivity experiments to confirm TZM-bl cell viability. MTT is a tetrazolium salt that is used to measure cell growth. After the infectivity assay, the

culture supernatant was removed from each well of the second set of plates and replaced with 50µL of serum-free media and 50µL of MTT labelling agent (0.5mg/mL) solution. Plates were incubated at 37°C, 5% $CO_2$ for 4 hours. After incubation, 150µL of MTT solvent (dimethyl sulfoxide or sodium dodecyl sulphate in diluted hydrochloric acid) was added into each well. Plates were wrapped in foil and shaken for 15 minutes. Absorbance was read at $OD_{590nm}$ using the BioTek microplate reader with Gen5™ Software (Agilent Technologies, California, United States). Data was analysed on Microsoft® Excel®.

## 2.9 Determine whether samples from the co-culture of *G. vaginalis* and *L. crispatus* in the presence of TFV and TDF, lower drug efficacy

### *2.9.1 Co-culture methodology and sample processing*

Bacterial cultures were individually grown in 15mL of NYCIII broth as described in section 2.4 and passaged at least 3 times prior to starting the co-culturing experiments. After 48 hours, cultures were diluted to a final 0.2 $OD_{600nm}$. A further 1:20 serial dilution was performed for *G. vaginalis*. The initial inoculum amount was $5x10^5$ CFU/mL for both species. The two bacterial cultures were grown individually and as a co-culture mixed at a ratio of 1:1 under anaerobic condition for 24 hours. For bacteria-drug experiments, $IC_{100}$ concentrations of each drug were added to the respective co-cultures and individual cultures. Culture medium and cell lysates were processed according to section 2.7.2.

### *2.9.2 Infectivity assay*

TFV and TDF are reverse transcriptase inhibitors and thus infectious viral particles, IMCs, were used in the infectivity assay. TZM-bl cells were prepared as outlined in section 8.2.1. IMCs stocks were thawed at room temperature, diluted and 200 $TCID_{50}$ units were added per well. Culture medium and cell lysate samples, prepared as outlined in section 2.9.1, were added to wells in triplicate. Protein concentrations were determined as described in section 2.7.2 and 10µg total protein of each sample was added per well. The infectivity assay and MTT was carried out as described in section 2.8.1 and 2.8.2, respectively. Three biological repeats were performed for each drug.

### *2.9.3 Validating the presence of G. vaginalis in the co-culture by quantitative PCR (qPCR)*

#### 2.9.3.1 Total genomic DNA isolation from TRIzol® organic phase

DNA was isolated from cell pellets obtained after 24 hours of culture in NYCIII broth supplemented with horse serum. DNA was isolated from 1mL of bacterial cell cultures at 4ºC from two timepoints, t0 and t24 hours. Cells were harvested by centrifugation and growth media was removed. TRIzol® reagent (Life Technologies, Unites States) was used to extract total genomic DNA according to the manufacturer's instructions.

#### 2.9.3.2 Presence of candidate genes determined by qPCR

qPCR was performed on extracted bacterial cell lysate genomic DNA to confirm the presence of *G. vaginalis* in the co-culture with *L. crispatus*. The candidate genes chosen were *G. vaginalis* specific and *L. crispatus* specific genes, namely, GV and LC. All qPCR reactions were performed using Applied Biosystems™ PowerUp™ SYBR™ Green master mix (Thermo Fisher Scientific™, Waltham, Massachusetts, United States) according to the manufacturer's protocol. All the reactions were amplified in the QuantStudio™ 3 System (Thermo Fisher Scientific™, Waltham, Massachusetts, United States) with the following cycling conditions: 50ºC for 2 minutes, 95ºC for 2 minutes, subsequently 40 cycles of 95ºC for 15 seconds, 55ºC for 15 seconds, 72ºC for 1 minute, and a final elongation step at 72ºC for 3 minutes. The primer sets listed in Table 2.2 were used in the qPCR experiments to confirm the presence of *G. vaginalis*.

#### 2.9.3.3 Analysis of qPCR data

The Design and Analysis software (version 2.6.0) was used to analyse the qPCR data. A melt curve analysis of the PCR amplicons was carried out to confirm that only one single specific product was amplified in each sample.

## 2.10 Determine uptake of antiretroviral drugs by mass spectrometry

Lysate and supernatant TFV samples were analysed and quantitated using a modified version of a validated liquid chromatography (LC) tandem mass spectrometry assay developed at the Division of Clinical Pharmacology, University of Cape Town, by Mr Anton Joubert.

The mass spectrometry for the MVC and ABC samples were kindly performed at the Division of Clinical Pharmacology, University of Cape Town, by Dr Daniel Watson.

### *2.10.1 High Performance Liquid Chromatography (HPLC) High Resolution Mass Spectrometry*

An AB Sciex® X500R QTOF (Sciex®, Framingham, Massachusetts, United States) mass spectrometer coupled to an AB Sciex® Exion LC system and a Kinetex® Phenyl-Hexyl column (2.6µm, 100Å, 50 mm x 4.6mm) were used. Samples injected into the HPLC-QTOF system were diluted by 1:20 in LC grade methanol for abacavir and maraviroc samples, and Millipore water for tenofovir samples and blanks. The mobile phases that were used are 1mM ammonium formate in water as the aqueous mobile phase and methanol with 0.05% formic acid as the organic mobile phase. The method used was a 12.5-minute run in which the organic mobile phase percentage was changed from 10% to 98% over 8.5 minutes, then returned to 10% to equilibrate the column for the remaining time. MS1 (unfragmented peptides) and MS2 (fragmented peptides) data were acquired using information dependent acquisition (IDA).

### 2*.10.2 Raw Spectra Processing*

Raw HRMS data produced by HPLC-QTOF was converted to mzXML format by ProteoWizard tool MSconvert (version 3.0.10051, Vanderbilt University, Tennessee, United States) [114]. The converted data was then analysed using the processing software MZMine2 [115]. The parameters set were based on the recommendations of MZMine2. After the raw data were imported, the peaks were detected using the Mass Detection feature. For MS1 detection, centroid data was selected, and the noise level cut-off was set to 1000. For MS2 peak detection, centroid data was also selected, and the noise level cut-off was set to 0. Next a chromatogram was created using the ADAP Chromatogram Builder feature [116]. To generate chromatograms the detected mass features were selected and the minimum group size in number of scans was set to 3, the group intensity threshold was set to 500, the minimum highest intensity was set to 1000 and the *m/z* tolerance was set to 0.1. At this point the *m/z* signals had been detected for MS1 and MS2, and a chromatogram had been created using these signals. Next, isobars (atoms of different chemical elements with the same number of nuclei) need to be removed by deconvoluting the chromatograms. This was achieved by using the Chromatogram

Deconvolution feature. The baseline cut-off algorithm was selected, and the minimum peak height was set to 1000, peak duration was set to 0.1-3.0 min and baseline level was 500. For the remaining settings, the *m/z* centre calculation was set to median, *m/z* range for MS2 scan pairing was set to 0.02 and the retention time range for MS2 scan pairing was set to 0.1 min. Next, isotopic peaks were grouped to remove redundancies, using the Isotope Peak Grouper function. The *m/z* tolerance was set to 0.1, the retention tolerance was set to 0.1 min and the maximum charge was set to 3. Finally, the processed spectra were aligned for comparison using the Join Aligner feature. The weight for *m/z* was 75 and the weight for retention time was 25, and the *m/z* and retention time tolerances were 0.1. The presence or/and relative intensity of maraviroc (*m/z* 514.3357) and abacavir (*m/z* 287.1620) were determined in each sample.

### 2.11 Statistical analysis

A one-way ANOVA with multiple comparison Dunnett's post-test were performed on the average of two independent experiments performed in triplicate. $IC_{50}$ values were determined by non-regression analysis of log inhibitor vs percent inhibition. Growth curves were compared using non-linear regression, exponential growth analysis. All statistical tests performed in this study were done in GraphPad Prism (Version 8, GraphPad Software, La Jolla, California, United States) and Microsoft® Excel®. Data were deemed significant if p-values were less than 0.05, 0.01, 0.001 and 0.0001, indicated as *, **, *** and ****, respectively.

# Chapter III

## Determining the effect of *G. vaginalis* on the efficacy of tenofovir, tenofovir disoproxil fumarate, maraviroc and abacavir

### 3.1 Introduction

The advent of ARVs transformed the treatment and prevention of HIV infection, and have contributed to the decline in AIDS-related deaths from 1.4 million in 2010 to 650,000 in 2021, a 52% decrease [1]. PrEP is an important component of the strategy to stem the HIV pandemic. Young women, however, continue to carry the burden of disease and although oral PrEP with TDF/FTC was efficacious in MSM it failed to consistently protect women against HIV infection [6, 117]. It has been suggested that the efficacy of PrEP is dependent on any factors that lower the half-life of the drugs, reducing their ability to protect against HIV infection. The reasons why the efficacy of PrEP is variable in women is thus likely multi-factorial and could include adherence, inflammation and/or the presence of STI [118]. Recent findings have highlighted the potential role that the FGT microbiome could play in reducing ARV PrEP efficacy in women [6].

A "healthy" FGT is described as one colonised by a homogenous microbial population belonging primarily to the *Lactobacillus* genus. These bacteria are thought to protect against pathogen infection including HIV [50, 66]. However, often these beneficial microbial populations are displaced by more diverse anaerobes, such as *P. bivia* and *G. vaginalis* which can lead to the onset of BV. BV is not only associated with a 60% increase in HIV acquisition rates [119-121], it has also been linked to reduction in PrEP efficacy [6]. Recently, it has been shown that *G. vaginalis* metabolises ARVs so that less active drug is available to inhibit HIV infection [109]. Up to now, studies have focussed on NRTIs: TFV, TDF, TAF and the NNRTI: DPV (dapivirine). Cheu *et al*., (2020) found that the microbial species present in the cervical lavage (CVL) of women with BV reduced the levels of extracellular TFV and DPV, whereas they had no effect on TAF [109]. In this study we investigated whether *G. vaginalis* was able to reduce the extracellular levels of the NRTIs, TFV, TDF and, ABC and the entry inhibitor, MVC and determined whether changes in concentration affected the ability of each ARV to inhibit HIV infection.

## 3.2 Results

### 3.2.1 Determine the $IC_{50}$ of ARV drugs

In order to determine the concentration at which to measure HIV infection in the presence or absence of bacterial samples, the $IC_{50}$ values for each ARV were identified (Figure 3.1) and the average of three biological repeats for each ARV was determined (Table 3.1). MVC seemed to be the more potent ARV with an $IC_{50}$ of $7x10^{-3}$ μM followed by TDF with an $IC_{50}$ of approximately 0.16μM, 22-fold higher than the $IC_{50}$ of TFV.

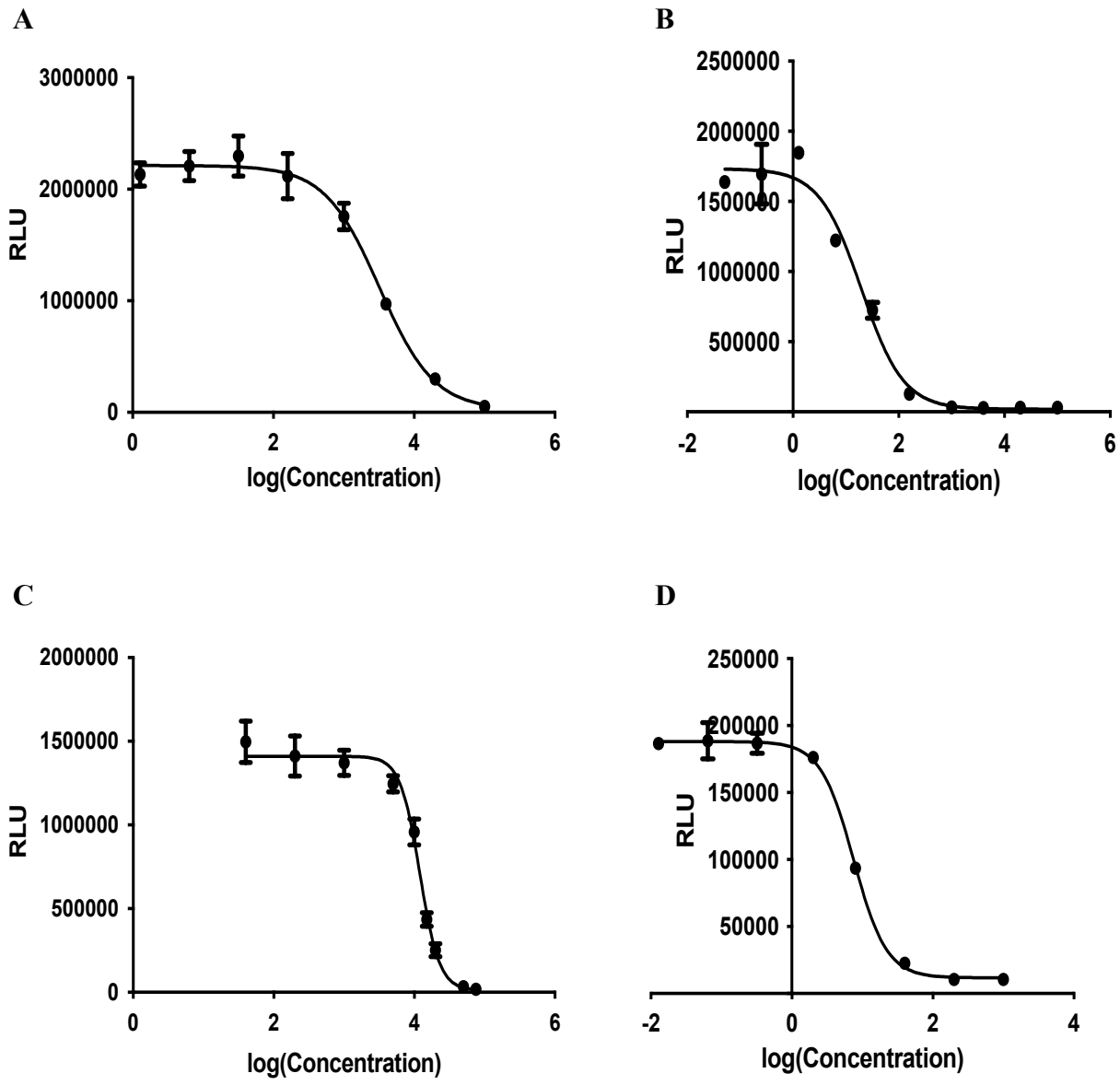



**Figure 3.1: $IC_{50}$s of tenofovir, tenofovir disoproxil fumarate, abacavir, and maraviroc. (A)** Tenofovir (TFV), **(B)** tenofovir disoproxil fumarate (TDF), **(C)** abacavir (ABC), and **(D)**

maraviroc (MVC) were serially diluted (Table 2.1), and added to infectious molecular clones (TFV, TDF, ABC) and pseudovirus (MVC) at 200$TCID_{50}$ before adding to the reporter cell line TZM-bl cells. Cells were incubated for 48 hours at 37°C and infection was measured using a luciferase assay and indicated as relative light units (RLU). The experiments were carried out independently with each of the three biological repeats done in triplicate. A representative of one biological repeat is indicated showing the average of triplicate values and standard deviation.

**Table 3.1: $IC_{50}$ and $IC_{100}$ values.**

| Drug | $IC_{50}$ (μM) (+/-SD) | $IC_{100}$ (μM) | Published $IC_{50}$ (μM) |
|---|---|---|---|
| Maraviroc | 0.007 (+/- 0.0006) | 1 | 0,0012 |
| Tenofovir | 3.1 (+/- 0.013) | 75 | 27.2 |
| Tenofovir disoproxil fumarate | 0.16 (+/- 0.005) | 4 | 0.09 |
| Abacavir | 12 (+/- 3) | 20 | 4.6 |

*Average of 3 independent biological repeats with standard deviation are indicated.

Although the $IC_{50}$ values were not similar to earlier reports, they also reported that MVC was more potent than TFV, TDF and ABC [122]. The differences between $IC_{50}$ values are likely due to variation in assay systems, including infectivity using PSVs or IMCs, and different host cells.

### 3.2.2 Optimisation of *G. vaginalis* culture

To optimise the culture method of *G. vaginalis* growth for ARV uptake assays, NYCIII media was inoculated with a two-day culture of the facultative anaerobe diluted to $OD_{600nm}$ readings of 0.15, 0.2, and 0,23. The growth of the three cultures was monitored by optical density over 48 hours. As expected, the culture initiated with the highest bacterial titre started plateauing earlier than the others (Figure 3.2). The growth curve with an inoculum of $OD_{600nm}$ 0.2 had a reduced lag phase, and growth started plateauing after 10 hours, indicating that the bacteria were at exponential growth between 0 and 10 hours. When the culture was serially diluted and plated on NYCIII plates, we found that culture with an $OD_{600nm}$ of 0.2 corresponded to 6.5 $x10^8$ CFU/mL *G vaginalis*. To maximise the detection of ARVs associated with cell lysates, this concentration was used for experiments going forward.

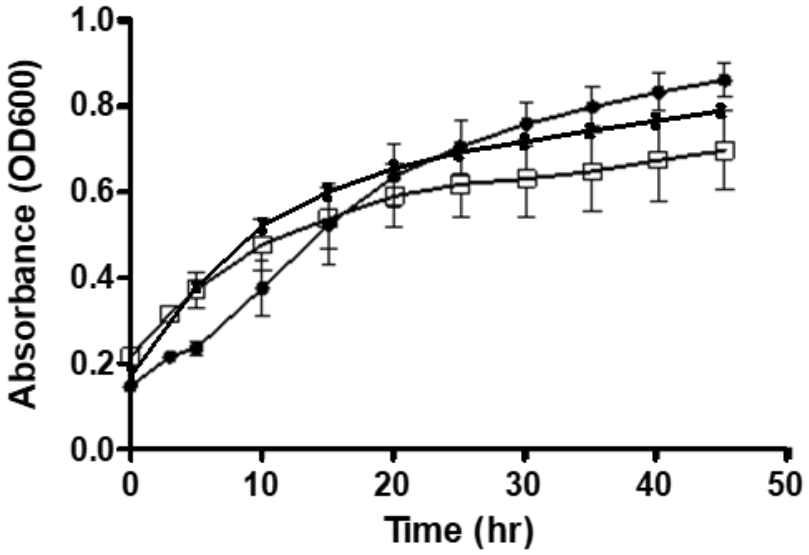



**Figure 3.2: *G. vaginalis* growth curve.** NYCIII medium was inoculated with cultures grown for 48 hours under anaerobic conditions corresponding to different optical densities determined at a wavelength of 600nm: 0.23 (open squares), 0.2 (closed squares), and 0.15 (closed circles). Growth was monitored for 48 hrs at a wavelength 600nm. Three independent biological repeats were performed in triplicate for each inoculum. The average of three biological repeats and standard deviation is indicated.

### 3.2.3 *G. vaginalis* culture medium affects pseudovirus infectivity assay

NYCIII was incubated under anaerobic conditions in the absence and presence of *G. vaginalis* for 48 hours before the culture was centrifuged and the supernatant (medium without cells) added to TZM-bl cells infected with PSV. After incubation for 48 hours at 37°C and 5% $CO_2$, NYCIII medium did not affect the infectivity assay (Figure 3.3). However, the *G. vaginalis* supernatant reduced PSV infectivity by approximately 60%. When the supernatant was boiled before adding to the PSV infectivity assay, the inhibitory effect of the supernatant was reduced, although infection was still lower than the PSV-only control. Interestingly, heating the NYCIII abiotic media caused a decrease in infectivity, although not significantly. An MTT assay was used to confirm that the supernatant was not affecting TZM-bl cell viability (data not shown). For all experiments the supernatant of *G. vaginalis* cultures were boiled to limit the effect of the culture medium on the infection assay.

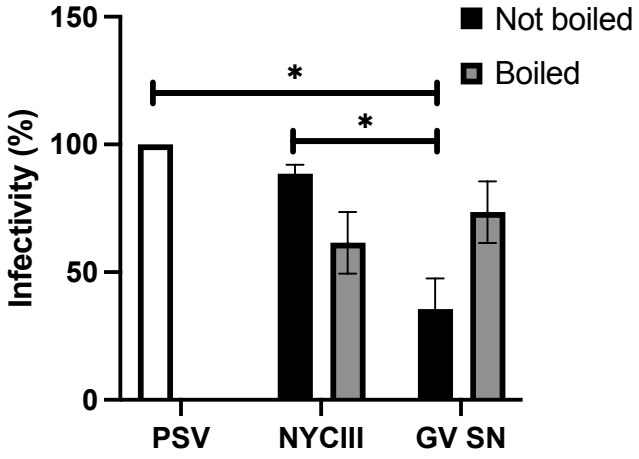



**Figure 3.3: Effect of *G. vaginalis* culture medium (GV SN) on HIV pseudovirus (PSV) infectivity.** NYCIII medium was inoculated with a culture of 6.5 x$10^8$ CFU/mL *G. vaginalis* and grown for 48 hours. The culture was centrifuged and the supernatant was either left untreated (black bars) or boiled (grey bars). TZM-bl cells were infected with 200 $TCID_{50}$ of pseudovirus and incubated for 48 hours at 37$^o$C in the presence of treated and untreated culture medium. Infection was measured using a luciferase assay and indicated as % infectivity relative to the positive control (white bar) (pseudovirus not exposed to culture medium). The graph represents averages of two biological repeats with standard deviation and One-way Anova, with Tukey post-test was used to compare averages. Infectivity was deemed statistically significant (*) if the p-value was less or equal to 0.05. Error bars represent standard error of the mean.

### 3.2.4 Uptake of ARV drugs by *G. vaginalis*

#### 3.2.4.1 Detection of intracellular and extracellular TFV after exposure to *G. vaginalis* in culture

Mass spectrometry (MS) was used to quantify TFV in the culture supernatant and cell lysate of *G. vaginalis* using a standard curve generated by TFV solutions at different concentrations (Supp Fig 2). Thereafter, TFV, prepared at 3 different concentrations: 0.16µM, 3.2µM, and 100µM, was quantified by mass spectrometry to confirm the accuracy of the mass spectrometry method. Using the analyte area under the curve we found that the analyte concentration of each independent experiment deviated from the expected concentration, except at high concentrations (Table 3.2). However, the results of the independent experiments were similar suggesting that preparation of the samples might have been inaccurate.

**Table 3.2: Confirmation of Mass spectrometry standards.**

| Sample | Analyte Area | Analyte concentration (µM) | BR1* Calculated concentration (µM) | BR2* Calculated concentration (µM) |
|---|---|---|---|---|
| $<IC_{50}$ | **20600** | **0.16** | **0.27** | **0.38** |
| $IC_{50}$ | **556000** | **3.2** | **3.08** | **2.97** |
| $>IC_{100}$ | **19000000** | **100** | **100** | **100** |

*BR1: Biological repeat 1, BR2: Biological repeat 2.

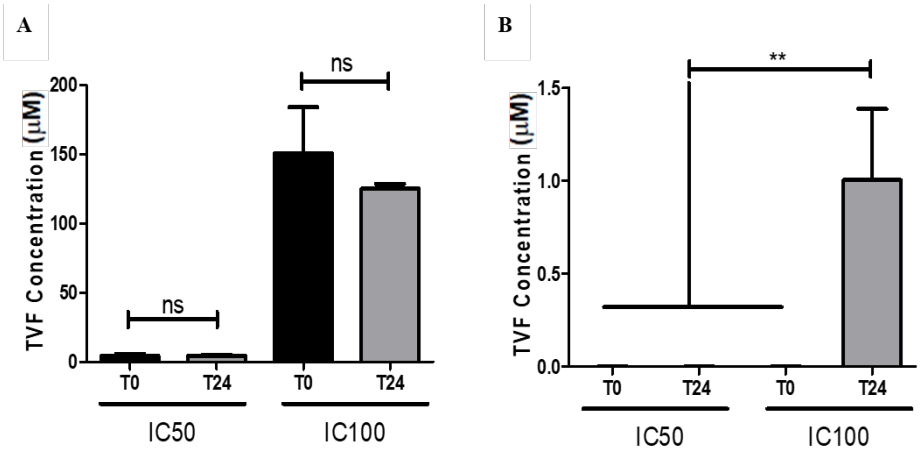



**Figure 3.4: Intracellular and extracellular tenofovir levels associated with *G. vaginalis* culture.** The ATCC 14018 *G. vaginalis* strain was incubated with tenofovir at two different concentrations: 3.2µM which corresponded to the $IC_{50}$ and 100µM which corresponded to a concentration greater than the calculated $IC_{100}$. The bacteria were exposed to TFV for 24 hours under anaerobic conditions at 37°C. At time zero (T0) (black bars) and after 24 hours (T24) (grey bars) the culture medium was harvested, and the cells lysed by sonication. The concentration of TFV in the **(A)** culture medium and **(B)** cell lysate was determined by mass spectrometry. The graph represents averages of two biological repeats with standard deviation and One-way Anova, with Tukey post-test was used to compare averages.

In order to determine TFV uptake, *G. vaginalis* was cultured for 24 hours in the presence of the drug at its $IC_{50}$ and $IC_{100}$ concentrations. TFV concentrations were measured intracellularly and extracellularly before (time 0: t0) and after 24 hours (t24) incubation with the drug. When TFV was added to the cell culture at 3.2µM, it was barely detectable at t0 and t24 in the supernatant and cell lysate (Figure 3.4A), suggesting that the concentration was too low to monitor uptake of the ARV. TFV, added at a concentration of 100µM was detected extracellularly at t0 at a concentration of approximately 150µM which is higher than the expected concentration, most likely due to intra-experimental variation. At 24 hours, TFV

concentration in the supernatant was similar to that at t0, suggesting that the bacteria had not internalised the ARV. However, TFV was detected in the cell lysates albeit at very low concentrations (1 μM) (Figure 3.4B). Therefore, due to the poor uptake of TFV, there was no apparent change in extracellular ARV.

### 3.2.4.2 Identify intracellular MVC and ABC associated with *G. vaginalis* cell lysates

To assess MVC and ABC uptake, *G. vaginalis* was grown in anaerobic conditions in the presence of $IC_{100}$ MVC and ABC concentrations for 24 hours. Cells were harvested, sonicated and prepared for further MS analysis. Although we were unable to quantify the amount of MVC and ABC taken up by *G. vaginalis*, MS detected the presence of the ARVs in the cell lysate samples (Figure 3.5).

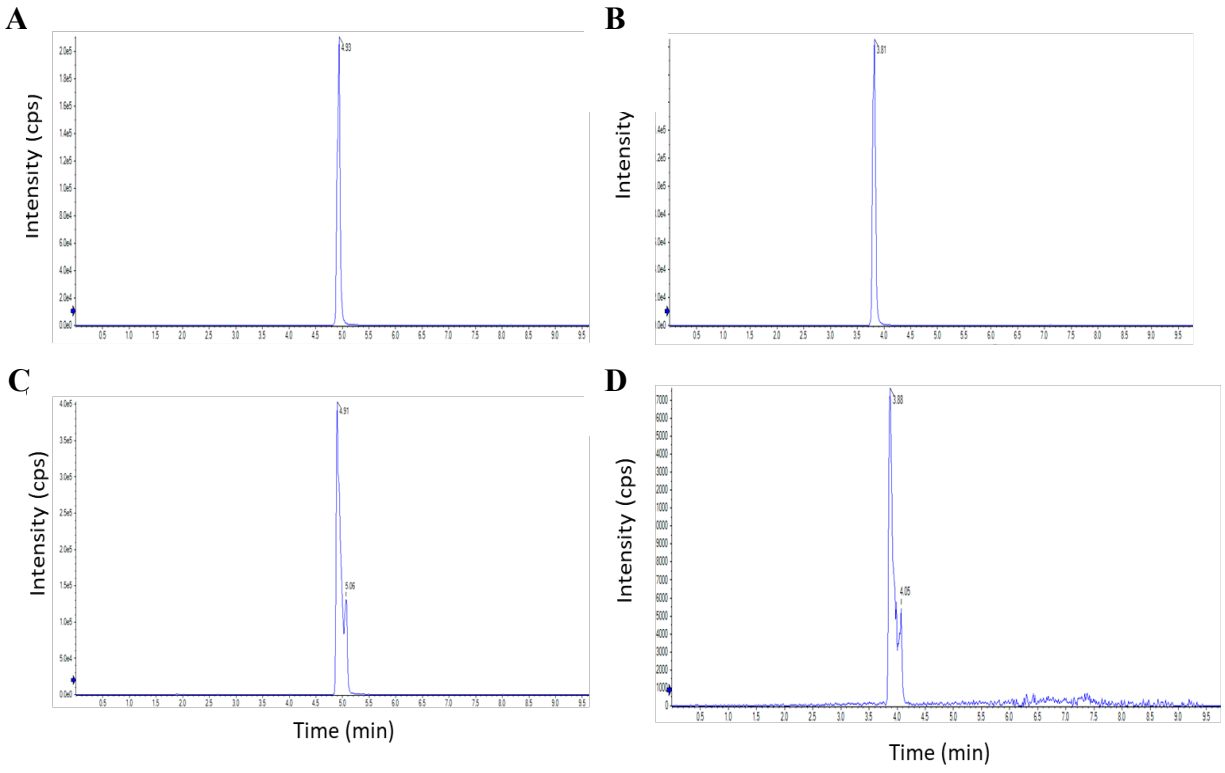



**Figure 3.5: Identification of MVC and ABC associated with *G. vaginalis* cell lysates.** Mass spectrometry was used to determine the standard spectra of **(A)** 100nM MVC and **(B)** 100nM ABC. The ATCC 14018 *G. vaginalis* strain was incubated with **(C)** MVC and **(D)** ABC for 24 hours under anaerobic conditions at 37°C before cells were lysed by sonication and the presence of the ARVs were detected by mass spectrometry and compared to the standards. The height of the peak represents counts per second (CPS) for MVC (m/z 514.3357) and ABC (m/z 287.1620). Chromatograms represent two independent biological repeats.

### 3.2.5 *G. vaginalis* influences ARV efficacy

We hypothesised that a decrease in the level of extracellular TFV, TDF, ABC, and MVC due to internalisation (or metabolism) by *G. vaginalis*, would reduce the inhibitory dose of the ARVs to below their effective range. Conversely, the presence of intracellular ARVs, taken up by the bacteria, would inhibit HIV infection. *G. vaginalis* was cultured in the presence of TFV, TDF, MVC, and ABC at their respective $IC_{100}$ concentrations before the supernatants and cell lysates were added to TZM-bl cells infected with either PSV or IMC. Infection with PSV was only used to test the efficacy of MVC as TFV, TDF, and ABC inhibited reverse transcription, a step after viral entry.

#### 3.2.5.1 ARVs associated with *G. vaginalis* cell lysates inhibit infection

When the cell lysate samples, obtained from exposure of *G. vaginalis* to TFV and TDF were added to TZM-bl cells and incubated for 48 hours there was no significant difference compared to the virus only control as seen in Figure 3.6. Therefore, although MS showed the presence of the drug in the cell lysates, the concentration of active TFV was likely too low to inhibit infection.

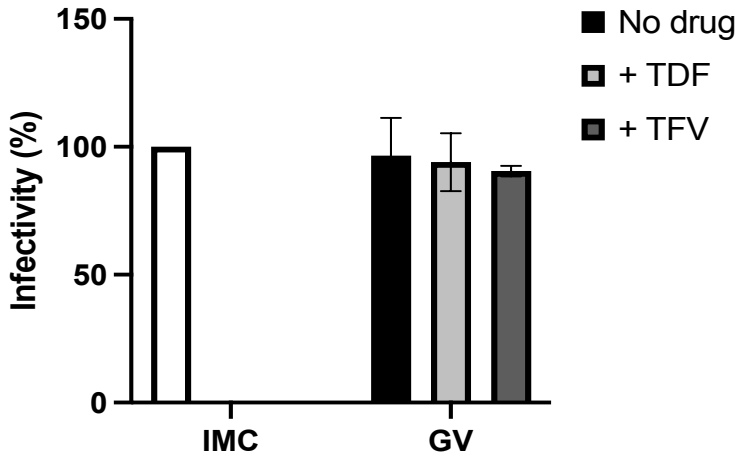



**Figure 3.6: Determine whether *G. vaginalis* cell-associated TDF and TFV inhibit IMC infection.** Bacterial cell lysate samples were diluted with sterile DMEM and 10µg of total protein was added to each well containing TZM-bl cells. The infectivity assay was performed in triplicate. Cells were incubated for 48 hours at 37°C and infection was measured using a luciferase assay and indicated as % infectivity. Control with ARV in the absence of bacterial sample is not included in the graph as it is consistently inhibited infection by 100%. Infectivity was deemed statistically significant (*) if the p-value was less or equal to 0.05. Error bars represent standard error of the mean.

### 3.2.5.2 *G. vaginalis* cell-free culture medium with ARVs impacts their efficacy

Firstly, ARVs were boiled in the absence of supernatant to confirm that the efficacy of the ARVs was not reduced by boiling (data not shown) because all supernatant samples were boiled to limit the impact of *G. vaginalis* culture medium on infection (Figure 3.3). Supernatants harvested at t0 inhibited infection by only 40% compared to the virus-only control, despite the concentration of TFV and TDF exceeding their $IC_{100}$ values (Figure 3.7). At t0, the supernatant was removed before incubation with *G. vaginalis* suggesting the low inhibition was not due to bacterial-specific degradation of the drugs. Instead, a soluble factor in the culture medium could be affecting inhibition.

After 24 hours of exposure to *G. vaginalis*, supernatant with TFV and TDF only inhibited infection by 20-25%. The decrease in inhibition from approximately 40% to 20% could be due to the uptake of the ARVs by *G. vaginalis*, decreasing their bioavailability. The effect seemed more marked for TDF which would suggest that less TDF was present in the culture medium than TFV, potentially because it was more efficiently internalised by *G. vaginalis*. However, without MS data, we cannot confirm that TDF was taken up by *G. vaginalis*. Supernatant from *G. vaginalis* culture with MVC showed a similar pattern although inhibition at t0 reached 50% (Figure 3.8). Similar to TFV and TDF, after exposure to *G. vaginalis* for 24 hours, the MVC supernatant only inhibited infection by 25%.

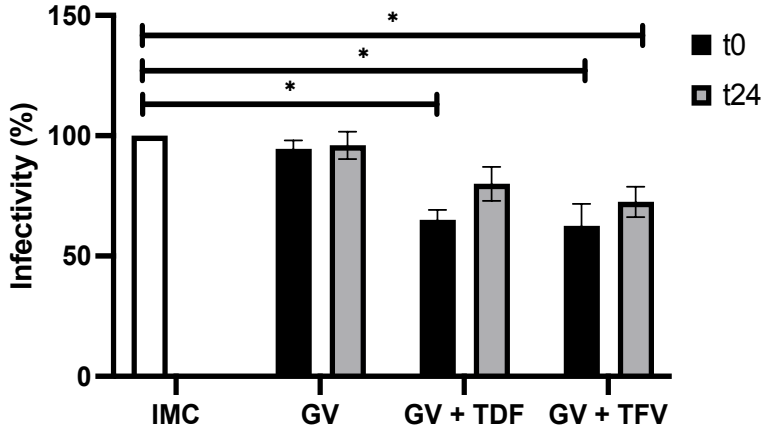



**Figure 3.7: Impact of *G. vaginalis* cell-free culture medium on IMC infection after incubation with TFV and TDF.** Bacterial cell culture medium samples were diluted with sterile DMEM and 10µg of total protein was added to each well containing TZM-bl cells. Infectivity assay was performed in triplicate. Cells were incubated for 48 hours at 37°C and

infection was measured using a luciferase assay and indicated as % infectivity relative to the positive control (IMC only). Control with ARV in the absence of bacterial sample is not included in the graph as it consistently inhibited infection by 100%. Infectivity was deemed statistically significant (*) if the p-value was less or equal to 0.05. Error bars represent standard error of the mean.

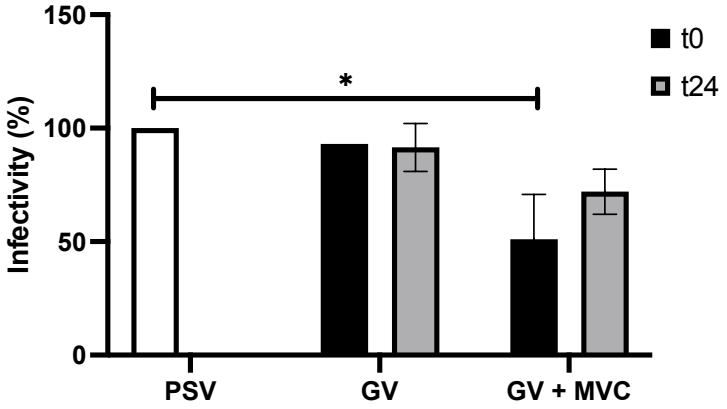



**Figure 3.8: Impact of *G. vaginalis* cell free culture medium on PSV infection after incubation with MVC.** Bacterial cell culture medium was diluted with sterile DMEM and 10µg of total protein was added to each well containing TZM-bl cells. Infectivity assay was performed in triplicate. Cells were incubated for 48 hours at 37$^{o}$C and infection was measured using a luciferase assay and indicated as % infectivity. Infectivity was deemed statistically significant (*) if the p-value was less or equal to 0.05. Error bars represent standard error of the mean.

### 3.3 Discussion

Only sustained concentrations of ARVs at the site of infection can prevent transmission and thus the ability of BVAB to metabolise TFV in the FGT, has severe consequences. Although the threshold concentration of ARVs in the FGT required to prevent HIV infection is not known, it has been suggested that cervicovaginal TFV concentrations greater than 0.466µM (1000ng/mL) protect against infection [36]. We determined the $IC_{50}$ of TFV as 3.2µM under our assay conditions, much higher than the predicted threshold, which suggested that the ARV at this concentration would prevent HIV infection. However, when 3.2µM TFV was incubated with *G. vaginalis*, the residual drug in the cell-free culture supernatant was not able to inhibit IMC infection of TZM-bl cells (data not shown). Therefore, the concentration of the ARVs used in this study exceeded their $IC_{100}$ to maximise detection of the ARVs in cell lysates by mass spectrometry and identify differences in infection.

Despite the high concentration of TFV added to the culture medium of *G. vaginalis*, the cell-free supernatant only inhibited IMC infection by 40% and after incubation for 24 hours, it inhibited infection by 20%. It is likely that a feature or component of the culture medium interfered with inhibition, although it is unlikely to be degradation because MS determined TFV concentration was approximately equivalent to its $IC_{100}$. The unexpectedly poor inhibition at t0 most likely interfered with the ability to detect any increase of infection after 24 hours due to uptake of TFV. To improve the detection of changes in inhibition, the supernatant could have been partially purified or the TFV extracted to remove soluble factors that could influence infection, however, this might have resulted in loss of the drug which might have interfered with accurate quantification of ARV uptake.

MS detected 1μM TFV in the bacterial cell lysates indicating that *G. vaginalis* internalised less than 1% of the total drug added (150μM). As the concentration of TFV taken up by *G. vaginalis* was so low, it stands to reason that we could not detect the small decrease in TFV concentration in the supernatant after 24 hours incubation. Furthermore, the low concentration of TFV in the cell lysates, was not sufficient to inhibit IMC infection. To confirm that 1μM was unable to inhibit HIV infection, or the level of inhibition was below the detection limit of the assay, cell lysates could have been spiked with TFV at 1μM and tested for inhibition of IMC infection.

Interestingly, the effect of TFV supernatant on HIV infection was similar for TDF and MVC samples, before and after incubation with *G. vaginalis*, only inhibiting IMC infection by 40% and 20%, respectively. A previous study has shown that TDF efficacy was not affected by *G. vaginalis*, and the authors suggest it was due to its passive uptake by host cells [14]. However, we consistently detected a 20% reduction in inhibition for TFV, TDF and MVC. It seems unlikely that the uptake of 1% TFV would translate into a 20% change in inhibition. It is thus more likely that an unknown, non-specific process associated with the supernatant samples is reducing inhibition despite high concentrations of ARVs. *G. vaginalis* and its components might have directly impacted the efficacy of the ARVs, including potentially modifying the drug and affecting its activity. Further research into the effect of boiling the *G. vaginalis* growth medium may help clarify what which elements have an effect on the ARVs in this study. To test this, we could have spiked boiled and not boiled t0 and t24 *G. vaginalis* cell-free culture supernatant samples with ARVs before adding to the infection assay.

It was previously reported that high pH and conversion of TFV to adenine in the culture medium by *G. vaginalis* inhibited endocytosis thereby decreasing its efficacy [6]. If this was the case in our assay, then we should observe close to 100% inhibition at t0. Instead, at 100μM, TFV only inhibited infection by 40% in the presence of supernatant from t0, suggesting the presence of a soluble factor(s) directly preventing inhibition or reducing HIV infection. The extent to which drugs were internalised by TZM-bl cells should have been tested to confirm the efficiency of TFV cell uptake. The source of the soluble factor is not clear because *G. vaginalis* cultures were diluted to an OD of 0.2 prior to adding the ARVs, most likely reducing the concentration of bacterial metabolites. Furthermore, the presence of the NYCIII medium had no effect on ARV inhibition.

It is possible that the poor inhibition observed at t0 and the apparent decrease in inhibition observed in the presence of t24 *G.vaginalis* supernatant was due to the design of the assay and that further optimisation is required. Increased concentrations of *G. vaginalis*, TZM-bl cells and ARVs should have been tested especially noting that much higher amounts were used in the uptake and infection assays outlined in Taneva *et al*. (2018). According to Taneva *et al*. (2018), the optical density at 600nm of 1 corresponded to $0.5 \times 10^9$ CFU/mL for *G. vaginalis* and bacteria were grown to stationary phase equivalent to an $OD_{600nm}$ of 4 in peptone yeast extract glucose broth (PYG-YG) or ATCC medium 1685. When the *G vaginalis* strain 14018 was cultured in NYCIII, an $OD_{600nm}$ reading of 0.2 corresponded to $15 \times 10^6$ CFU/mL, 400-fold lower than what we found [123]. This suggests that growth conditions and inaccurate inoculum concentrations might have affected experimental outcomes. Furthermore, other studies infected cells in the presence of ARVs in the presence and absence of either the supernatants of *G. vaginalis* culture, whole cells or CVL samples, whereas we first incubated the drugs with *G. vaginalis* before adding the cell lysate and supernatant to the infection assay. Lastly, the MS method did not allow for the quantification of TDF, ABC, and MVC, and we could not confirm the relative amounts of ARVs in the supernatant and cell lysates. Therefore, variation in culture conditions and study design might have impacted experimental results, making it difficult to compare to other studies.

Despite conflicting results and potential caveats of the experimental approach, mass spectrometry showed that that TFV, ABC, and MVC were internalized by *G. vaginalis*.

Uncovering more information on the mechanism of how TFV is internalized by *G. vaginalis* could increase the understanding of the role it plays in HIV infection.

# Chapter IV

## Impact of *Lactobacillus* on uptake of antiretrovirals by *G. vaginalis*

### 4.1 Introduction

It has been suggested *Lactobacillus* spp. such as *L. jensenii, L. crispatus, and L. mucosae*, would be ideal probiotics because of their potential anti-inflammatory properties, and antipathogen features [104]. However, as the FGT microbiome has been associated with reduced efficacy of TFV, the potential benefit of *Lactobacillus* spp. during PrEP requires further investigation. Klatt *et al*. (2017) showed that when women with *Lactobacillus*-dominated FGT microbiomes were given TFV-containing PrEP, they were less likely to become HIV infected than those with microbial communities dominated by BVAB [6]. Recently, it was shown that CVL with high levels of *Lactobacillus* spp. degraded TFV inefficiently and increased the uptake of TFV by Jurkat cells, and that these processes were associated with increased HIV infection *in vitro*. Furthermore, the effect on HIV infection was also observed in the presence of co-cultured *G. vaginalis* and lactobacilli but not when single cultures of GV were added [109].

It was also reported that *Lactobacillus* spp. reduced the viability of *G. vaginalis* under co-culture conditions, with some strains of the same species more toxic than others [124]. Therefore, *Lactobacillus* spp. could be highly beneficial as probiotics during PrEP as it could reduce the viability and hence outgrowth of BVAB such as *G. vaginalis* thereby preventing degradation of the ARV and increasing the uptake of TFV by HIV permissive cells. In this study we wanted to determine whether the residual drug left in the culture medium after uptake by *G. vaginalis*, was sufficient to inhibit HIV infection and if *L. crispatus* could rescue the impact that *G. vaginalis* has on ARV levels and thus drug efficacy.

### 4.2 Results

#### 4.2.1 Culture conditions of *G. vaginalis* and *L. crispatus*

Prior to co-culture of *L. crispatus* and *G. vaginalis* the titre of *L. crispatus* single cultures was determined to be 8.6 x $10^6$ at an $OD_{600nm}$ of 0.2, 100-fold less than that of *G. vaginalis* (Table 4.1). Surprisingly, when the two species were grown separately with a starting $OD_{600nm}$ of 0.2, there was no significant difference between the slopes of the growth curves (Figure 4.1). It is

broadly accepted that the OD of bacterial culture at 600nm is directly proportional to CFU/mL, however this relationship only holds true when the number of bacteria is low ($OD_{600nm}$<0.2) and even then, the relationship is affected by medium composition and cell size [125]. Although we would have expected *G. vaginalis* to grow faster than *L. crispatus* due to the greater number of bacteria present at an $OD_{600nm}$ of 0.2, differences in shape and size of the bacterial species and variation between media composition, likely contributed to the apparent equal growth rates observed and thus, all co-culture experiments were carried out normalised to CFU/mL.

**Table 4.1: CFU/mL for bacterial strains corresponding to an $OD_{600nm}$ of 0.2.**

| Bacterial strain | $OD_{600nm}$ | Average CFU/mL |
|---|---|---|
| *Gardnerella vaginalis* (ATCC 14018) | 0.2 | $6.35x10^8$ |
| *Lactobacillus crispatus* | 0.2 | $8.66x10^6$ |

CFU/mL is the average of three independent biological repeats.

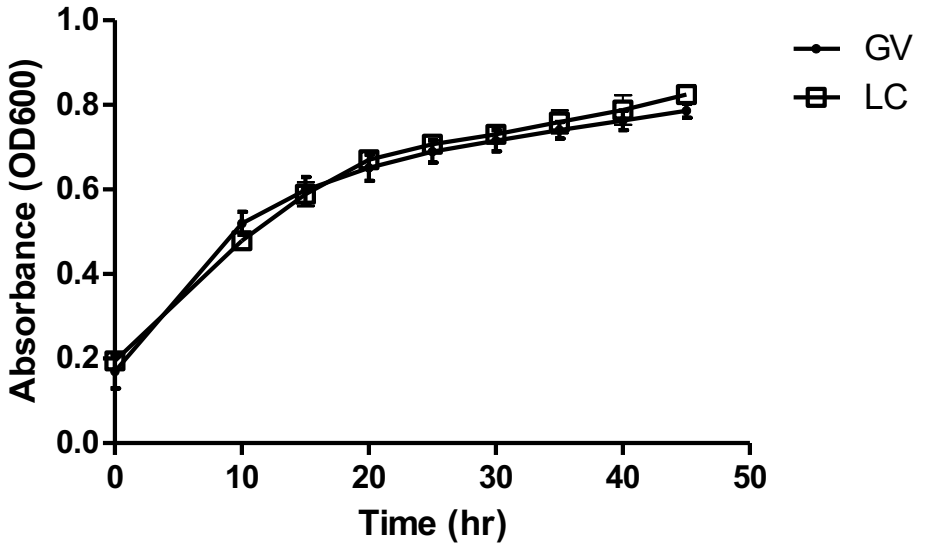



**Figure 4.1: Growth curves of *G. vaginalis* and *L. crispatus*.** NYCIII media was inoculated with *G. vaginalis* (closed circles) and *L. crispatus* (open squares) at concentrations of $6.35x10^8$ and $8.66x10^6$ CFU/mL and grown separately for 48 hours under anaerobic conditions. Absorbance readings were taken at regular intervals at a wavelength of 600nm. Three independent biological repeats were performed for each species. Exponential decay growth curve analysis was carried out using GraphPad Prism. Error bars represent standard error of the mean.

### 4.2.2 Growth of *G. vaginalis* and *L. crispatus* during co-culture

Bacterial cultures were diluted so that each species' concentration was at $5x10^4$ CFU/mL and were grown separately and together for 24 hours. To determine the presence of both species, gram stains of bacterial culture samples were carried out after 24 hours. The gram variable *G. vaginalis* cocci and gram-positive *L. crispatus* bacilli were visible in the single cultures and co-culture samples, confirming that both species were present after 24 hours (Figure 4.2). It appeared as though there was a decrease in number of both species after co-culture, possibly due to nutrient depletion. However, the relative amount of each species in the co-culture was difficult to ascertain and we thus did qPCR using primers specific to *G. vaginalis* and *L. crispatus* 16S rRNA gene to determine whether the one had outgrown the other [126]. However, the qPCR using universal 16S rRNA primers did not detect *L. crispatus* despite being shown to detect a broad range of *Lactobacillus* species and we could not normalise the qPCR of *G. vaginalis* and *L. crispatus* relative to total DNA [127]. Therefore, Ct values for each set of primers could not be compared to confirm whether *L. crispatus* had outgrown *G. vaginalis* or vice versa.

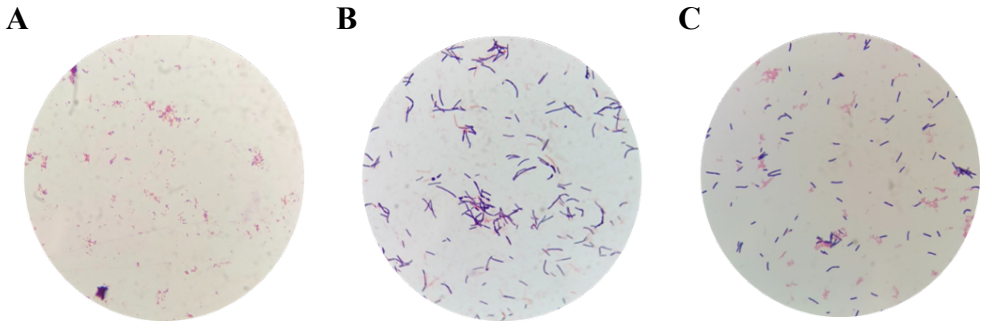



**Figure 4.2: Gram stain of *G. vaginalis* and *L. crispatus* in single and co-culture** *G. vaginalis* **(A)** and *L. crispatus* **(B)** were grown separately and together **(C)** in NYCIII media for 24 hours before a 5μL of bacterial culture was gram stained and photographed.

The primer sets were highly specific as amplification was only detected from the single cultures of the respective species. Amplification of the *L. crispatus*-specific 16S rRNA gene occurred later than that for the *G. vaginalis*-specific primers, potentially suggesting that *L. crispatus* titre was lower than that of *G. vaginalis*. However, without controlling for primer-binding efficiency, consistent DNA extraction and total genomic DNA (gDNA), no conclusion can be made [128]. Amplification signal was detected for *G. vaginalis* single culture and co-culture samples using the *G. vaginalis*-specific primer set (Figure 4.3A). Interestingly, the

amplification signal from the co-culture was detected at a later cycle compared to that of the single culture. This could suggest that the amount of *G. vaginalis* was reduced in the presence of *L. crispatus* relative which would support the results of the gram stain. The qPCR signal using the *L. crispatus*-specific primers appeared at the same time for both the single and co-culture samples, suggesting that the number of bacteria did not change in the presence of *G. vaginalis*. However, without determining total gDNA, we could not quantify the relative levels of the bacterial species and thus could not determine whether *L. crispatus* outgrew *G. vaginalis* in the co-culture.

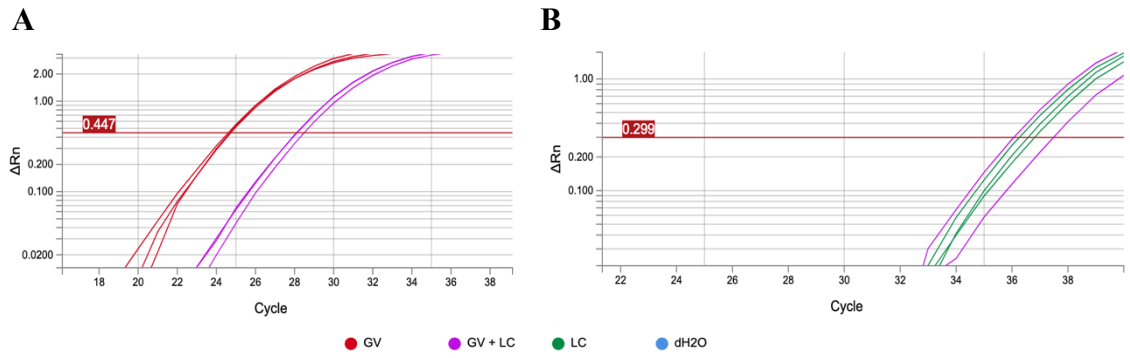



**Figure 4.3: Detection of species-specific genes in the co-culture of *L. crispatus* and *G. vaginalis* by qPCR.** *G. vaginalis* and *L. crispatus* single (GV or LC) and co-cultures (GV + LC) were grown for 24 hrs before genomic DNA (gDNA) was extracted. **(A)** The gDNA from the LC and GV + LC cultures was amplified using LC-specific qPCR primers LcrisF and LcrisR. **(B)** The gDNA from the GV and GV + LC cultures was amplified using GV-specific qPCR primers R-GV3 and F-GV1.The negative control ($dH_2O$) did not yield a signal.

### 4.2.3 Uptake of ARVs by *G. vaginalis* in the presence of *L. crispatus*

As shown, *G. vaginalis* takes up TFV, TDF, MVC, and ABC (Figures 3.4 and 3.5) and the decrease in ARV concentration in the supernatant appeared to coincide with an increase in infection (Figure 3.6). As *L. crispatus* has been shown to have a beneficial effect in the FGT, it was determined whether the lactobacilli could reduce *G. vaginalis* uptake of ARVs.

#### 4.2.3.1 The effect of *L. crispatus* on levels of TFV associated with *G. vaginalis* cell lysate

Samples were analysed by MS as outlined in the materials and methods section. Similar to a previous study, TFV was identified in the *L. crispatus* cell lysates albeit approximately 7-fold lower than that of *G. vaginalis* (Figure 4.4A), indicating that *L. crispatus* was less efficient at uptake than *G. vaginalis*. The concentration of TFV associated with the cell lysates from single cultures were similar to that of the co-cultures even though there should be twice the number of cells. However, if there was a reduction of bacterial numbers in co-culture as suggested by gram stain, we would expect the level of TFV to be similar in single and co-cultures. Although, there were lower levels of TFV in the supernatant of *L. crispatus* single cultures compared to both *G. vaginalis* culture and co-culture samples, this is likely due to experimental error. Therefore, there was no apparent effect of *L. crispatus* on *G. vaginalis* uptake in co-culture despite being able to take up TFV itself.

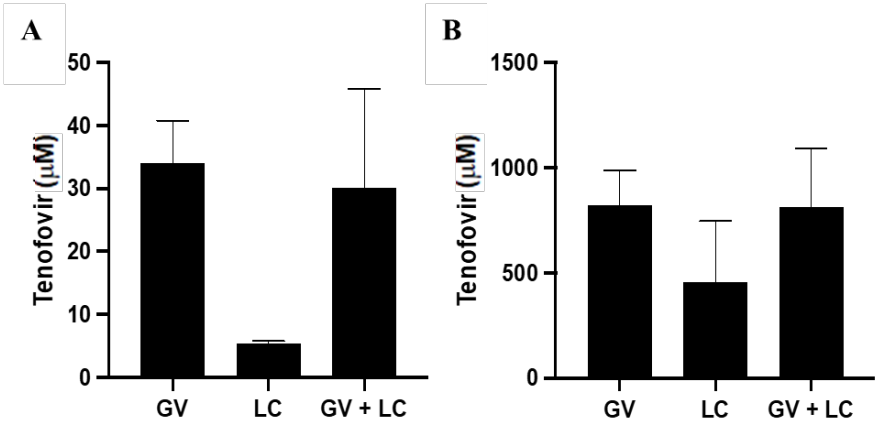



**Figure 4.4. Effect of *L. crispatus* on TFV uptake by *G. vaginalis* in co-culture.** The concentration of **(A)** internalised TFV and **(B)** extracellular drug in the cell-free supernatant was assessed by HPLC-tandem mass spectrometry. *G. vaginalis* and *L. crispatu*s single (GV or LC) and co-cultures (GV+LC) were tested. Bars represent the average of two independents biological repeats and error bars represent standard error of the mean.

#### 4.2.3.2 Detection of MVC and ABC associated with *L. crispatus* cell lysate

Cheu *et al.* (2020) showed that lactobacilli-dominated cervical lavages increased the uptake of TFV and DAP by Jurkat cells but had no effect on TAF [109]. The authors suggested that the effect of the FGT microbiome on ART drugs could be ARV-dependent. We showed that *G. vaginalis* is able to take up MVC and ABC and as *L. crispatus* cell lysates were positive for

TFV, we determined whether the species was also able to take up ABC and MVC. As illustrated in Figure 4.5, the MS results revealed the presence of MVC and ABC in *L. crispatus* cell lysates. Therefore, *L. crispatus* might also be able to affect the efficacy of ABC and MVC if provided as a probiotic during PrEP.

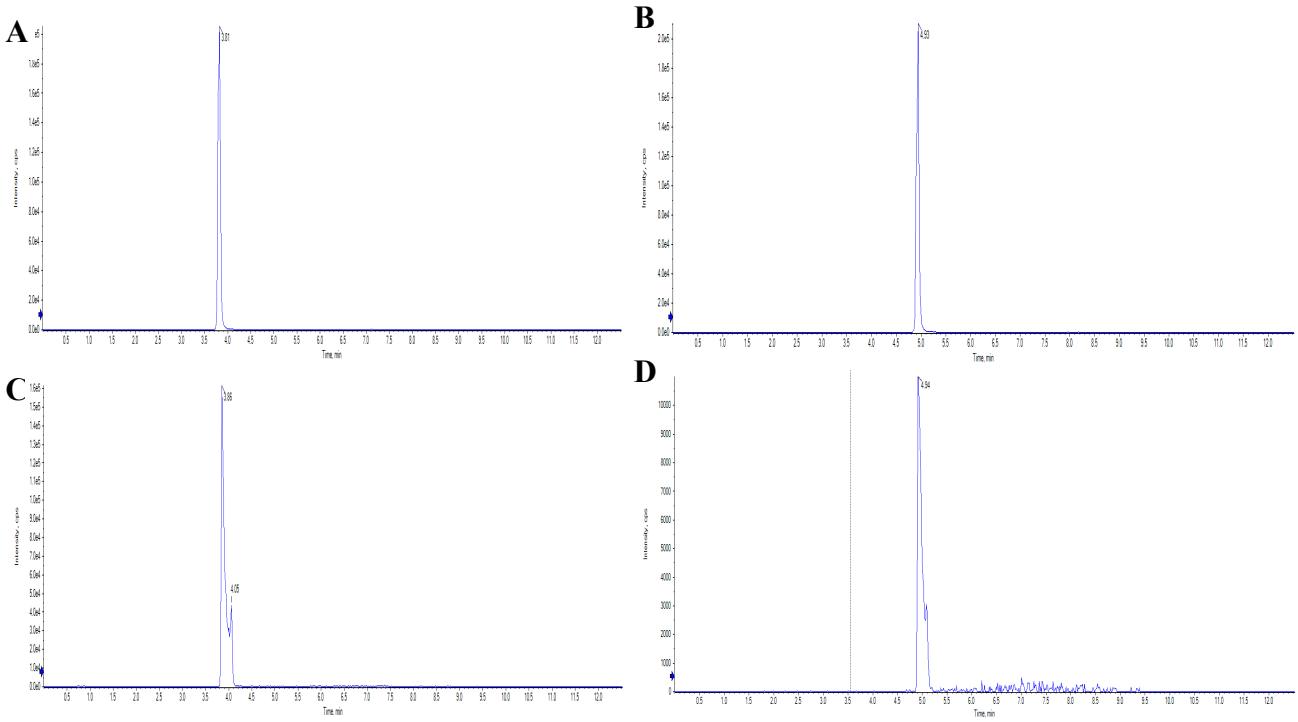



**Figure 4.5. Identification of ARVs associated with *L. crispatus* cell lysates.** MVC and ABC uptake was determined by mass spectrometry. **(A)** Spectra was determined for ABC (100nM), **(B)** MVC (100nM) standards, Spectra of **(C)** *L. crispatus* cell lysate after 24 hrs culture in the presence of ABC, and **(D)** *L. crispatus* cell lysate grown for 24 hrs in the presence of MVC. Retention of the standards was used to identify the presence of the ARVs in the samples.

### 4.2.4 Impact of *L. crispatus* and *G. vaginalis* co-culture on ARV efficacy

As observed previously (Figure 3.6), the addition of *G. vaginalis* supernatant harvested after culture with TFV and TDF for 24 hours reduced relative infectivity by approximately 20-25% (Figure 4.6A). *L. crispatus* supernatant had the same effect, suggesting that the lactobacilli reduced TFV and TDF to similar levels as *G. vaginalis*. However, even though *L. crispatus* and *G. vaginalis* were both present, ostensibly both internalizing TFV and TDF, the change in TFV concentration in the co-culture was the same as that observed for the single cultures. As suggested before, this could be because of the decrease in bacterial numbers during co-culture

as a result of nutrient depletion and/or inter-bacterial growth effects resulting in one species displacing the other such as decreased pH limiting the growth of *G. vaginalis*. When the cell lysates of *L. crispatus* and *G. vaginalis*, grown either singly or as co-culture, were added to the infection assay, there was no difference compared to when the drug was absent (Figure 4.6C). This supported previous data (Figure 3.7) that either the concentration of the ARV taken up by the bacteria was too low to inhibit infection or cellular factors were affecting ARV activity.

MVC is an entry inhibitor and we showed that *G. vaginalis* (Figure 3.5) and *L. crispatus* (Figure 4.5) take up the ARV. Furthermore, we found that the addition of MVC at its $IC_{100}$ concentration to the infection assay resulted in approximately 50% inhibition at t0 (Figure 3.8). Once exposed to *G. vaginalis* for 24 hours in culture (t24), MVC in the supernatant reduced HIV infectivity by approximately 20%. Similar to TFV and TDF, the change in inhibition from 50% to 20% could be due to bacterial uptake decreasing MVC bioavailability in the supernatant or soluble factors influencing the efficacy of MVC.

On the contrary, exposure of MVC to *L. crispatus* in culture had no effect on its ability to inhibit infection (Figure 4.6B). This result is unexpected because MS determined the presence of MVC in *L. crispatus* cell lysates, suggesting uptake. Perhaps, the amount of MVC internalized was too low to affect its bioavailability in the supernatant. Interestingly, the supernatant of the co-culture also inhibited infection by 50%, suggesting that the presence of *L. crispatus* prevented *G. vaginalis* uptake or metabolism of MVC or inhibited the action of soluble factors present in the supernatant. Similar to *G. vaginalis*, the *L. crispatus* cell lysates had no effect on HIV infection, suggesting the concentration of the internalized drug was too low to inhibit PSV entry (Figure 4.6D). Unfortunately, the MS method did not allow for quantification of the concentration of MVC taken up by *G. vaginalis* and *L. crispatus*.

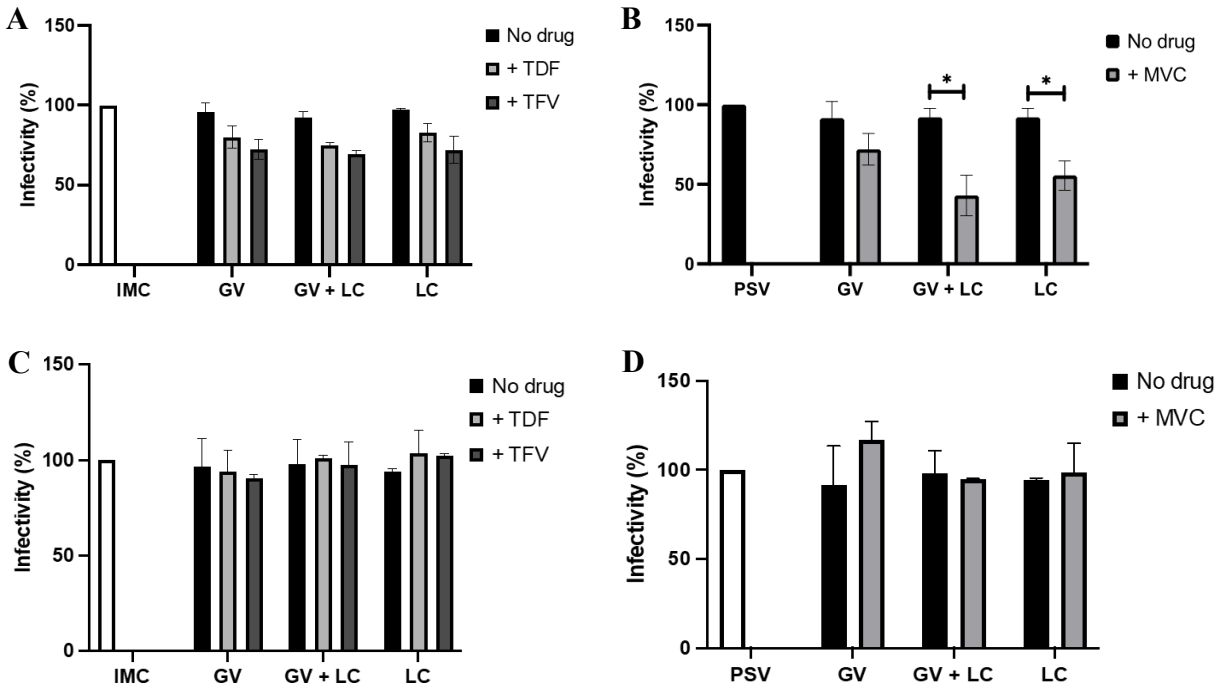



**Figure 4.6: Effect of *L. crispatus* and *G. vaginalis* co-culture on HIV infectivity in the presence of ARVs.** *L. crispatus* and *G. vaginalis* were grown separately and together and the growth media was centrifuged before the cells were lysed by sonication. The supernatant **(A and B)** and cell lysates **(C and D)** were diluted and 10µg of total protein was added to each well containing TZM-bl cells infected with either **(A and C)** infectious molecular clones (IMC) or **(B and D)** pseudovirus (PSV). Luciferase assay was performed to assess HIV infectivity *in vitro* and relative light units (RLU) were normalised to the positive control (infection in the absence of bacterial sample and ARVs) and shown as percentage infection. TZM-bl cells were infected with IMC in the presence of *L. crispatus* and *G. vaginalis* supernatants from cultures grown for 24 hrs separately (GV or LC, respectively) and together (GV+LC) in the presence of **(A and C)** tenofovir disoproxil fumarate (TDF) or tenofovir (TFV) and **(B and D)** maraviroc **(**MVC) at their $IC_{100}$ concentrations. Bars represent the average of three independent biological repeats with error bars representing standard deviation.

### 4.3 Discussion

*Lactobacillus* species are typically associated with "healthy" vaginal microbiota. They contribute to the maintenance of an acidic environment in the female genital tract, which is less conducive to the growth of harmful bacteria. *Lactobacillus* spp., specifically *L. crispatus*, has been shown to be associated with lowered HIV infection. *Lactobacillus* spp. have also been shown to inhibit *G. vaginalis* growth, adhesion and biofilm formation, indicating that it could play a very important role in preventing BV [129]. Therefore, co-culture of *L. crispatus* and *G.*

*vaginalis* should result in a decrease in the titre of the latter. Gram stain and qPCR suggested that the growth of GV was slowed but the results were inconclusive as to whether the one species was able to displace the other. However, importantly, we showed that after 24 hours of co-culture both *L. crispatus* and *G. vaginalis* were present. This had implications when investigating the extent at which the ARVs were taken up by the bacteria during co-culture.

The composition of the FGT microbiome impacts the i*n vivo* efficacy of TFV as shown in the FAME 04 and CAPRISA 004 studies, where women were less likely to become HIV infected if the microbiome was dominated by *Lactobacillus* spp. [6, 130]. Cheu *et al.* (2020) went further and reported that lactobacilli seemed to enhance the uptake of TFV and DAP by Jurkat cells and were less able to degrade ARVs compared to BVAB-dominated CVL [109]. Overall, this would suggest that *Lactobacillus* spp. could counter the effect of BVAB on the efficacy of ARVs in the FGT. Conversely, another study found that *L. crispatus* actively internalised and metabolised TFV, which would ostensibly reduce the bioavailability of the drug [42]. The CONRAD 128 study reported that continuous delivery of TFV through an intravaginal ring maintained high concentrations of TFV in the cervicovaginal fluid that met the criteria for inhibiting HIV-1 [131], regardless of whether the microbiome was dominated by *Lactobacillus* spp. or anaerobes associated with BV. This suggested that both species did not have an effect on the bioavailability of drugs if high concentrations of ARVs were maintained in the FGT.

Therefore, our study aimed to confirm the uptake of TFV by *G. vaginalis* and determine whether the presence of *L. crispatus* in co-culture would counter the effect of *G. vaginalis* on the bioavailability of TFV, TDF, ABC, and MVC. Similar to a previous study, we detected TFV in the cell lysates of *L. crispatus* by MS [42]. The uptake of TFV by *L. crispatus* was less efficient than that of *G. vaginalis*, but there was high variation between experiments potentially due to culture conditions. Analysis of vaginal samples identified the density of *Lactobacillus* spp. as $10^7$ – $10^8$ CFU/mL [132] and the density of *G. vaginalis* was either lower or higher than 2 x $10^7$ CFU/mL depending on whether the individuals were BV negative or positive, respectively [133]. Single cultures of *G vaginalis* were initiated with an inoculum of 6.5 x$10^8$ CFU/mL to maximise detection of internalized ARVs and reflect physiologically relevant bacterial titres. However, after 24 hours, growth had plateaued, suggesting that bacterial cell death could be affecting accurate determination of intracellular and extracellular ARV concentrations. Therefore, to investigate the effect of co-culture on ARV efficacy, single and dual cultures were initiated with 5x$10^5$ CFU/mL of both *L. crispatus* and *G. vaginalis* which

was similar to a previous report [134]. Cultures were inoculated with lower CFU/mL to ensure exponential growth of the bacteria and thus maximise the bacterial metabolism of the ARVs. However, the bacterial titre might have been too low to detect TFV in the cell lysates and also influence the ability to detect small changes in TFV concentration in the supernatant.

One would expect that the total amount of TFV in the cell lysates of co-cultures would be higher than single cultures given that both *L. crispatus* and *G. vaginalis* are taking up the ARV. Conversely, supernatants of co-cultures should have lower levels of TFV compared to single cultures. However, the TFV levels in *G. vaginalis* lysates and supernatants were similar to that of the co-culture, suggesting that *L. crispatus* was not affecting the amount of TFV taken up by *G. vaginalis*. It is possible that the MS method was not sensitive enough to detect small changes in total TFV because the amount of TFV taken up by *L. crispatus* was too low or the method underestimated the level of internalised ARVs. Alternatively, as *L. crispatus* and *G. vaginalis* were shown to degrade TFV, it is possible that the disappearance of intact TFV due to variable rates of degradation affected the levels in the samples [109]. Finally, *L. crispatus* has been shown to reduce the viability of *G. vaginalis* which would affect the total TFV taken up by the bacteria in culture [129].

To determine whether decreased concentration of ARVs in the supernatant led to decreased inhibition of HIV infection, and conversely, if internalised ARVs were able to inhibit infection, the supernatants and cell lysates of *L. crispatus* and *G. vaginalis* cultures were added to TZM-bl cells infected with either PSV or IMCs. Prior to incubation with *L. crispatus* and *G. vaginalis* i.e. t0, TFV, TDF and MVC at concentrations greater than their $IC_{100}$ values, only inhibited infection by 40-50%, suggesting that the culture medium, NYCIII was having an effect on its activity. However, we rationalised that any decrease in inhibition to below 40% would suggest a decrease in ARV efficacy. When *L. crispatus* and *G. vaginalis* cell lysates were added to TZM-bl cells after incubation with TFV, TDF, and MVC (t24) there was no effect on infectivity despite confirmation of their presence by mass spectrometry. This suggested that either concentration was too low because of poor uptake, internalisation affected their activity or factors present in the cell lysates interfered with inhibition.

When supernatants with TFV and TDF from single *L. crispatus* and *G. vaginalis* cultures were added to the assay, infection was inhibited by approximately 25% and 20%, respectively. The increase in IMC infection in the presence of TFV and TDF indicated a decrease in efficacy. As

*L. crispatus* and *G. vaginalis* were able to internalise TFV and TDF, it is likely that the decrease in inhibition is due to bacterial uptake. The co-culture of *L. crispatus* and *G. vaginalis* also reduced TFV and TDF inhibition by 20% and thus had the same effect as the single cultures. Overall, this suggests that both *L. crispatus* and *G. vaginalis* have a negative effect on the ability of TFV and TDF to inhibit IMC infection i*n vitro*. However, the effect of *L. crispatus* and *G. vaginalis* culture on TFV and TDF efficacy was not statistically significant and thus the assay needs to be optimised. Despite adding the ARVs in excess, TFV and TDF did not completely inhibit infection. Therefore, the effect of the bacteria on ARV bioavailability might have been masked by other factors present in the culture medium. Potentially, partial purification and enrichment of the ARVs in the supernatants would allow for improved quantification and removal of negative effectors.

*G. vaginalis* had the same effect on MVC as it had on TDF and TFV, suggesting that despite very different structures and uptake mechanisms, *G. vaginalis* was still able to internalise the entry inhibitor and reduce its efficacy, albeit non-significantly. According to our knowledge, we are the first to report that *L. crispatus* is able to internalise ABC and MVC, similar to that of *G. vaginalis*. However, in the case of MVC, this did not translate into reduced ARV efficacy because supernatants of *L. crispatus* culture inhibited infection to the same extent as MVC without incubation with the lactobacilli i.e. 50%. Interestingly, the supernatant of the co-culture also had no effect on MVC efficacy, suggesting that *L. crispatus* was able to mitigate the effect of *G. vaginalis* on the efficacy of the drug. Therefore, MVC might be a better candidate for PrEP and microbicide formulations as its efficacy might not be compromised in the FGT dominated by *Lactobacillus* spp.

# Chapter V

## 5.1 Conclusions

BV has been associated with suboptimal concentrations of ARV drugs in the genital tract, potentially impacting HIV transmission [6]. Additionally, the presence of BV has been linked to inflammation in the female genital tract, which can further complicate the effectiveness of TFV and other antiretroviral drugs [135, 136]. Previous studies have provided conflicting evidence as to the mechanism by which *G. vaginalis* affects the efficacy of TFV. These include bacterial uptake, inhibition of host cell endocytosis, conversion to adenine, or other metabolites, and degradation. In our study we did not aim to determine the mechanism by which *G. vaginalis* decreases the efficacy of TFV but confirm that *G. vaginalis* was able to take up TFV, and that after internalisation, the concentration of extracellular TFV would be too low to inhibit HIV infection. For this reason, our methodological approach did not include TFV in infection assays in the presence of *G. vaginalis* samples. Instead, we first exposed the ARV to *G. vaginalis* for 24 hours before adding the cell culture supernatant and bacterial cell lysates to the infection assay. Similarly, we determined whether *L. crispatus* affected the efficacy of TFV, in the absence and presence of *G. vaginalis*. Despite the potential need to optimise the assays used in this study, we showed that *L. crispatus* and *G. vaginalis* are able to internalise TFV, MVC, and ABC. According to our knowledge the uptake of MVC has not previously been reported and the finding adds a new dimension to the role of *G. vaginalis* in ARV efficacy as MVC does not need to be internalised to prevent infection, contrary to TFV and ABC. Furthermore, the presence of *L. crispatus* in co-culture seemed to abrogate the effect of *G. vaginalis* on the efficacy of MVC. These findings suggest that PrEP microbicide formulations containing MVC in conjunction with a *L. crispatus* probiotic could provide an alternative means to prevent HIV transmission. However, changes in ARV efficacy in the presence of *L. crispatus* and *G. vaginalis* was not significant, indicating that further analysis is important.

Despite these concerns, it is tempting to speculate that the differential effect of *L. crispatus* and *G. vaginalis* co-culture on the efficacy of MVC vs TFV, TDF and ABC could be due to differences in uptake mechanisms or site of activity. TFV is taken up by active transport or endocytosis and TDF by passive diffusion whereas MVC is active extracellularly. Further investigations on uptake mechanisms should be carried out in order to determine specific

transporters involved in the uptake and metabolism of ARVs by *L. crispatus* and *G. vaginalis*. Understanding how different species of bacteria can affect drug availability and effectiveness can aid in the development of targeted therapies to improve treatment outcomes in the medical field, especially in women's health.

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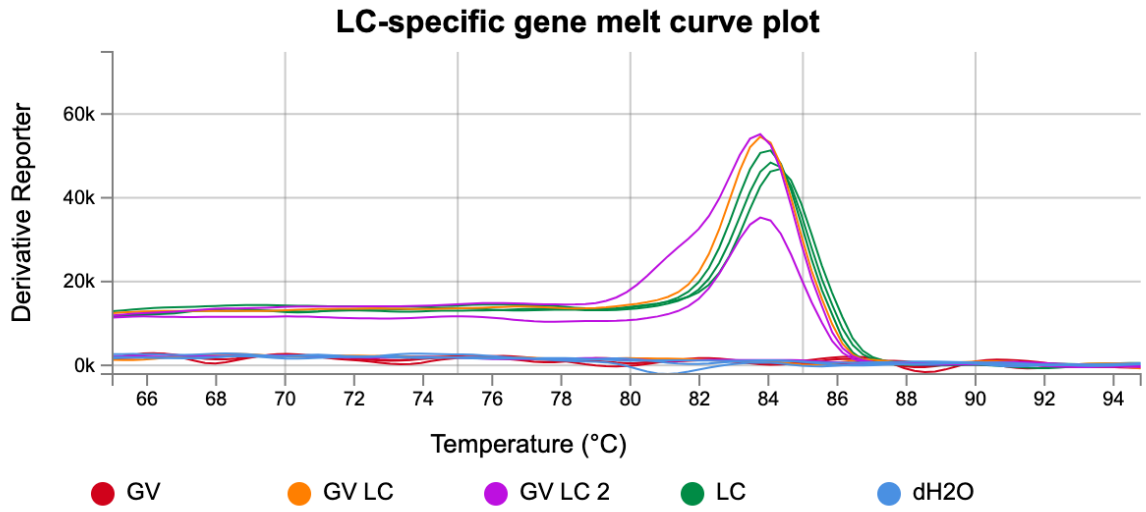
## Supplementary Data

**A**




**B**

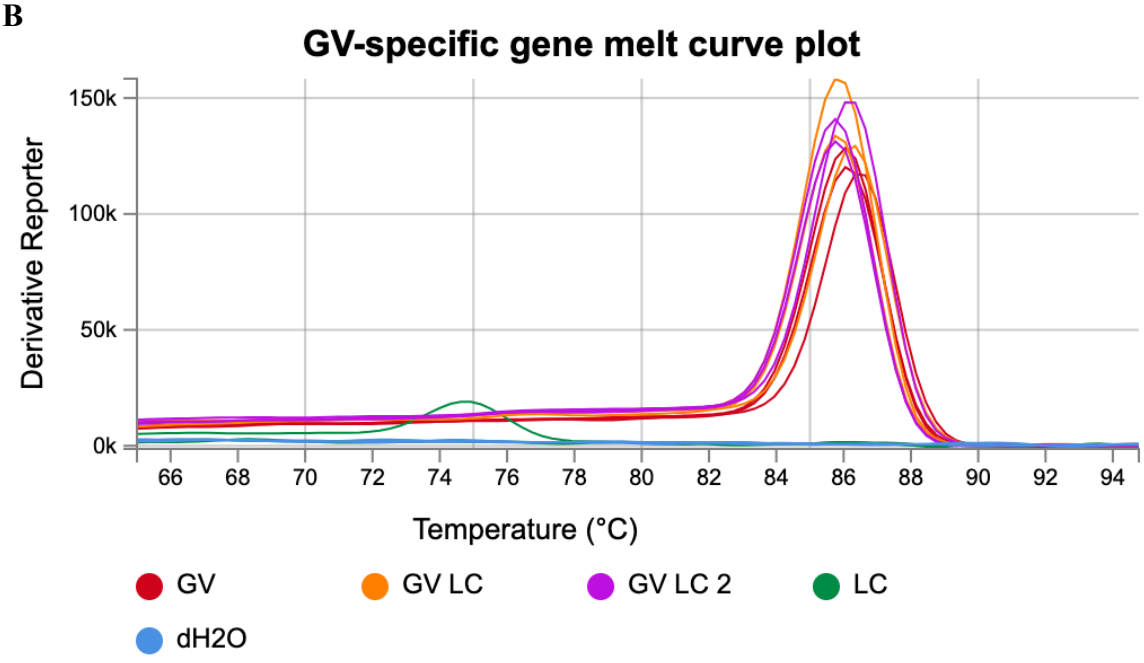



**Figure 5**: **qPCR melting curves.** *G. vaginalis* and *L. crispatus* single (GV or LC) and co-cultures (GV + LC) were grown for 24 hrs before genomic DNA (gDNA) was extracted. **(A)** LC-specific and **(B)** GV-specific genes. The negative control ($dH_2O$) did not yield a signal.