Investigation of the interaction between antiretroviral drugs and the mucosal microbiome in African women.

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Preface

The research contained in this dissertation was completed under the Discipline of Biochemistry, School of Life Sciences at the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg campus, South Africa. The research was carried out from January 2020 to December 2022, under the supervision of Dr. Pamela P. Gumbi and co-supervision of Prof. Theresa H.T. Coetzer. The contents of this work are original and have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate. Where use has been made of the work of others, it has been duly acknowledged in the text.

[redacted] _____

Signed: Nomacusi Sibeko

Date: 02/12/2022

As the candidate's supervisors, we agree to the submission of this dissertation.

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Signed: Dr. Pamela P. Gumbi

Supervisor

Date:02/12/2022

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Signed: Prof. Theresa H.T. Coetzer

Co-supervisor

Date:02/12/2022

Declaration: Plagiarism

I, Nomacusi Sibonganjalo Lindiwe Sibeko, declare that:

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Abstract

The use of antiretroviral drugs (ARVs) as both a treatment and pre-exposure prophylaxis (PrEP) against the human immunodeficiency virus (HIV) has increased exponentially over the past few years. Each ARV drug works by targeting HIV at different phases of its replication cycle, however, their off-target effects, especially for the drugs in the pipeline, remain unknown. Previous studies have shown that there is some evidence of interaction between ARVs and the mucosal microbiota, even though this relationship is not completely understood. This study aimed to isolate gut *Lactobacillus* species and to characterise beneficial gut and vaginal *Lactobacillus* species (spp.) from HIV-negative South African adolescent girls and young women. The antibacterial activity of selected antibiotics and ARVs on the vaginal and gut microbiota associated with healthy microbiota was also investigated. Gut microbiota *Lactobacillus* spp. were isolated from rectal swabs of n=11 South African women aged 14 to 24 years. Bacterial isolates (n=10) were identified by 16S rRNA gene sequencing and four morphologically distinct bacterial isolates, (*Enterococcus faecium, L. fermentum, L. plantarum,* and *Lacticaseibacillus rhamnosus*) were selected for further analysis. In addition, previously isolated *Lactobacillus* species (*L. crispatus, L. gasseri, L. jensenii, L. mucosae,* and *L. vaginalis*) from the vagina and gut-derived American Type Culture Collection (ATCC) strains (*Bifidobacterium (B.) animalis, B. breve, E. faecalis, E. faecium, L. gasseri,* and *L. lactis*), were included for further evaluations. The bacteria were characterised by testing the production of lactic acid, and hydrogen peroxide (H_2O_2) using colorimetric assays. The ability to reduce culture pH was measured using a pH meter. Finally, the antibacterial activity of various antibiotics and ARVs against the bacteria was also tested using minimum inhibition assays. The growth of *L. rhamnosus* and the ATCC of *E. faecium* was not affected by pH changes, while *L. plantarum* was susceptible to most pH values. The vaginal *Lactobacilli* produced more D-lactate compared to L-lactate, while the gut bacteria produced L-lactate and did not produce significant amounts of D-lactate. *Lactobacillus vaginalis* produced significantly lower levels of both D- and L-lactate compared to the other vaginal species which produced similar amounts of both conformations of lactate. *Lactobacillus rhamnosus* produced significantly higher levels of both D- and L-lactate compared to other gut *Lactobacillus* spp., *Bifidobacterial spp.* and *Enterococcal* spp., which produced similar amounts of both conformations of lactate. Only the vaginal bacteria produced significant amounts of H_2O_2, and the vaginal bacteria were more susceptible to antibiotics compared to gut bacteria. The production of lactic acid and H_2O_2 by the gut isolated *E. faecium* was comparable to the ATCC strain. However, the gut isolated *E. faecium* was more susceptible to antibiotics compared to the ATCC strain. Dolutegravir (DTG) exhibited the greatest

potency followed by the tenofovir/lamivudine/dolutegravir (TLD) combination, tenofovir disoproxil fumarate (TDF), and to a lesser extent, tenofovir alafenamide (TAF), exhibiting growth inhibitions that were more than 50% in some cases. Cabotegravir (CAB) was the least potent and only inhibited the growth of the ATCC strain of *E. faecium.* Overall, *E. faecium* and *L. gasseri* were the most susceptible species of the gut and vagina, respectively, while *L. fermentum* and *L. rhamnosus* were the least susceptible to any of the ARVs. The present study identified *L. rhamnosus* as an ideal probiotic over the other species due to its ability to produce the most L-lactate, resistance to pH changes, antibiotics and ARVs. This study demonstrated the antibacterial activity of ARVs *in vitro,* implying that the ARVs may lead to dysbiosis which may lead to adverse health outcomes. However, the mechanism that these ARVs use to inhibit the growth of bacteria remains unknown as the ARVs had no impact on pH, suggesting a different mechanism of inhibition. Further *in vitro* and *ex vivo* studies are necessary to investigate the antibacterial effects of ARVs and the mechanisms that these drugs use to inhibit the growth of beneficial bacteria.

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List of abbreviations

Abbreviation	Full name
3TC	Lamivudine
AIDS	Acquired immunodeficiency syndrome
P	P-value
ART	Antiretroviral therapy
ARV	Anti-retroviral
ATCC	American Type Culture Collection
AZT	Zidovudine
BV	Bacterial vaginosis
C-section	Caesarean section
CAB	Cabotegravir
CCR5	C-C chemokine receptor type 5
CFU	Colony forming unit
CXCR-4	C-X-C chemokine receptor type 4
BHI	Brain heart infusion
d4T	Stavudine
ddI	2′,3′-dideoxyinosine
DMPA	Depot medroxyprogesterone acetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTG	Dolutegravir
EFV	Efavirenz
EtOH	ethanol
FDA	The Food and Drug Administration
FTC	Emtricitabine
Gag	Group-specific antigen
gp	Glycoprotein
HIV	Human immunodeficiency virus
HSV	Human simplex virus
IL	Interleukin
INSTIs	Integrase strand transfer inhibitors

LAB	Lactic acid-producing bacteria
LPS	Lipopolysaccharides
MeOH	Methanol
MRS	De Man, Rogosa and Sharpe
NAD	Nicotinamide adenine dinucleotide
Nef	Negative regulatory factor
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside-analogue reverse transcriptase inhibitors
NVP	Nevirapine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIs	Protease inhibitors
PrEP	Pre-exposure prophylaxis
RNA	Ribonucleic acid
RTIs	Reverse transcription inhibitors
SCFA	Short-chain fatty acids
STI	Sexually transmitted infection
TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
TFV	Tenofovir
Th	Helper T-cells
TLD	Tenofovir/lamivudine/dolutegravir
TMAO	Trimethylamine N-oxide
TNF	Tumour necrosis factor
UNAIDS	The Joint United Nations Programme on HIV/AIDS
Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
WHO	World health organisation

CHAPTER 1

Introduction

1.1. Background

According to the Joint United Nations Programme on HIV/AIDS (UNAIDS) 2022 report there were 38.4 million [33.9 million–43.8 million] people living with HIV in 2021 (UNAIDS, 2022). It is estimated that 54% of all people living with HIV were women and girls. Additionally, girls and young women aged 15–24 years are twice as likely to be living with HIV as young men in sub-Saharan Africa. Eastern and Southern Africa have the highest number of people living with HIV in the world 20.6 million [18.9 million– 23.0 million] with an estimated 670 000 [530 000– 900 000] new infections in 2021. Moreover, AIDS-related deaths have been reduced by 68%. The reduction in AIDS-associated morbidity and mortality is mainly due to the development of a large repertoire of anti-retroviral (ARV) drugs. Access to antiretroviral therapy (ART) has increased exponentially since 2010 from 7.8 million to 28.7 million people accessing ART at the end of 2021 (UNAIDS, 2022; UNAIDS, 2022).

Although remarkable progress has been made in changing the face of HIV infection from a progressive deadly infection to a chronic, mostly manageable condition, the number of new HIV infections remains high. Therefore, in addition to the use of ART for people infected with HIV, ARVs can be taken by HIV-negative people as pre-exposure prophylaxis (PrEP). Pre-exposure prophylaxis is a biomedical intervention against HIV transmission with highly variable efficacy (Günthard *et al.*, 2016). Tenofovir disoproxil fumarate (TDF) alone or in combination with emtricitabine (FTC), which blocks HIV reverse transcription, is used widely for both treatments of HIV and as daily oral PrEP. Adherence to daily intake of antiretroviral drugs is key for optimal PrEP efficacy. Alternatives to avoid the need for daily oral PrEP dosing and to minimise long-term side effects of TDF/FTC are now in the pipeline (Landovitz *et al.*, 2018). These options include tenofovir alafenamide (TAF), a new prodrug of tenofovir that can prevent HIV infection at a lower dose than TDF because it delivers tenofovir more efficiently to cells than TDF (Arribas *et al.*, 2017). As a possible alternative option to TDF, TAF implant is currently being explored in mice models (Gunawardana *et al.*, 2022). Another option in the pipeline for HIV prevention is

Cabotegravir (CAB), a long-acting injectable antiretroviral drug that is a potent integrase strand transfer inhibitor (Zhou *et al.*, 2018).

Although the main purpose of ARVs in the context of HIV is to treat and/or prevent viral infection with different classes of ARVs acting at various stages of the HIV life cycle, there is some evidence for the interaction between ARVs and the commensal microbiota. The microbiota refers to all the microorganisms that inhabit a particular environment while the microbiome is all the microorganisms and their genes, proteins, lipids, secretions and excretions in a particular environment (Berg *et al.*, 2020). Firstly, some ARVs may have an antibacterial effect as shown by an *in vitro* study by Shilaih *et al*. (2018) where it was demonstrated that efavirenz (EFV), zidovudine (AZT), and 2′,3′-dideoxyinosine (ddI) may have antibacterial effects against *Bacillus subtilis* and/or *Escherichia (E.) coli* in the human gut (Shilaih *et al.*, 2018). Secondly, there is some evidence that microbiota composition may impact ARV metabolism which may impact ARV/PrEP efficacy (Klatt *et al.*, 2017). Importantly, the efficacy is impacted by the composition of both the gut and the vaginal microbiota. Several bacterial enzymes such as cytochromes and oxidases are known to play a role in drug pharmacokinetics and metabolism (Swanson, 2015; Taneva *et al.*, 2018). A *Gardnerella vaginalis*-dominated vaginal microbiota was associated with poor efficacy of topical PrEP (TDF gel), and other microbes of the vaginal microbiota were able to directly metabolise TDF *in vitro* (Klatt *et al.*, 2017; Cheu *et al.*, 2020). Other bacteria, which are also found in the gut, such as *Prevotella (P.)* species and *E. coli*, can metabolise tenofovir (TFV) *in vitro*, indicating that ARV metabolism may be impacted by bacteria commonly found in the gut and vagina, particularly bacteria that are associated with poor health (Klatt *et al.*, 2017). The role of the vaginal microbiota in modulating drug pharmacokinetics was further confirmed *in vitro* by Taneva and colleagues. (2018). They observed that TFV was absorbed and metabolised to adenine by human Jurkat cells, *L. crispatus* and *G. vaginalis* (Taneva *et al.*, 2018). However, this was not true for TDF and TAF as they were not taken up by either the cells or the bacteria (Taneva *et al.*, 2018).

The effect of ARVs on the mucosal microbiota across the human population may vary due to the microbial diversity that exists. Several studies have demonstrated a vast microbial diversity in both the female genital tract and gut that is highly variable both over time and across the human population (Eckburg *et al.*, 2005). In addition to genetic differences, the microbiota also differs as

a result of several factors including environmental factors, hygiene, diet, ethnicity, sexual practices, hormone levels, smoking, antibiotic and probiotic use (Lozupone *et al.*, 2012; Bayigga *et al.*, 2019).

The vaginal microbiota of healthy women is mainly dominated by *Lactobacillus* species such as *L. crispatus, L. gasseri,* and *L. jensenii* which are associated with healthy pregnancy outcomes and reduced risk of sexually transmitted infections (STIs), including HIV and bacterial vaginosis (BV) (Ravel & Brotman, 2016). This description of a healthy genital tract may not be perfectly applicable to women of African descent (Anahtar *et al.*, 2015). A multiracial study conducted in North America reported differences in the composition of the vaginal microbiota of women of different races, where 39% and 34% of black and Hispanic women, respectively, had vaginal microbiota dominated by anaerobic microorganisms such as *Gardnerella vaginalis* and *P. bivia* (Jespers *et al.*, 2017). Similar studies reported that the vaginal microbiota of women of European descent was dominated by *L. crispatus* whilst those of women of African descent were mainly dominated by *L. iners* (Borgdorff *et al.*, 2014; Gosmann *et al.*, 2017).

Contrary to the vaginal microbiota, the gut microbiota is diverse and is dominated by anaerobes. The major microbiota taxa commonly shared in the gut include *E. coli, Faecalibacterium praunsnuitzi, Roseburia intestinalis, Bacteroides uniformis, Shigella, Ruminococcus* spp.*, Treponema* spp.*,* and *Xylanbacter* spp. (Lozupone *et al.*, 2012). The most notable variability in gut microbiota is the differences in the abundance of *Prevotella* and *Bacteroides* which are impacted by diet (Cronin *et al.*, 2021). *Prevotella* is associated with diets that are fibre-rich while *Bacteroides* are associated with protein-rich diets (Wu *et al.*, 2011; De Filippis *et al.*, 2016; Klimenko NS, 2018; Jiang *et al.*, 2022). Several studies undertaken in America characterised the gut microbiota of women of African and European descent. They discovered that women of African descent had gut microbiota dominated by *Prevotella* with lower levels of *Bacteroides*, while women of European descent had the inverse ratio of gut microbiota, where *Bacteriodes* were more abundant than *Prevotella* (Ou *et al.*, 2013; Roager *et al.*, 2014; Gorvitovskaia *et al.*, 2016).

ARV drugs themselves have been found to cause microbial dysbiosis (Liu *et al.*, 2019) and distinct ARV combinations could have different effects on the vaginal and gut microbiota. These data suggest that there is a need for studies that will further evaluate the impact of ARVs on vaginal and gut microbiota. Performing these studies with bacterial strains of African origin is essential since the mucosal microbiota has been proven to be a dominant factor in the transmission and pathogenesis of HIV and geographic differences in microbiota composition have been described. This study aims to evaluate the *in vitro* interaction between selected ARVs used in South Africa and those in the pipeline for the treatment and prevention of HIV on a number of the vaginal and gut microbiota species that are associated with healthy mucosal microbiota.

1.2. Aims and objectives

1.2.1. Aim 1

The first aim of this study was to isolate gut *Lactobacillus* spp. and to characterise gut and vaginal *Lactobacillus* spp. from HIV-negative South African adolescent girls and young women between the ages of 14-24 years.

1.2.1.1. Objectives

-To isolate gut *Lactobacilli* spp. such as *L. rhamnosus, L. crispatus, L. casei, L. acidophilis,* and *L. reutei* from stool specimens collected from HIV-negative South African females between ages 14 and 24 years.

-To extract DNA from the isolated *Lactobacillus* followed by 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) to confirm the species of isolated bacteria.

-To evaluate the characteristics of the gut and previously isolated vaginal *Lactobacillus* species from South African women by assessing growth at varying pH-values, production of D-/L-lactate and H_2O_2

1.2.2. Aim 2

The second aim was to investigate the antimicrobial activity of five antiretroviral drugs against the isolated vaginal and gut bacterial species and the American Type Culture Collection (ATCC) gut bacterial species associated with a healthy microbiota.

1.2.2.1. Objectives

-To examine tenofovir disoproxil fumarate, cabotegravir, tenofovir alafenamide, and dolutegravir individually, and tenovifor/lamivudine/dolutegravir combination for their antibacterial activity against vaginal species, *L. crispatus, L. jensenii, L. gasseri, L. vaginalis and L. mucosae* using a broth microdilution assay.

-To examine tenofovir disoproxil fumarate, cabotegravir, tenofovir alafenamide, and dolutegravir individually, and tenofovir/lamivudine/dolutegravir for their antibacterial activity against gut microbiota (isolated *Lactobacilli* and ATCC *Lactobacillus gasseri*, *Bifidobacterium* and *Enterococcus* strains) using a broth microdilution assay.

Hypothesis:

Antiretrovirals, namely: tenofovir disoproxil fumarate, cabotegravir, tenofovir alafenamide , and dolutegravir individually, and tenofovir/lamivudine/dolutegravir have an *in vitro* antibacterial effect against bacterial species commonly found in the gut and vaginal microbiota of South African women.

Chapter 2

Literature review

2.1. HIV epidemiology

The human immunodeficiency virus (HIV) remains an ongoing concern worldwide, with no cure presently available. The 2022 UNAIDS report estimated 1.5 million new infections reported globally in 2021, totalling 38.4 million people living with HIV by the end of 2021 (UNAIDS, 2022). HIV claimed approximately 650 000 lives in 2021 alone and about 84.2 million since the epidemic started in 1981 (UNAIDS, 2020; UNAIDS, 2022). An earlier report stated that on average, there is an estimated 4 000 new infections reported globally daily, with more than half (60%) owing to the sub-Saharan region. About half (51%) of these infections are in women (UNAIDS, 2019). The global HIV prevalence in 2021 is shown in Figure 2.1, with the sub-Saharan Africa region showing the highest number of people living with HIV. During this period, the region saw an estimated 860 000 new infections, totalling 20.7 million people living with HIV, and 420 000 AIDS-related deaths (UNAIDS, 2022). Figure 2.2 shows the top twenty countries with the highest number of people living with HIV globally, 75% (15) of these falls under the sub-Saharan region.

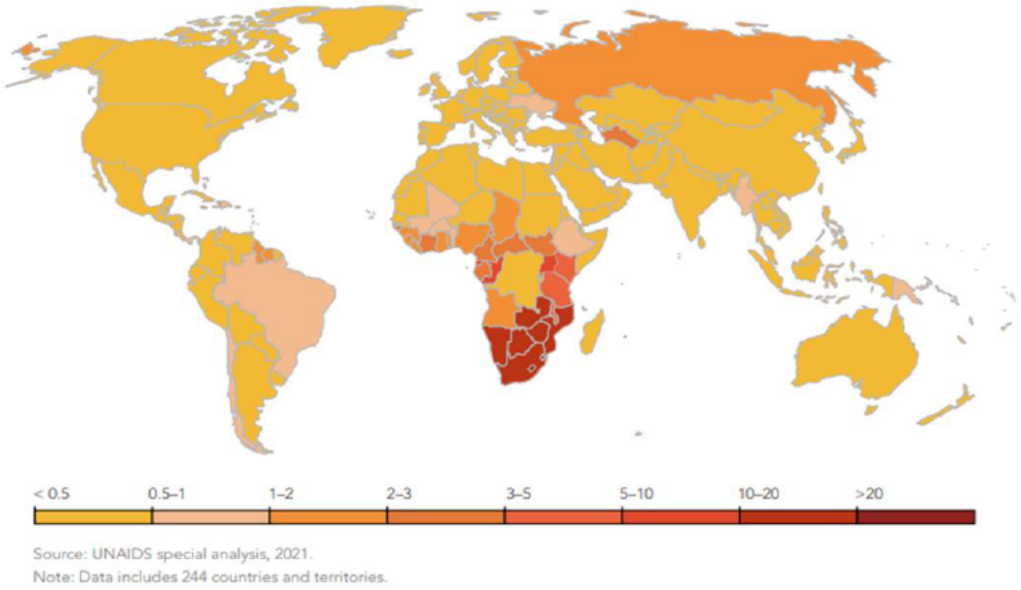

Figure 2.1: Map showing the global HIV prevalence by 2021. The countries coloured in darker colour have the highest percentage of people living with HIV, while those that have a lighter colour had the lowest number of people living with HIV (UNAIDS, 2021).

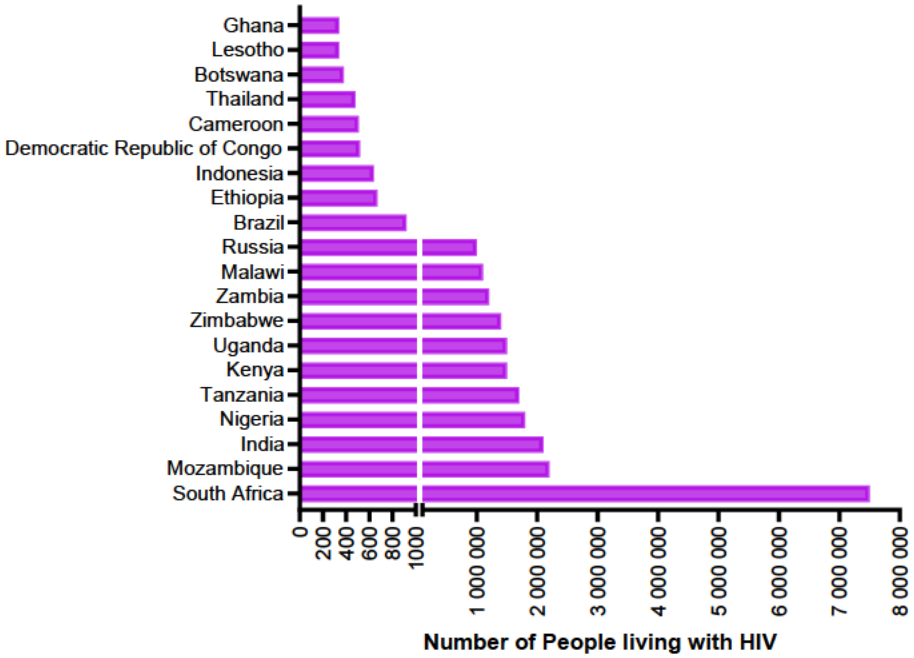

Figure 2.2: Countries with the highest number of people living with HIV in 2018. The countries that reported the top 20 highest number of people living with HIV by the end of 2018. Adapted from (Ritchie, 2018).

South Africa has the highest number of people living with HIV globally, with an estimated 7.6 million people living with HIV in 2021 (Figure 2.2) (UNAIDS, 2022). There were 210 000 new infections and 51 000 AIDS-related mortalities reported by UNAIDS for South Africa in 2021 alone (UNAIDS, 2020). Mirroring the global figures, women accounted for more than half (59%) of the new HIV cases in South Africa and are disproportionately affected by HIV, with 61.5% of the cases in women aged 15 and over. There are 60 000 more new infections in women than in men (130 000 compared to 70 000) (UNAIDS, 2020; UNAIDS, 2022).

2.2. Factors that influence HIV acquisition risk in women

Women are at a higher HIV acquisition risk compared to their male peers (Naicker *et al.*, 2015). The vulnerability of women to HIV infection cannot only be explained by behavioural factors, such as early sexual debut, vaginal insertion product use, having multiple or older partners (Hilber *et al.*, 2010; George *et al.*, 2022). Rather, some of the factors are biological, in particular, women have a larger mucosal surface area that is exposed to contact and subsequent infection with HIV (Ramjee & Daniels, 2013). The disruption of the epithelial mucosa may be linked to the acquisition of HIV. The disruption of the delicate mucosal barrier means that other pathogens may infect the female genital tract and cause STIs such as herpes, syphilis, gonorrhoea, and chlamydia, which have all been associated with an increased HIV acquisition risk (Cohen *et al.*, 2019; Lowe *et al.*, 2019). Infection with any of the causative pathogens is often accompanied by coinfection with other pathogens or re-occurrence of infection (Menezes *et al.*, 2018). It is harder to diagnose STIs and they are often asymptomatic in women compared to men, thereby making women more susceptible to HIV. Pathogenic invasion by STI-causing bacteria is accompanied by immune activation. This involves the secretion of proinflammatory cytokines for example interleukin (IL)-8, tumour necrosis factor (TNF)-α, and monocyte chemotactic protein (MCP)-1, resulting in genital inflammation. The elevation in these cytokine levels results in the recruitment of HIV target cells (CD4+T-cells), thereby increasing the vulnerability of women to HIV (Masson *et al.*, 2015; Passmore *et al.*, 2016). The susceptibility to infection is further increased by the fact the HIV co-receptor C-C chemokine receptor type 5 (CCR5) is overly expressed in women compared to men (Meditz *et al.*, 2012).

Sexual activity can also alter a very key component of vaginal health, i.e. the vaginal microbiota (Abdool Karim *et al.*, 2019). This is a community of microorganisms that govern what species may inhabit this environment by numerous mechanisms which are explained later in this chapter. Therefore its disruption may result in the growth of undesirable pathogens including STI-causing pathogens, thereby increasing the susceptibility to HIV (Eastment & McClelland, 2018). There are also concerns that certain contraceptives, such as the depot medroxyprogesterone acetate (DMPA) injection, may increase the HIV acquisition risk in women (Haddad *et al.*, 2014). One reason for this is that the injections do not prevent infection, therefore exposure to STI-causing pathogens may increase HIV acquisition in women (Haddad *et al.*, 2014; Abbai *et al.*, 2016; Wessels *et al.*, 2018). A second reason would be that hormone-based contraceptives may disrupt the vaginal microbiota which has also been associated with an increase in the HIV risk (Haddad *et al.*, 2014; Wessels *et al.*, 2018). The third possible reason is that inflammatory immune responses may be induced at the injection site (Weinberg *et al.*, 2016). Contrary to studies that support the notion that contraceptives increased HIV acquisition risk, the Evidence for Contraceptive Options and HIV Outcomes (ECHO) HIV prevention study did not find significant differences in HIV risk between women using DMPA and those using other contraceptives (Ahmed *et al.*, 2019).

Behavioural factors that increase the risk of HIV acquisition in women include early sexual debut, defined as 15 years old or younger. Women who initiated sexual activity early were reported to be at a higher HIV and other STIs acquisition risks as this expose them to riskier sexual behaviour such as having more lifetime sexual partners (Wand & Ramjee, 2012). During a questionnaire-based interview and STI testing at each visit during the three-year-long study, Wand and Ramjee (2012) found that women who had an early sexual debut accounted for most (74%) of HIV infection at baseline and they reported a high seroconversion rate. The early sexual debut also means a higher probability of cervical ectopy and that the epithelial barrier is not fully developed, therefore engaging in sexual activities would lead to subsequent infection (Gonzalez *et al.*, 2019). Women who have multiple sexual partners had more than a two-fold increased HIV risk compared to women with a single partner (Naicker *et al.*, 2015). Other behavioural factors include transactional sex, which was shown to increase HIV acquisition by about 13%, gender based violence and inconsistent condom use (Cicconi *et al.*, 2013; Lyons *et al.*, 2020).

2.3. Viral replication of HIV in the human host

2.3.1. Structure of HIV

Both known HIV strains (HIV-1 and -2) target the human immune system and often lead to a complete immune collapse and leads to acquired immunodeficiency syndrome (AIDS) (Peruski, 2020). HIV-2 is the less common strain because of its lower transmissibility and reduced likelihood of progression to AIDS (Nyamweya *et al.*, 2013). Within HIV-1, there are three HIV-1 groups: major (M,), outlier (O), and nonmajor and nonoutlier (N). Group M is the most common circulating HIV-1 group. Group M is subdivided into several subtypes which include A1, A2, A3, A4, B, C, D, F1, F2, G, H, J, and K. The interaction of HIV subtypes with human host is different, and this can determine how easily transmittable and infectious the virus strains will be. The HIV strains that use the chemokine coreceptor CCR5 (R5 viruses) are transmitted easily than strains that use the CXCR4 coreceptor (X4 viruses) (Taylor *et al.*, 2008). Although X4 viruses are less frequently transmitted, once they have infected the host, they are likely to cause rapid progression to AIDS. Although all HIV-1 subtypes can use both coreceptors, the ability of attaching to CCR5 and CXCR4 chemokine coreceptors is different between subtypes, for example, subtype D is a mixed tropic virus, meaning that it includes a combination of R5-tropic virus and X4-tropic virus. On the other hand, subtype C mainly uses CCR5 compared to subtype B which mainly uses CXCR4 (Taylor et al., 2008).

The HIV virus typically has an outer envelope that has glycoprotein (gp) receptors extracellular gp120 and transmembrane gp41 on the surface which play vital roles in viral binding and fusion, respectively (Prabakaran *et al.*, 2007). The HIV virus has an inner compartment called the capsid which encloses two copies of viral RNA which are coupled with four enzymes that are involved in replication; reverse transcriptase, integrase, protease and RNase H (Schultz & Champoux, 2008; Campbell & Hope, 2015).

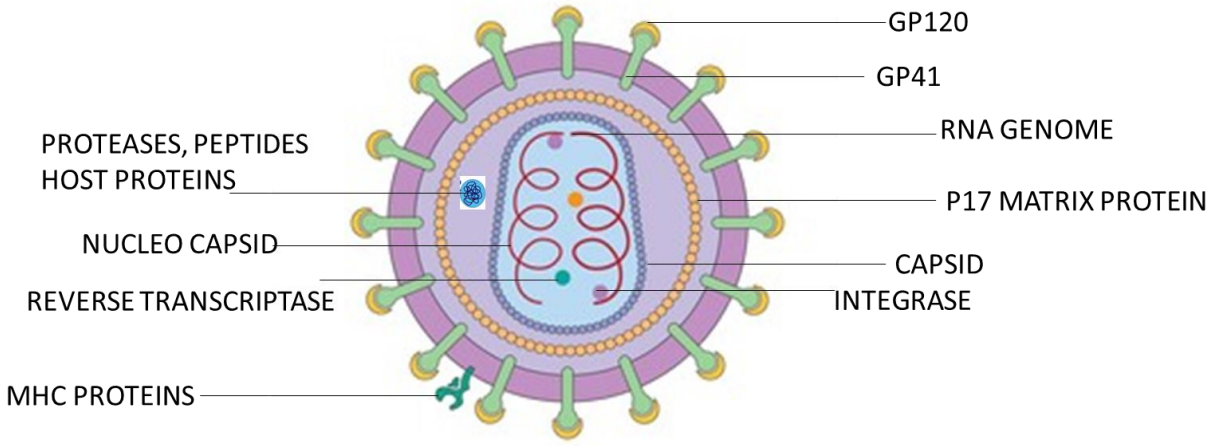

Figure 2.3: The structure of the human immunodeficiency virus. HIV consists of an envelope that is made up of a lipid membrane that is coated with glycoproteins (gp) 41 and 120. These surface proteins are important for HIV attachment to the host cell. Inside the virus there are two copies of the viral RNA genome. The viral enzymes important for reverse transcription are protease, integrase, and reverse transcriptase enzymes which lie inside the viral core. Created with BioRender.com.

2.3.2. Receptor binding

The binding of gp120 to CD4 results in conformational changes in gp120 leading to the exposure of the binding site for a secondary coreceptor, which is either of the chemokine receptors CCR5 (R5) or CXCR4 (X4) (Kwong *et al.*, 1998; Wilen *et al.*, 2012; Kirchhoff, 2021; Schiff *et al.*, 2021) (Figure 2.4 step 1).

2.3.3. Fusion

Gp41 N-terminus also known as the fusion peptide facilitates fusion of the viral and host membranes. In the cell membrane, the virus uncoats and release viral contents into the cell (Wilen *et al.*, 2012; Chen, 2019; Ramdas *et al.*, 2020). Upon cell entry, the capsid is thought to be transported by the actin of the cytoskeleton and facilitates interaction with host proteins, resulting in a protective pre-integrase complex (PIC) that shields the capsid contents from cytoplasmic and lysosomal degradation as the nucleic acid is being transported to the nucleus. (Delaney *et al.*, 2017; Ramdas *et al.*, 2020; Ingram *et al.*, 2021). In the nucleus, the capsid releases viral RNA

copies and other viral enzymes such as reverse transcriptase and integrase (Fredericksen *et al.*, 2002) (Figure 2.4 step 2).

2.3.4. Transcription and Integration

Once, the capsid contents are released, the motor protein kinesin family member 5B (KIF5B) facilitates viral RNA and proteins into the nucleus via the nuclear pore (Dharan *et al.*, 2016). The reverse transcriptase enzyme transcribes the single stranded HIV RNA into double stranded DNA (Davis *et al.*, 2008). The newly synthesised viral DNA (known as a provirus) is incorporated into the provirus into the host's genome (Ramdas *et al.*, 2020) (Figure 2.4 step 4).

2.3.5. Replication

The host's RNA polymerase II transcribes DNA, including the incorporated viral DNA, to mRNA which is translated into protein by the ribosomes on the rough endoplasmic reticulum with the aid of ribosomal (r) DNA (Ajasin & Eugenin, 2020). During transcription, several short viral mRNA copies are synthesised which code for viral replication regulatory proteins, a regulator of expression of virion proteins (Rev), and a Trans-Activator of transcription (Tat) (Stoltzfus, 2009; Karn & Stoltzfus, 2012). Once sufficient Rev and Tat proteins are synthesised, Tat promotes transcription by binding the 5' trans-acting responsive (TAR) hairpin of the HIV genome, while Rev binds the Rev-responsive element, which then transports fully and partially spliced mRNA to the ribosomes where they are translated to important viral proteins (Fernandes *et al.*, 2012; Rice, 2017). The encoded viral proteins such as gp160, negative regulatory factor (Nef), virion infectivity factor (Vif), viral protein R (Vpr), and viral protein U (Vpu) are used to assemble a virion, which is an immature version of HIV, at the terminal stages of the HIV replication cycle (Ramdas *et al.*, 2020) (Figure 2.4 step 5).

2.3.6. Assembly

The tetrameric viral group-specific antigen (Gag) protein, previously encoded, is a key player in virion assembly, budding and ultimate maturation. The nucleocapsid domain of uncleaved Gag initiates virion assembly by recruiting the provirus to the phospholipid bilayer membrane and fuses the provirus to it (Freed, 2015) (Figure 2.4 step 6).

2.3.7. Budding and maturation

The p6 domain of uncleaved Gag proceeds to facilitate budding into virions using the host's membrane, which serves as a primary virion envelope (Weiss & Göttlinger, 2011; Freed, 2015) (Figure 2.4 step 7). As the virion leaves the cell, it destroys the cell and the trans-activator of transcription (Tat) protein can also initiate apoptosis. Post budding, Gag undergoes viral protease cleavage and produces $Pr55^{Gag}$ which is also a tetramer, which activates virion maturation into viruses by changing the arrangement of proteins around RNA, encapsulating it to form a mature virus, which goes on to infect other cells (Sundquist & Kräusslich, 2012; Freed, 2015) (Figure 2.4 step 7).

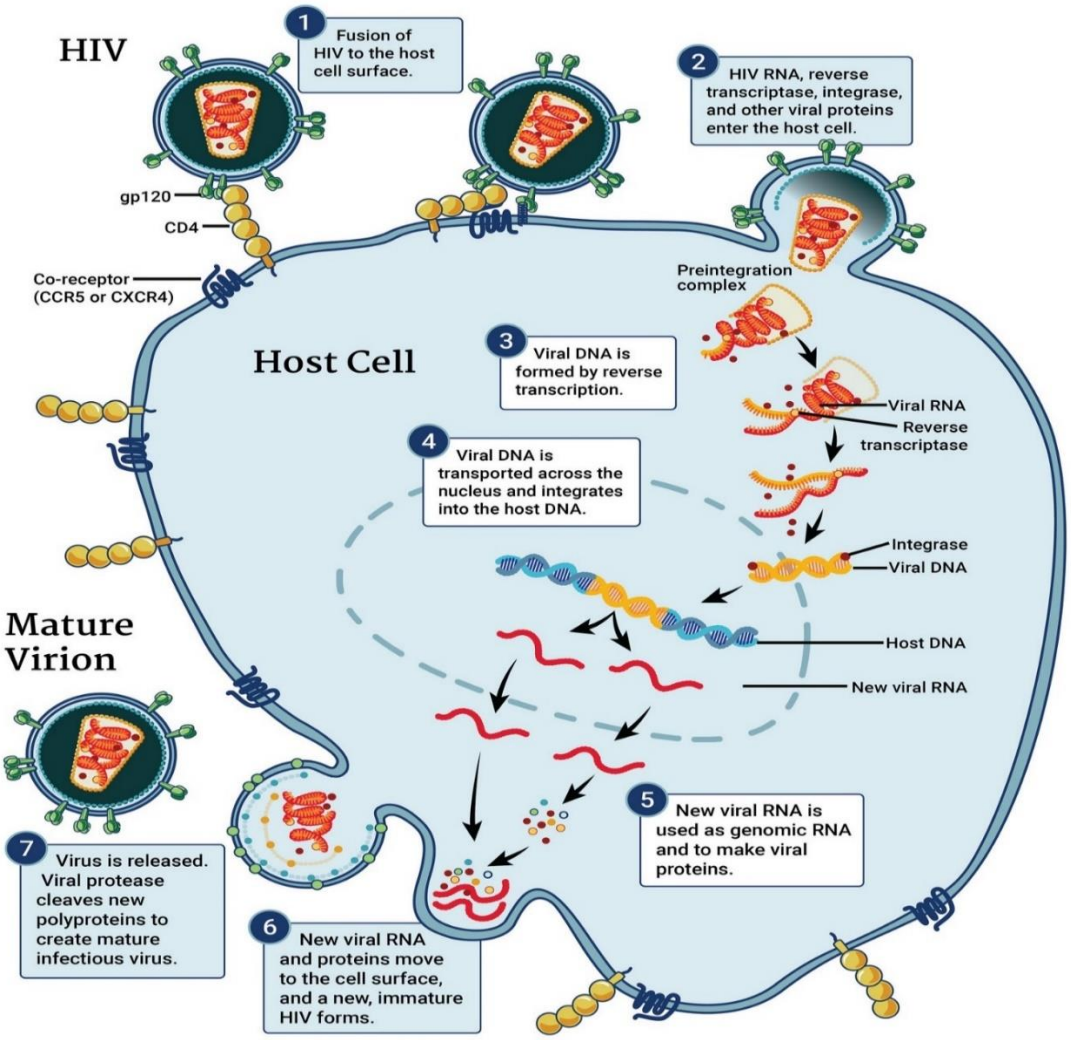

Figure 2.4: The replication of the human immunodeficiency virus (HIV). The HI virus uses glycoproteins (gp) 120 on the CD4 receptor, allowing gp41 to bind onto the co-receptor chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR-4) (X4 and X5 tropic, respectively) 1). The viral cell fuses and enters into the T-cell by endocytosis (2) and the envelope is degraded, while the viral RNA and proteins are kept intact and transported into the nucleus (3). The viral RNA is reverse-transcribed into DNA by reverse transcriptase and is incorporated into the host's DNA by integrase (4). The host's DNA along with the viral DNA is translated into protein (5). The viral proteins assemble into an immature viral cell called the virion (6) and escape the cell via exocytosis and mature into an infectious virus (7) (NIH, 2010).

2.4. Treatment of HIV using antiretroviral therapy

The ability of HIV to escape the host immune surveillance has led to investment in synthetic inhibitors such as ART that can limit viral progression. Antiretroviral drugs constitute a lifelong treatment that has significantly improved the quality of life and life expectancy of HIV-positive individuals by restoring CD4 cell counts, reducing viral replication, and reducing plasma viral load (Ramjee & Daniels, 2013; Bhatti *et al.*, 2016). Primarily, ART was designed to inhibit viral enzymes, leading to the development of reverse transcriptase inhibitors, zidovudine (AZT), and later stavudine (d4T), and lamivudine (3TC), which belong to the nucleoside-analogue reverse transcriptase inhibitors (NRTIs) class. They are competitive inhibitors of thymidine deoxynucleoside, preventing the binding of this nucleoside to viral reverse transcriptase, thus halting chain elongation. (Sierra-Aragón & Walter, 2012). Since then, other classes of ARVs have been developed to target certain phases of the HIV life cycle which are receptor binding, fusion, reverse transcription, integration, viral replication, assembly, and budding, later discussed in this section (Figure 2.4) (Arts & Hazuda, 2012).

To date, there are six main classes of antiretroviral drugs which have been approved by the Food and Drugs Administration (FDA): (i) chemokine receptor antagonists (ii) fusion inhibitors (iii) NRTIs, (iv) non-nucleoside reverse transcriptase inhibitors (NNRTIs), (v) integrase strand transfer inhibitors (INSTIs) (vi) and protease inhibitors (PIs) (Arts & Hazuda, 2012; Desai *et al.*, 2012; Sierra-Aragón & Walter, 2012; Pinto-Cardoso *et al.*, 2018). Chemokine receptor antagonists are entry inhibitors as they inhibit viral entry into cells that express CCR5 and CXCR-4. The chemokine receptor antagonist binds to HIV co-receptors CCR5 or CXCR-4. This class of drugs is non-competitive inhibitors that bind to the allosteric site, inducing conformational changes of CCR5 or CXCR-4, such that g120 cannot bind on the receptor, thereby preventing viral entry (Desai *et al.*, 2012; Bhatti *et al.*, 2016; Atta *et al.*, 2019). Maraviroc (MVC) is the only FDA-approved chemokine receptor antagonist, which binds CCR5 (Venuti *et al.*, 2017) (Table 2.1). Fusion inhibitors also known as entry inhibitors prevent the fusion of viral and cellular membranes. This drug inserts itself into gp41 as it unfolds in preparation to facilitate fusion, therefore preventing gp41 into forming the hex helical structure, such that it cannot insert the fusion peptide into the cell (Malik *et al.*, 2017). There is currently only one FDA-approved fusion inhibitor, enfuvirtide (T-20) (Malik *et al.*, 2017) (Table 2.1).

The NRTIs are classified as nucleoside-reverse transcriptase inhibitors, targeting the reverse transcriptase function which serves to reverse the transcription of RNA to DNA, therefore, preventing viral replication (Atta *et al.*, 2019). The NRTIs are orally-administered nucleosides and the later formulated prodrugs, such as tenofovir disoproxil fumarate (TDF) and are activated in the cell through 5' phosphorylation by kinases to tenofovir (TFV) diphosphate (Topalis *et al.*, 2015). Post-phosphorylation, the resultant structure of the drug lacks the 3' hydroxyl (OH) group and is a competitive inhibitor incorporated into the viral genome by reverse transcriptase during reverse transcription (Zapor *et al.*, 2004; Arts & Hazuda, 2012). The absence of the 3' OH prevents the formation of a phosphodiester bond between the nucleoside and the 5' triphosphate (PO_3^{3-}) group of the following nucleotide base, thereby initiating chain termination, halting viral DNA elongation (Patel, 2022). The NNRTIs like NRTs are also RTIs that are non-competitive inhibitors of reverse transcriptase which bind on the hydrophobic allosteric site changing the conformation of the active site such that deoxynucleotide triphosphates cannot bind and be reverse transcribed to DNA (Arts & Hazuda, 2012). The limitation is that NNRTIs can only inhibit HIV-1 reverse transcriptase but not HIV-2 reverse transcriptase. This specificity is due to the hydrophobic pocket only present in the allosteric site of HIV-1 that can bind the hydrophobic NNRTIs. Compared to NRTIs, NNRTIs have a higher bioavailability and a longer half-life (Desai *et al.*, 2012). The NRTIs have more adverse side effects compared to NNRTIs. These include, renal tubule failure, lactic acidosis, hepatic steatosis, pancreatitis, severe rash, cardiomyopathy, lipodystrophy, myopathy, bone density decrease and peripheral neuropathy, while NNRTIs are known to cause liver toxicity (Holec *et al.*, 2017; Yoder, 2020).

Similar to NRTIs and NNRTIs, INSTIs prevent viral replication. This class achieves this by inhibiting the enzyme integrase which incorporates the viral genome into the human genome (Bhatti *et al.*, 2016). These ARVs are competitive inhibitors that act by binding to the active site of integrase and prevent the incorporation of the newly synthesised viral DNA onto the 3' terminus of the host's DNA (Arts & Hazuda, 2012; Anstett *et al.*, 2017). The class of protease inhibitors, as the name suggests, inhibits important viral proteases that are essential for Gag cleavage during the maturation of HIV (Atta *et al.*, 2019). This class of inhibitors is competitive inhibitors that bind reversibly to the active site of the protease, preventing the binding of Gag-Pol during budding and maturation (Patick & Potts, 1998; Lv *et al.*, 2015). Generally, PIs have a high genetic barrier for drug resistance, however, once resistance occurs through the introduction of mutations, this

results in multiple drug resistance (Desai *et al.*, 2012). This class of drugs is usually used in combination with RTIs and INSTIs (Lv *et al.*, 2015).

The combination formulation of antiretroviral drugs is also common where emtricitabine (FTC) and tenofovir (TFV) are concerned. The current world health organisation (WHO) recommendations suggest that people living with HIV should start on ARVs immediately after diagnosis (Günthard *et al.*, 2016) in the form of the first-, second, and third-line ART regimen (Bhatti *et al.*, 2016). The first-line ART regimen requires that lamivudine (3TC) be taken with efavirenz (EFV), TDF, or FTC. Alternatively, EFV should be prescribed with either 3TC, zidovudine (AZT), 3TC, nevirapine (NVP), or TDF. In case the first-line ART regimen efficacy fails for whatever reason, the second-line ART regime is prescribed as a combination that includes AZT + 3TC or TDF + 3TC /FTC. Should both the first- and second-line ART regimes fail, the third-line ART regime is prescribed, which is any other drug with minimal side effects (Bhatti *et al.*, 2016).

Reverse transcriptase inhibitors regime posed major challenges such as its compatibility with other drugs and drug resistance (Desai *et al.*, 2012; Sierra-Aragón & Walter, 2012). This led to the development of highly active antiretroviral therapy (HAART), a “cocktail” of ARVs, consisting of three or more HIV drugs (Sierra-Aragón & Walter, 2012). This form of treatment has been used for both the treatment and prevention of HIV (Eggleton, 2022). Table 2.1 lists all the currently available ARVs and the classes they belong to (Pinto-Cardoso *et al.*, 2018; Gumede *et al.*, 2022). Of note, the significantly suppressed viral replication and resulted in lower mortality and morbidity rates (Arts & Hazuda, 2012).

Table 2.1: A summary of all the FDA-approved ARVs and their classification into the six classes.

NRTIs[a, c]	NNRTIs[d]	INSTIs[e]	PIs[f]	Chemokine receptor antagonist[d]	Fusion inhibitors[c]
Abacavir (ABC)	Delaviridine (DLV)	Dolutegravir (DTG)[b]	Amprenavir (APV)	Maraviroc (MVC)	Enfuvirtide (T-20)
Didanosine (ddI)	Efravirenz (EFV)	Elvitegravir (EGV)	Atazanavir (ATZ)		
Emtricitabine (FTC)[b]	Etravirine (ETR)	Raltegravir (RAL)	Darunavir (DRV)		
Lamivudine (3TC)	Nevirapine (NVP)		Fosamprenavir (FPV)		
Stavudine (d4T)	Rilpivirine (RPV)		Indinavir (IDV)		
Tenofovir (TFV)[b]			Nelfinavir (NFV)		
Zalcitabine (ddC)			Ritonavir (RTV)		
Zidovudine (AZT)			Saquinavir (SQV)		
			Tipranavir (TPV)		

[a] First approved ARV class

[b] approved for both treatment and prevention of HIV

[c] Arts & Hazuda (2012); [d] Desai *et al.* (2012); [e] Sierra-Aragón & Walter (2012); [f] Pinto-Cardoso *et al.* (2018)

2.4.1. Tenofovir disoproxil fumarate (TDF)

Tenofovir disoproxil fumarate (TDF) is an adenosine monophosphate analogue belonging to the nucleotide reverse transcriptase inhibitors (NRTI) used for HIV and hepatitis B treatment. It is one of the first FDA-approved combinations used for the treatment of HIV (Gallant & Deresinski, 2003). It is also the most widely prescribed antiretroviral agent as it has a longer plasma and cellular half-life (14-17 and more than 60 hours, respectively), is more tolerable and is less capable of causing viral resistance mutations (Atta *et al.*, 2019). Tenofovir DF is an orally administered prodrug of TFV. Upon entering the plasma, this drug is converted to the active TFV

in less than a minute and is rapidly intracellularly metabolised into TDF which subsequently competes with deoxyadenosine 5'- triphosphate initiating chain termination, thereby impairing reverse transcription of viral RNA to DNA (Ray *et al.*, 2016; Atta *et al.*, 2019). Side effects include nausea, headache, fatigue, dizziness, abdominal pain, rash, and liver cirrhosis. Even more concerning side effects are bone density depletion and renal toxicity (Sax *et al.*, 2015). The renal failure caused by TDF presents as impairment of tubular function, and failure to clear urea nitrogen and creatinine. Therefore, the tubular functioning should be assessed prior to its prescription and should be stopped immediately when there is some renal impairment detected (Ray *et al.*, 2016; Venter *et al.*, 2018). The implications of TDF on the kidneys and bone density makes TAF a safer ARV to use.

2.4.2. Tenofovir alafenamide fumarate (TAF)

Like TDF, TAF is a prodrug of tenofovir, following the same mode of action as TDF. It is hydrolysed by cathepsin A to TFV and subsequently phosphorylated to TFV-DP (Atta *et al.*, 2019). It is combined with other drugs for the treatment of HIV and is used alone to treat hepatitis B. It is also combined with FTC to form FTC/TAF (FTAF), often used as a pre-exposure prophylaxis (PrEP). This prodrug is more efficient in inhibiting reverse transcription and is clinically safer than TDF. The use of TAF instead of TDF lowers the risk of bone and renal diseases (Sax *et al.*, 2015; Antela *et al.*, 2016). Moreover, it has a shorter plasma and intracellular half-life (90 and 28 minutes, respectively), making TAF a more stable TFV drug than TDF (Atta *et al.*, 2019). However, this is a newer, recently FDA-approved, and understudied drug.

2.4.3. Dolutegravir

Dolutegravir is a new, once-daily, orally administrated ARV belonging to the family of integrase inhibitors. It is currently a crucial part of the first line regimen and is used for the prevention of HIV to replace EFV in Sub-Saharan Africa (Phillips *et al.*, 2020; Bangalee *et al.*, 2022). It is often used as a first-line regimen and is seldom used as a second-line regimen. The advantage of DTG over EFV is that it is less prone to resistance in patients, more tolerable and no significant drug-drug interaction has been observed (Venter *et al.*, 2020). Furthermore, DTG can be combined with other ARVs such as abacavir, TAF, FTC, and lamivudine. Dolutegravir-containing regimens have proven to effectively reduce viral load with limited side effects (Kandel & Walmsley, 2015).

However, the use of DTG by pregnant women has been reported to result in neural tubes failure of foetuses (WHO, 2019).

2.4.4. Cabotegravir

Cabotegravir (CAB) is a DTG analogue, belonging to the INSTI class, being investigated for both HIV treatment and prevention. It is available as an orally administrable drug and as a long-acting injectable drug that is delivered either intramuscularly or subcutaneously (Fernandez & van Halsema, 2019). The oral formulation of CAB has a half-life of approximately 40 hours (Whitfield *et al.*, 2016). The injectable formulation can be co-administered with rilpivirine. Some side effects reported so far include vertigo, fatigue, rash, indigestion, and even neurological effects. However, it remains poorly investigated and much research needs to be done to provide insights into the potential adverse outcomes this drug might have.

2.4.5. Tenofovir/lamivudine and dolutegravir

The new rollout TFV, 3TC, (both NRTIs) and DTG combination (TLD) was traditionally the combination of TFV, 3TC, and EFV (TLE) and it is currently used as part of the first line defence regimen (WHO, 2018). However, DTG has since replaced all EFV-containing regimens. As aforementioned, the replacement of EFV with DTG makes this regimen even more potent, reducing drug resistance and increasing its tolerability, ensuring that it has fewer side effects (Kandel & Walmsley, 2015; Mendelsohn & Ritchwood, 2020; Phillips *et al.*, 2020; Venter *et al.*, 2020).

2.5. The impact of ART and PrEP on the HIV epidemic

The last decade saw substantial progress being made in reducing the number of new HIV infections and AIDS-related mortalities. UNAIDS reported a 31% and 47% reduction in new HIV infections and AIDS-related deaths, respectively since 2010 (UNAIDS, 2020). A further quarter (25% reduction in new infections was observed in women aged 15-24 years. These improvements were due to more people accessing ARVs and PrEP. There are over 25.4 million people receiving

ARVs currently compared to 6.4 million people that were receiving ARVs in 2010 (UNAIDS, 2019). Furthermore, 845 000 individuals had access to PrEP in 2020 (UNAIDS, 2020). It is also worth noting that only 1.5 million new infections were reported in 2020 compared to 2.8 million that was reported in 2010 (UNAIDS, 2020), showing the effectiveness of PrEP in preventing HIV acquisition, thus serving its purpose. As a consequence of the increased use of ARVs and PrEP, a 43% reduction in AIDS-related deaths was observed (UNAIDS, 2021). In addition, the tremendous decline in new HIV infections in Sub-Saharan Africa can be attributed to the UNAIDS 90-90-90 goal, which was set with the aim to contribute towards ending the epidemic. It stipulated that by 2020, 90% of people know their status, 90% are accessing ARVs and 90% of people receiving ARVs are virally suppressed. In 2020, UNAIDS recorded that there were 73% of people knew their HIV status, 87% were receiving treatment and 68% of people receiving ARVs were virally suppressed, having missed the 90-90-90 goal (UNAIDS, 2020). Although this goal was not met, it improved the response to the epidemic. This strategy was countered by UNAIDS with the 2030 95-95-95 goal, which aims that by 2030, 95% of people know their status, 95% are accessing ARVs and 95% of people receiving ARVs are virally suppressed (UNAIDS, 2021). Figure 2.4 shows the impact of ARV/PrEP on the HIV epidemic. The global decline of 31% since 2010 suggests that there is still a long way to go to combat HIV.

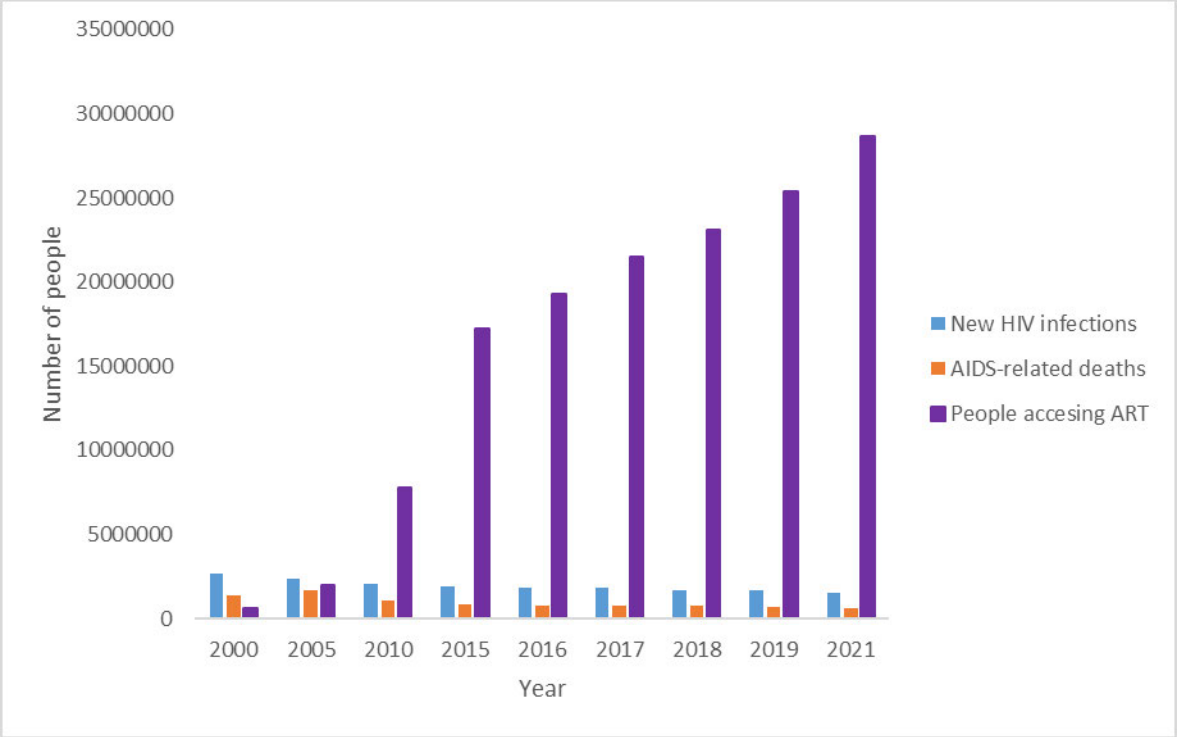

Figure 2.5: The impact of ARVs on the HIV pandemic. The reduction of new HIV infections and the number of people living with HIV by the end of 2019 as a result of more people accessing ARVs between 2000 and 2021 as reported by UNAIDS. (UNAIDS, 2020; UNAIDS, 2021). The graph was generated in MS Excel.

Pre-exposure prophylaxis is a WHO-recommended preventative combination of antiretroviral drugs taken daily, either orally or non-orally, aimed to prevent HIV-negative people from contracting HIV. The non-oral formulation was tested during the CAPRISA-004 trial, where Abdool Karim and colleagues tested the efficiency of a vaginally administrated 1% TFV gel in the prevention of HIV acquisition in South African women. About half (54%) reduction of HIV incidence was observed in women with high gel use adherence and about a third (39%) in women with low adherence (Abdool Karim *et al.*, 2010). The Partners' PrEP clinical trial aimed to test the efficacy of the oral formulation of PrEP. The study recruited heterosexual couples from Kenya and Ghana, where one partner was HIV positive (male or female), and the other was negative. There were 1586 couples who received TDF, another 1586 received TDF/FTC combination, while the remaining 1586 received a placebo. The risk of acquiring HIV decreased by 69% and 75% in women using TDF and TDF/FTC, respectively. The difference in reducing the risk of acquiring HIV between the TDF and TDF/FTC groups was not significantly different. Therefore, the combination did not render more protection than one drug alone (Baeten *et al.*, 2012). Moreover,

the TD-2 trial randomly assigned TDF/FTC to young HIV-negative- men and women who had HIV-positive partners while others received a placebo that they had to take orally once daily. There were only nine patients who seroconverted in the FDF/FTC group and 24 in the placebo group, with a 62% reduction in HIV acquisition risk, showing that the use of PrEP reduces susceptibility to HIV (Thigpen *et al.*, 2012). Choopanya and colleagues also showed that TDF effectively reduced infection with a 49% reduction observed in injection drug users (Choopanya *et al.*, 2013). All these studies reported that both oral and non-oral formulations of PrEP were effective in reducing HIV susceptibility in both men and women.

Contrary to the aforementioned findings, another study suggested that TDF-FTC was ineffective in preventing infection or progression of HIV in HIV-negative South African, Tanzanian, and Kenyan women (van Damme *et al.*, 2012). These results agreed with those in the FEM-PrEP (vaginal and oral Interventions to control the Epidemic and Pre-exposure Prophylaxis Trial for HIV Prevention among African Women) trials, where TDF-FTC was found to be ineffective in reducing HIV susceptibility. Furthermore, two independent studies demonstrated that women who had a dysbiotic vaginal microbiota or bacterial vaginosis (BV) were likely to have a compromised PrEP and ARV efficacy, resulting in a lesser HIV reduction (Klatt *et al.*, 2017; Cheu *et al.*, 2020). Bacterial vaginosis is a condition that arises from a dysbiotic vaginal microbiota which often results in genital inflammation, vaginal discharge that is grey, and an unpleasant fish-like smell (Redelinghuys *et al.*, 2020; Abou Chacra *et al.*, 2022). Often, BV clears without any treatment, therefore making it harder to detect and often results in coinfection with STIs, but when diagnosed, it is treated with antibiotics such as orally-administered metronidazole as well as clindamycin oral and vaginal cream (Workowski & Bolan, 2015; Bagnall & Rizzolo, 2017). These antibiotics are effective against reinfection as bacteria such as *G. vaginalis* acquire resistance, in that case, a more potent regimen such as tinidazole is required (Workowski & Bolan, 2015; Bagnall & Rizzolo, 2017).

2.6. The human microbiota

The human microbiota refers to all the commensal, symbiotic, and pathogenic microorganisms that live within the human body (Valdes *et al.*, 2018; Berg *et al.*, 2020). This includes bacteria, viruses, fungi, and protozoa which can be found on the skin, nasal cavity, oral cavity, digestive tract, and female genital tract (Biswas *et al.*, 2015; Arweiler & Netuschil, 2016; Ravel & Brotman,

2016; Valdes *et al.*, 2018; Berg *et al.*, 2020). Symbiotic microbes play a vital role in human health, homeostasis, and immunity, thus a study of these microbes is essential (Valdes *et al.*, 2018).

2.6.1. Characteristics of a healthy vaginal microbiota and its implications for health

A healthy vaginal microbiota is less diverse and is characterised by the presence of *Lactobacilli spp.* such as *L. crispatus, L. iners, L. gasseri,* and *L. jensenii* (Ravel & Brotman, 2016). These bacteria serve to protect the cervicovaginal environment by (i) producing lactic acid which maintains an acidic environment of pH ≤4.5 that is ideal for preventing invading pathogens from growing in the cervicovaginal environment (Amabebe & Anumba, 2018), (ii) secreting bacteriocins that inhibit the growth of pathogenic bacteria (Abdool Karim *et al.*, 2019), (iii) production of hydrogen peroxide which prevents catalase-lacking microbes from growing (Barrons & Tassone, 2008), (iv) secreting adhesins that enable *Lactobacilli* to adhere to the female genital tract (Bayigga *et al.*, 2019) and (v) regulating immunity by reducing genital inflammation (Anahtar *et al.*, 2015) (Figure 2.6). *Lactobacillus crispatus* and *L. vaginalis* play important roles in vaginal health as they are the major producers of lactic acid (Jespers *et al.*, 2017). The composition of the vaginal microbiota can also vary from person to person and from time to time in a person as a result of several factors such as age, diet, hormone levels, sexual practices, and the use of antibiotics/probiotics (Hilber *et al.*, 2010; Ravel & Brotman, 2016).

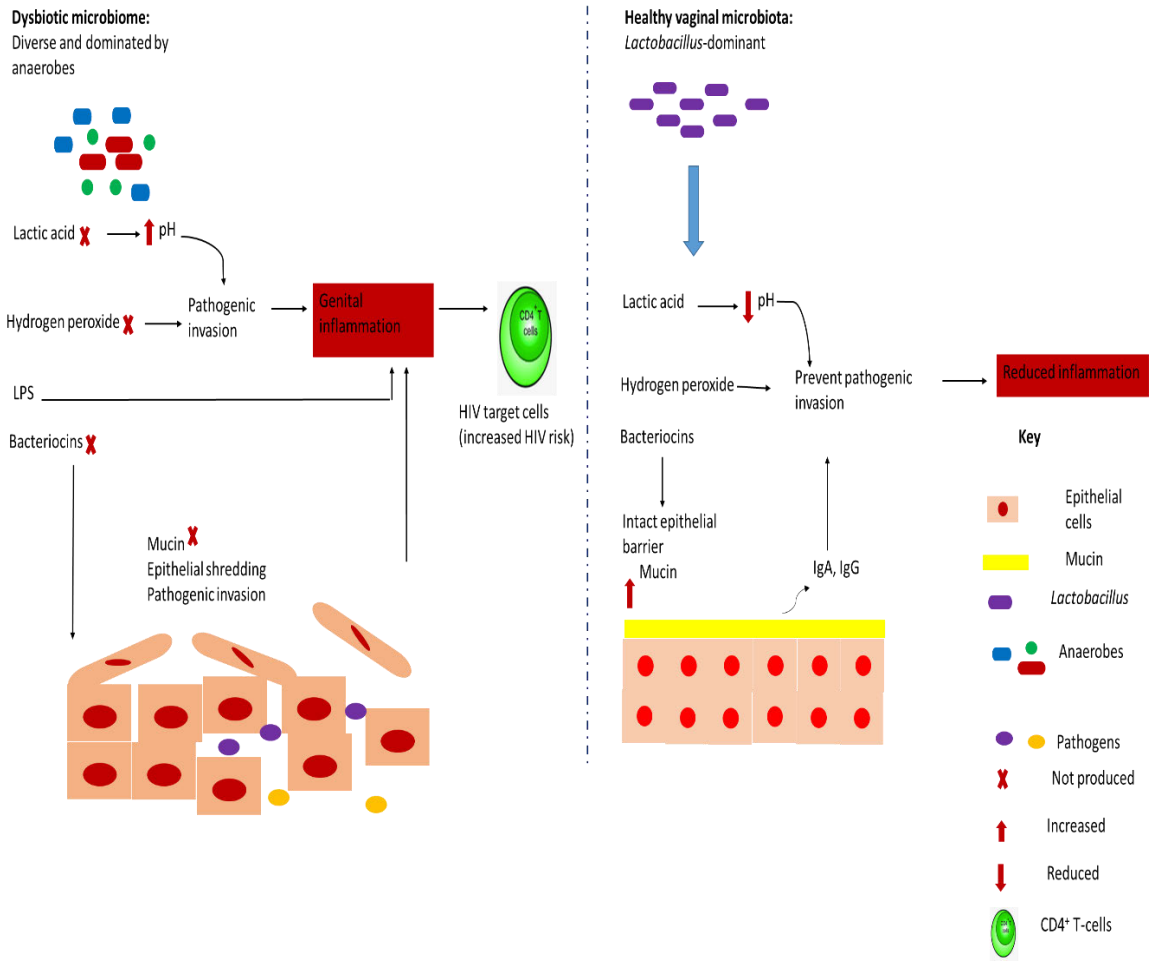

Figure 2.6: Characteristics of a healthy vaginal microbiota. The vaginal microbiota is essential in maintaining a healthy vaginal environment. A diverse mixed anaerobic microbiota is considered one that is dysbiotic as it results in a more basic pH which enables the growth of pathogens. Anaerobic bacteria cannot produce hydrogen peroxide and bacteriocins which are essential for inhibiting the growth of pathogens and keeping the epithelial barrier intact. The lack of these induces genital inflammation. Genital inflammation is also induced by the production of lipopolysaccharides (LPS), ultimately, recruiting HIV target cells. A favourable, healthy microbiota is dominated by *Lactobacillus* species and is characterised by an acidic pH, reduced inflammation, and an intact epithelial barrier, achieved by the production of lactic acid, bacteriocins, and hydrogen peroxide. An intact barrier prevents the invasion of pathogens by secreting immunoglobulins (Ig) A and G. (Bayigga *et al.*, 2019). The figure was generated in MS PowerPoint.

Genetics also plays a pivotal role in shaping the composition of the vaginal microbiota. Women in sub-Saharan Africa and those of African descent have been reported to have dysbiotic and more diverse vaginal microbiota compared to other women. A multiracial study conducted in the United States revealed that vaginal microbiota is affected by a genetic component. The findings of this study discovered that BV was most prevalent in Black and Hispanic women (Nugent score 7-10), having 39% and 34% anaerobic domination, respectively compared to Asian and white women

who had 17.6% and 9.3% of anaerobic species (Ravel *et al.*, 2011). This HIV-associated condition affects 29% of women in the United States and 52% in sub-Saharan Africa (McKinnon *et al.*, 2019). Another study that evaluated BV showed that African Americans had a higher BV incidence (33%) compared to Hispanics (31%) and white women (24%) as per the Amsel criteria and Gram staining. This increased the risk of STI acquisition in black women (Hazard Ratio=2.86) (Peipert *et al.*, 2008). Similarly, a multi-racial study in the Netherlands revealed differences in microbial microbiota among Dutch, Moroccan, Ghanaian, South Asian Surinamese, African Surinamese, and Turkish women. This study reported that the vaginal microbiota of Dutch women was dominated by *L. crispatus,* while that of South Asian Surinamese, Turkish, and Moroccan women was dominated by *L. iners*. On the contrary, the vaginal microbiota of African Surinamese and Ghanaian women were dominated by *G*. *vaginalis* (Borgdorff *et al.*, 2017).

A dysbiotic microbiota can lead to numerous adverse outcomes, the most common being BV (Liebenberg *et al.*, 2017). Prevention of this condition is essential as anaerobes like *G vaginalis* and *Prevotella* spp. can directly disrupt the epithelial lining by secreting enzymes and toxins that degrade the epithelial cells, increasing the host's susceptibility to vaginal infections (Bayigga *et al.*, 2019). This condition enables the permeation of STIs like herpes, syphilis, chlamydia, gonorrhoea*,* and HIV (Bayigga *et al.*, 2019). This condition has also been associated with other adverse health outcomes like pre-term births and miscarriages (Africa *et al.*, 2014). Changes in this microenvironment can also impact immunity. The acidic environment that is maintained by *Lactobacilli spp.* promotes an anti-inflammatory nature, thus reducing the disruption of the cervicovaginal mucosal epithelial barrier, which is the first line of defense against pathogenic microbes (Amabebe & Anumba, 2018). When the integrity of the mucosal barrier is compromised, pathogenic invasion elicits inflammatory responses. The cytokines produced include IL-1α IL-β, IL-2, IL-6, IL-8, IL-10, TNF-α, and interferon (IFN)-γ resulting in the recruitment of CD4+ T-cells which are HIV target cells (Masson *et al.*, 2015; Passmore *et al.*, 2016). An inflammatory response can also cause abrasions of the vaginal epithelial cells and the secretion of IL-33, which inhibits T-cell migration (Oh *et al.*, 2016). Further, the mucosal barrier serves to retain immunoglobulin A and G (IgA and IgG) which serve to protect against infection (Amabebe & Anumba, 2018). *Ureaplasma urealyticum* reduces vaginal immunity by secreting IgA protease which cleaves the antibody, thereby increasing the host's susceptibility to genital infections including HIV (Africa *et al.*, 2014). *Sneathi amnii, Prevotella amnni, S. anguinegens,* and *Mobiluncus* spp. elevated the

levels of IL-1α, IL-1β, and IL-8, which activated CD4 T-cells, thereby increasing the HIV acquisition risk (Ravel & Brotman, 2016). *Lactobacilli* dominated microbiota is associated with reduced levels of IL-1α IL-β and IL-8 (Heinemann & Reid, 2005).

There are various systems that genital anaerobes use to aid in STIs progression and transmission, including HIV. Once the anaerobes colonise the vagina, they can form biofilms and secrete adhesins, therefore becoming drug-resistant and they can mask themselves against the host immune system (Bayigga *et al.*, 2019). Moreover, biofilms formed by the anaerobes may act as reservoirs of pathogens that cause other STIs such as chlamydia*,* gonorrhoea*, and* syphilis*,* thereby aiding the penetration of several STI-causative microorganisms which may result in concurrent infection. Secondly, the BV-associated anaerobes can produce short-chain fatty acids (SCFA) that can either induce the reactivation of latent HIV even when the viral load has become undetectable or enable HIV-1 to interact with CD4 T-cells (Das *et al.*, 2015). Thirdly, the presence of BV-associated anaerobes can alter innate and adaptive immunity by manipulating different pathways. It was reported that *P. bivia* produces lipopolysaccharides (LPS), an endotoxin that induces genital inflammation by recruiting proinflammatory cytokines such as IL-6 which will activate the adaptive immune system, recruiting HIV target cells, thus increasing the HIV acquisition and transmission risk (Hummelen *et al.*, 2010). The LPS-induced activation of the p38 Mitogen-Activated Protein Kinase (MAPK) pathway is essential for viral progression (Medders & Kaul, 2011). The expression of viral proteins such as p38 can increase inflammatory responses via the MAPK pathway, which may activate adaptive responses, thereby recruiting HIV target cells (Furler & Uittenbogaart, 2010). *Prevotella bivia* and *A. vaginae*, but not *L. crispatus* and *L. iners* upregulated inflammatory cytokines TNF-α, IL-6, and IL-8 *in vitro* by manipulating the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway (Doerflinger *et al.*, 2014). Fourthly, Gram-negative anaerobes can increase CD4+ T-cell expression of the HIV-1 receptor CCR5 *in vitro* (Dillon *et al.*, 2016) and induce viral shedding, thereby allowing it to infect numerous cells at a time (Mitchell *et al.*, 2012; Borgdorff *et al.*, 2014). It was also observed for *L. iners* but not for *L. crispatus.*

2.6.2. Characteristics of healthy gut microbiota and its implication on health

Contrary to the vaginal microbiota, the gut microbiota is complex and diverse, encoding roughly three million genes (Rinninella *et al.*, 2019). The gut microbiota comprises microorganisms belonging to the Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Verrucomicrobia, and Fusoacteria phyla. Examples include *Faecalibacterium praunstzii, Escherichia coli, Ruminococcus faecis, Enterococcus faecium,* and *L. reuter* (Rinninella *et al.*, 2019). This microbiota is responsible for several functions within the gastrointestinal tract (GIT) beyond the digestion of indigestible foods, including the secretion of vitamins, innate immunity regulation, and weight management (Valdes *et al.*, 2018). This microenvironment achieves this by secreting vitamins such as vitamin K and vitamin B12 which are precursors of the blood clotting factor thrombin, red blood cells, and DNA (Narayanan, 1999; Napolitano *et al.*, 2010; Mohammed *et al.*, 2020). The colon is primarily responsible for the digestion of substances that were not degraded by prior digestion processes. For example, humans lack enzymes capable of degrading complex carbohydrates such as starch, cellulose, and hemicellulose, therefore microorganisms such as *Ruminococcus* spp*.* are responsible for producing such enzymes as cellulases which are responsible for the hydrolysis of cellulose (Flint *et al.*, 2008; Flint *et al.*, 2012). Another example is the degradation of pectin. Humans lack a mechanism to use pectin, however, *F. prausnitzii* is responsible for the degradation of pectin through fermentation (Lopez-Siles *et al.*, 2017). The involvement of the gut microbiota in digestion enables it to produce metabolites that impact several other pathways as these metabolites act as signalling molecules or hormones. Some gut microbiota species can produce SCFA such as acetate, butyrate, and propionate, which play key roles in immunity. Butyrate plays a key role in preventing pathogen invasion of the gut by creating an anaerobic microenvironment during oxygen-dependant metabolism, thus reducing gut inflammation and increasing the gut barrier integrity (Lopez-Siles *et al.*, 2017; Valdes *et al.*, 2018; Chen & Vitetta, 2020). Butyrate can also halt proliferation of cancerous colonocytes and stimulates apoptosis of cancerous cells via the MAPK pathway (Valdes *et al.*, 2018; Geng *et al.*, 2021). Gram negative bacteria have a cell wall that comprises lipopolysaccharides (LPS) that have been implicated in aiding inflammatory immune responses by binding to TLR-4 on macrophages, activating the secretion of pro-inflammatory cytokines TNF-α, IL-6, and IL-8 (Wang *et al.*, 2020). Further, SCFAs are responsible for homeostasis by regulating inflammatory responses, using several methods. Butyrate reduces the secretion of MCP-1, IL-8, and IFN-γ protein-10, thereby reducing inflammation (Vinolo *et al.*, 2011). The SCFAs are also able to inhibit the production of LPS-induced inflammatory cytokines such as IL-10 and TNF-α by inhibiting the

production of prostaglandin E_2 (PGE_2), thereby reducing inflammation (Cox *et al.*, 2009; Vinolo *et al.*, 2011). In addition to digestion and hot's immunity, microbiota SCFAs are also able to regulate weight control using different mechanisms as studies have reported a decrease of obesity in the presence of SCFAs (Valdes *et al.*, 2018). Acetate ad propionate promotes the lipogenesis of lipids such as cholesterol, thus reducing excess fats which are associated with increased obesity (Beylot, 2005; Valdes *et al.*, 2018). Acetate, along with propionate can control appetite by acting on the hypothalamus via suppression of the AMP-activated kinase (AMPK) pathway while propionate does this by promoting gluconeogenesis (Beylot, 2005; De Vadder *et al.*, 2014; Valdes *et al.*, 2018; Hernández *et al.*, 2019). Butyrate is an energy source for epithelial cells lining the colon called colonocytes by inducing gluconeogenesis (Valdes *et al.*, 2018). However, excessive butyrate production can compromise the gut barrier integrity by initiating apoptosis of even healthy colonocytes (Peng *et al.*, 2007). Propionate is responsible for signalling satiety (Valdes *et al.*, 2018).

Several genetic and environmental factors have been implicated in shaping the gut microbiota such as type of birth delivery, age, breastfeeding, diet, and antibiotics/probiotics use (Thursby & Juge, 2017; Rinninella *et al.*, 2019). In an infant's early life, the type of delivery is a major determinant of the infant's gut microbiota. Vaginally-delivered infants have gut microbiotas dominated by species of the vaginal microbiota *Lactobacillus* and *Provotella,* while those that were delivered by caesarean (C-) section had gut microbiotas dominated by species belonging to *Streptococcus, Staphylococcus, Enterococcus,* and *Cutibacterium* genera which are bacterial species of the placenta and amniotic fluid (Zimmermann & Curtis, 2018; Hasan & Yang, 2019). It is also suggested that mothers can transfer their gut microbiota such as *Clostridium* spp*., E. coli,* and *Enterococcus* spp. through breastfeeding (Zimmermann & Curtis, 2018; Hasan & Yang, 2019). An adult's gut is more diverse and stable, having numerous species. However, it can be changed by environmental factors such as diet and drug use. *Prevotella* is associated with fiber-rich diets*,* while *Bacteroides* spp. is associated with protein-rich diets. Certain drugs have also been shown to shape gut microbiota. For example, probiotics increase the diversity of this community, promoting the growth of essential species, while antibiotics can disrupt this microenvironment, promoting the growth of pathogens such as *Clostridium difficile* and leading to a dysbiotic condition (Hasan & Yang, 2019). Similar to antibiotics, ARVs can promote the dysbiotic condition that is coupled with HIV-1 infection (Dillon *et al.*, 2016; Pinto-Cardoso *et al.*, 2018; Liu

et al., 2019). In addition, previous studies have shown that ARVs cannot restore the microbial community after HIV-1-induced dysbiosis (Pinto-Cardoso *et al.*, 2018).

Dysbiosis of this microbiota can have adverse implications on health, immunity, and weight management issues, summarised in Figure 2.6. Individuals with low microbiota diversity have been shown to have a risk for cardiovascular diseases (CVDs) as well as insulin resistance type 1 and 2 diabetes (Valdes *et al.*, 2018). These adverse health outcomes result from the loss of specific microorganisms that produce certain metabolites that may directly or indirectly affect health. For example, an increase in trimethylamine N-oxide (TMAO), a by-product of the microbial metabolism of dietary carnitine, choline, and betaine, has been directly associated with atherosclerosis (Yang *et al.*, 2019). Another scenario would be the growth of pathogens such as *C. difficile*, which releases two *C. difficile* toxins (TcdA and TcdB), that damage the mucosal epithelial. The mucosal barrier damage will allow pathogen invasion, which will elicit inflammatory responses which will lead to adaptive immune activation, thereby recruiting HIV target cells (El Feghaly *et al.*, 2013). This leads to a condition known as inflammatory bowel disease (Sahibdeen *et al.*), which occurs when T-cells are activated by TNF-α and other chemokines (de Mattos *et al.*, 2015). An increased LPS level can also induce inflammation by T-cell activation and insulin resistance (Muscogiuri *et al.*, 2019; Trøseid *et al.*, 2020). *Faecalibacterium prausnitzii* can also modulate inflammatory responses by preventing the secretion of IL-8 via the NF-$_K$B pathway (Lopez-Siles *et al.*, 2017). Further, *Christensenella minuta* was found to be dominant in lean people while the *Oscillospira* and *Akkermansia* spp. are responsible for an increase in visceral fat and were found in people with a higher body mass index (BMI) (Beaumont *et al.*, 2016).

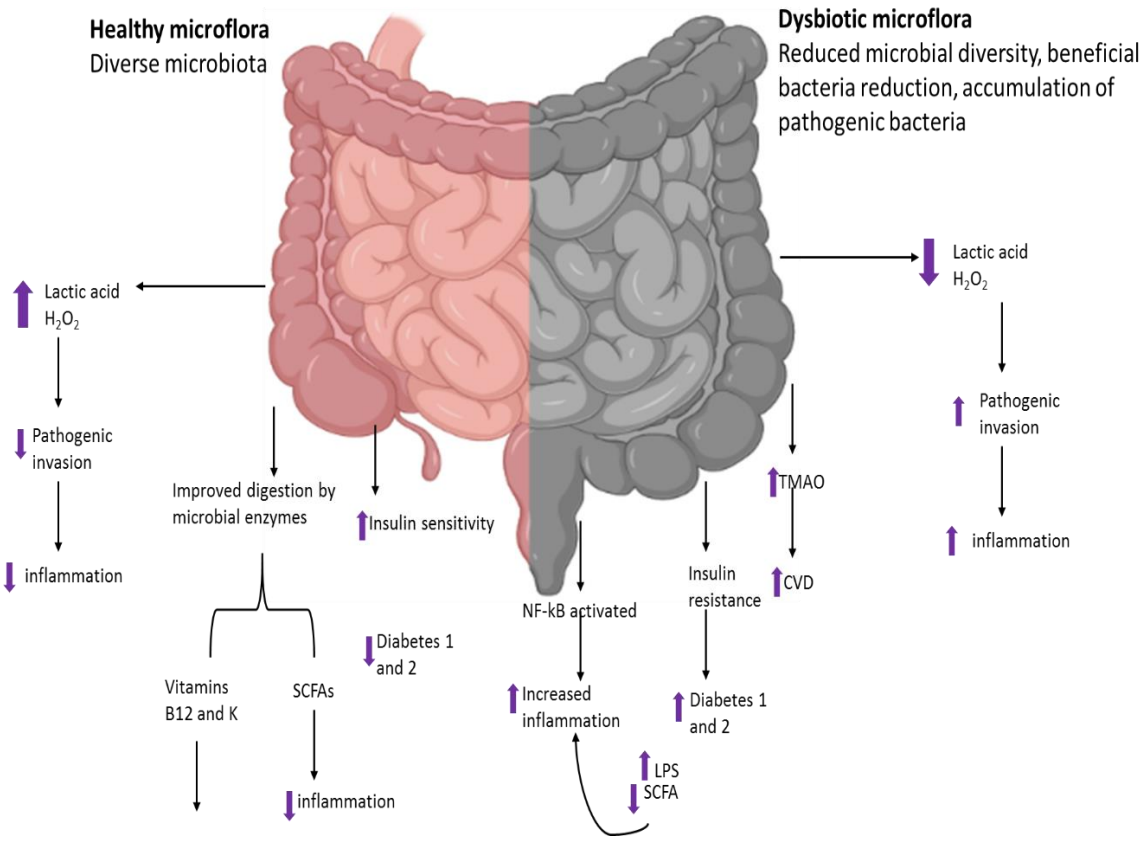

Figure 2.7: The impact of microbiota on human health. Healthy gut microbiota is diverse and plays a pivotal role in human health. The presence of certain microorganisms improves digestion which releases vitamins such as B12 and K. Vitamin B12 is required in the formation of red blood cells and vitamin K is required for clotting along with clotting factors. The gut bacteria can also produce lactic acid during digestion via fermentation. Lactic acid prevents pathogenic invasion and reduces inflammation. Short-chain fatty acids (SCFAs) are also secreted during digestion, these are essential in reducing inflammation. Microbes of a healthy microbiota can also increase insulin sensitivity and uptake by cells, thus reducing the incidence of diabetes 1 and 2. Additionally, the bacteria produce hydrogen peroxide (H_2O_2) which also inhibits the growth of catalase-negative pathogens. On the contrary, the dysbiotic gut microbiota is characterised by the reduction of species richness and the accumulation of pathogenic microbes. The presence of pathogenic species activates inflammatory responses via the NF-$_k$B pathway, resulting in increased inflammation. Gram-positive bacteria have a large peptidoglycan layer on their cell walls which consists of lipopolysaccharides (LPS) that induces inflammation. This inflammation cannot be resolved as the production of SCFAs is reduced. Other adverse health outcomes caused by a dysbiotic gut microbiota such insulin resistance resulting in diabetes 1 and 2 and cardiovascular disease (CVD) resulting from the production of trimethylamine N-oxide (TMAO). Adopted from (Valdes *et al.*, 2018).

2.7. The impact of antiretroviral drugs on the mucosal microbiota

Despite the effectiveness of ARVs against HIV, the interaction between ARVs and the mucosal microbiota has not been fully elucidated. Some studies have reported that ARVs may induce dysbiotic conditions (Cheu *et al.*, 2020; Imahashi *et al.*, 2021). Evidence has shown that the

mucosal microbiota plays an important role in the pathogenesis and transmission of HIV. Although HIV treatment may be effective in the treatment and prevention of HIV, the antimicrobial activities of ART on the vaginal microbiota are not entirely understood. In a study where the *in vitro* antibacterial activity of 25 antiretroviral drugs, including three-drug combinations, were investigated on the gut-associated bacteria, *E. coli* (Gram-negative) and *B. subtilis* (Gram-positive) only EFV demonstrated antibacterial activity against *B. subtilis,* while AZT and 2'3' dideoxyinosine were active against *E. coli.* The EFV, FTC, and TDF combination on the other hand exhibited antibacterial activity against both bacteria (Shilaih *et al.*, 2018). These findings were consistent with an earlier study where it was found that women on TFV and ritonavir-boosted atazanavir (ATV) had less diverse microbiota dominated by *Lactobacilli* (Carlson *et al.*, 2017). On the contrary, other studies found that nevirapine had no antimicrobial activity (Jackson *et al.*, 2009), ART initiation did not impact the vaginal microbiota (Liu *et al.*, 2019), and there was a reduction in gut microbiota diversity in EFV-treated individuals (Pinto-Cardoso *et al.*, 2017; Liu *et al.*, 2019). In the latter case, members of the Ruminococcaceae family, including *F. prausnitzii*, were affected the most (Pinto-Cardoso *et al.*, 2017). These contrasting reports on the antibacterial properties of ARV drugs necessitate further investigation.

2.8. The roles of gut and vaginal *Lactobacilli*

Some microbes such as *Prevotella, E. coli, Streptococcus, Bifidobacteria,* and *Gardnerella* inhabit both the vaginal and gut microbiota. These bacteria are introduced into the vagina by women wiping in the opposite direction or by the condition known as "leaky gut" whereby the gut bacteria and metabolites enter the bloodstream (Amabebe & Anumba, 2020). One such genus is *Lactobacillus.* This genus plays a significant role in health maintenance in both microenvironments. Vaginal *Lactobacilli* are suspected to have originated from the gut and were introduced into the vagina by any one of the aforementioned means. Noteworthy, babies delivered vaginally had more *Lactobacillus* than those delivered by C-section, further proving that there is a possibility of bacterial translocation (Amabebe & Anumba, 2018). *Lactobacilli* can also be ingested as probiotics and can treat diarrhoea and irritable bowel syndrome (Wilkins & Sequoia, 2017; Sadrin *et al.*, 2020). Perhaps the most distinguishing factor between *Lactobacilli* and the other bacteria that inhabit both these microenvironments is that *Lactobacilli* play a major role in the homeostasis and health of the vagina, while the other bacteria are pathogenic, often resulting in BV. *Lactobacillus* is the most prevalent of all the lactic acid bacteria, species including *L.*

rhamnosus, L. gasseri, L. casei, L. crispatus, L. reutei, L. acidophilis and *L. plantarum* amongst others (Pessione, 2012; Azad *et al.*, 2018). The key roles *Lactobacilli* play in these microenvironments include immunomodulation and prevention of pathogenic invasion (Petrova *et al.*, 2013; Tachedjian *et al.*, 2017; Jang *et al.*, 2019; Armstrong & Kaul, 2021).

Firstly, this genus serves to preserve the epithelial barrier of both the gut and vagina, which prevents CD4+ activation-associated pathogenic invasion (Amabebe & Anumba, 2020). The mucus that lines the epithelial cells serves to protect both microenvironments from pathogenic invasion, including HIV (Mall *et al.*, 2017). The mucus can trap and removes pathogens and also act as an anchor for the 'good' bacteria, therefore, shaping the microbial composition (Petrova *et al.*, 2013). Both the gut and vaginal microbiota influence mucus functioning. The gut bacteria secrete SCFA which upregulates the expression of the *MUC2* gene, which encodes for mucus-secreting mucins, and vaginal D-lactate improves the trapping of HIV by mucus (Lai *et al.*, 2009; Hald, 2015; Armstrong & Kaul, 2021). However, some gut *Lactobacilli* impact mucin production, which is required to form the protective intestinal barrier over the gut wall.

Secondly, both the production of lactic acid and H_2O_2 by LABs can inhibit the growth of pathogens in both these microenvironments (Knaus *et al.*, 2017; Tachedjian *et al.*, 2017). The lactic acid produced can regulate inflammatory responses, which is important since inflammation is associated with an increased HIV acquisition risk (Masson *et al.*, 2015). Previous studies showed that in cervicovaginal cell lines, both D- and L-lactic acid can reduce IL-6 and IL-8-mediated inflammatory responses. Additionally, lactic acid was able to elicit IL-1 receptor antagonist (IL-1RA), preventing activation of IL-1 (Hearps *et al.*, 2017; Delgado-Diaz *et al.*, 2020).

Lactic acid production maintains an acidic pH. The maintenance of pH is essential as it plays a major role in the homeostasis of inflammatory responses. The pH has been shown to have an impact on T cells and natural killer cells, which play a part in tumor apoptosis, by preventing the activation of CD4+ and CD8+ T cells *in vitro* (Huber *et al.*, 2017; Erra Díaz *et al.*, 2018). Additional studies have also shown that immune cells are sensitive to changes in pH. At neutral pH (7), no anti-inflammatory cytokines were elicited, but rather pro-inflammatory cytokines such as TNF-α

were secreted via the TLR of the NF-$_k$B pathway (Delgado-Diaz *et al.*, 2020). Overall, pH values between 5.8 and 7, impair the function of immune cells such as T-cells, natural killer cells, monocytes, macrophages, and dendritic cells (Martínez *et al.*, 2007; Calcinotto *et al.*, 2012; Jancic *et al.*, 2012). Therefore, the maintenance of pH is essential for optimal immune responses and the prevention of tumors. Additionally, the production of lactic acid by gut *Lactobacilli* shows that these bacteria are also involved in digestion as lactic acid is produced via fermentation of various hexoses such as galactose and lactose (Pessione, 2012). *Lactobacilli* have also been implicated in reducing insulin resistance by preventing inflammation that is linked to insulin (Salles *et al.*, 2020).

2.9. Conclusion

The human microbiota is an important community of microbes that live in symbiosis with the host (Valdes *et al.*, 2018; Bayigga *et al.*, 2019). This community plays a pivotal role in several host mechanisms and aid in health. In the past, evidence for the interaction of the gut and vaginal microbiota with drugs has been presented. ARVs, in particular, have been suspected of exhibiting antibacterial effects against the microbiota, including species that are essential to health and whose depletion could result in adverse outcomes (Pinto-Cardoso *et al.*, 2018). Moreover, in recent years, ARVs have been used not only as HIV treatment tools but also for prevention known as PrEP. Their impact on the mucosal microbiota, especially those in the pipeline, remains understudied, therefore, more research is necessary. (Klatt *et al.*, 2017; Taneva *et al.*, 2018). Understanding the differential interactions between ARVs and the gut and the vaginal bacteria could help inform researchers of the unintended effects of ARVs on mucosal microbiota.

Chapter 3

Methodology

3.1. Biomedical ethics approval statement

The present study was approved by the University of KwaZulu-Natal Biomedical Research Ethics Committee (Approval number BREC/00003679/2021; Appendix A). The present study was under the Mucosal Injury from Sexual Trauma (MIST) parent study. The parent study recruited South African women from the Vulindlela area in rural KwaZulu-Natal and Philippi Township in peri-urban Western Cape. The inclusion criteria included: women between the ages of 14 – 19 years old or 25 – 35 years old, willing and able to provide informed consent and/or assent to participate in the study, not pregnant, not having taken antibiotics in the past month, and HIV negative.

3.2. Bacterial isolation and handling

3.2.1. Handling of vaginal *Lactobacilli*

Vaginal *Lactobacillus* species used in this study were previously isolated at the University of Cape Town. The bacterial isolates received were *L. crispatus, L. gasseri, L. jensenii, L. mucosae* and *L. vaginalis.* These samples were isolated from cervicovaginal secretions collected as part of the completed WISH cohort study of HIV-negative South African (Cape Town) adolescent girls aged 15-19 years and their identity was confirmed by polymerase chain reaction (PCR) and 16 S rRNA genome sequencing (Happel *et al.*, 2020). Study participants were tested for STIs caused by *Chlamydia trachomatis*, type 2 human simplex virus (HSV-2), *Neisseria gonorrhoea, Mycoplasma genitalium,* and *Trichomonas vaginalis,* as well as bacterial vaginosis (BV). In this study, only bacterial isolates from women who tested negative for the aforementioned STI-causing organisms and BV as per the Nugent score (7-10) were used. The Nugent score measures BV on a scale of 0-10 by assessing the vaginal microbiota composition. A 0-3 score indicates a *Lactobacilli* dominated microbiota, a score of 4-6 indicates an intermediate microbiota and a score of 7-10 indicates BV (Amegashie *et al.*, 2017). The bacterial isolates were streaked onto pre-reduced De Man, Rogosa, and Sharpe (MRS) agar plates (Merck, Kenilworth, NJ, US), supplemented with 0.05 g/L L-cysteine (Glentham, Corsham, United Kingdom) and 1% (v/v)Tween 80 (Glentham,

Corsham, United Kingdom) and anaerobically incubated for 48 hours at 37°C. Single colonies were re-suspended with pre-reduced MRS broth (Merck, Kenilworth, NJ, US) and anaerobically incubated overnight at 37°C, using an Oxoid anaerobic jar (Thermo Fisher Scientific™, Waltham, MA, US) and Anaerogen sachet (Thermo Fisher Scientific™, Waltham, MA, US). The bacterial isolates were inoculated with 60% (v/v) glycerol (Sigma-Aldrich, Darmstadt, Germany) and stored at -80°C until further use. An aseptic technique was observed throughout when handling the *Lactobacillus* isolates.

3.2.2. Isolation and handling of gut bacteria

Stool samples from eleven consenting adolescent girls and young women of ages 14-24 years from Durban, South Africa were collected using a dry swab and stored at -80°C until further isolation could be conducted. The swabs were suspended in 50 mL pre-reduced MRS broth supplemented with L-cysteine and Tween 80, and vortexed briefly. Five swabs inoculated in broth were incubated micro-aerobically using an Oxoid anaerobic jar and the remaining swabs were incubated anaerobically using an Oxoid anaerobic jar, in the presence of Anaerogen sachets at 37°C for 48 hours before ten serial doubling dilutions of the cultures were performed. The dilutions were streaked out onto pre-reduced MRS agar (supplemented with L-cysteine and Tween 80) and incubated for 48 hours at 37°C. Subcultures were done by re-suspending distinct single colonies in pre-reduced MRS broth and incubated overnight at 37°C and re-streaking onto pre-reduced MRS agar. The bacterial isolates were sub-cultured a total of three times. Purity was confirmed by Gram staining using a previously described method (Bartholomew & Mittwer, 1952), discussed in detail in section 3.3.

3.2.3. Identification and confirmation of putative gut bacteria

To confirm that the isolated bacteria were indeed lactic acid-producing bacteria (LABs) the16S ribosomal (r) DNA gene targeted colony polymerase chain reaction (PCR) was performed using the primer pair 15F, 5'-GCTCAGGATGAACGCTGG-3' and 687R 5'-CACCGCTACACATGTACTTC-3'. The initial denaturation was set for 30 seconds at 94ºC, followed by 30 cycles of denaturation for 30 seconds at 94ºC, annealing for 30 seconds at 50ºC,

extension for 30 seconds at 68ºC, and final extension for 5 minutes at 68 ºC. The resultant amplicons were analysed on a 1% (w/v) agarose gel (Thermo Fisher Scientific™, Waltham, MA, US) prepared in 1X TAE buffer (Thermo Fisher Scientific™, Waltham, MA, US). The total nucleic acid of the confirmed LABs was isolated using the Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research, Irvine, CA, USA, Catalogue No. D6005) following the manufacturer's protocol. Briefly, single colonies were resuspended in 100 µL ultrapure water produced by the Milli-Q Plus Ultra-pure water system (Millipore, Marlboro, USA) and diluted with 750 µL BashingBead buffer (Zymo Research, Irvine, CA, USA). The bacteria were lysed using the BeadBug™ bead beater (Benchmark Scientific, Sayreville, NJ, USA) at 4 000 rpm for five minutes and centrifuged at 10 000 x g for one minute using a Spectrafuge™ 16M (Sigma-Aldrich, Darmstadt, Germany). The supernatant was centrifuged at 8 000 x g for one minute and genomic lysis buffer was added to repeat the centrifugation step and wash the resultant DNA. The 16S rRNA V3-V4 region of the isolated DNA was amplified using the primer pair 16S-27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 16S-1492R 5'-CGGTTACCTTGTTACGACTT-3' (Inqaba Biotech™, Durban, South Africa). The amplicons were visualised on a 1% (w/v) agarose gel and stained with EZ-Vision© Bluelight DNA dye (Avantor, Pennsylvania, USA). Thereafter the amplicons were sent to Inqaba Biotechnical Industries (Pty) Ltd for sequencing. The BLASTn analysis was done on the NCBI website to find matching sequences in the database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences with an identity threshold of 92% were considered a match.

3.2.4. ATCC strains

Gut ATCC strains *E. faecalis,* NCTC 13379 (catalogue number 714197)*, E. faecium,* ATCC 27270 (catalogue number 679556)*, L. gasseri,* ATCC 19992 (catalogue number 127236)*, L. lactis,* ATCC 49032 (catalogue number 980454)*, B. animalis,* ATCC 25527 (catalogue number 10921231) and *B. breve*, ATCC 15700 (catalogue number 175441) were obtained from Microbiologics (St. Clouds, MN, USA) as KwikStiks. To culture these, the KwikStiks were left to thaw at room temperature and the vials at the top were broken and the pellets at the bottom were crushed. The *Enterococcus* and *Lactobacillus* swabs were rolled onto pre-reduced MRS agar and the *Bifidobacterium* swabs were rolled onto brain heart infusion (BHI) agar (Condalab, Madrid, Spain) and incubated anaerobically at 37°C for 48 hours. Distinct single colonies were inoculated in pre-reduced MRS broth (*Enterococcus* and *Lactobacillus*) and BHI broth (*Bifidobacterium*) and

incubated anaerobically at 37°C overnight. These were inoculated with 60% (v/v) glycerol and stored at -80°C until further use.

3.3. Gram staining

The bacterial morphology was confirmed employing Gram staining, using the Gram stain kit (77730-1KTF) (Sigma-Aldrich, Buchs, Switzerland) as previously described (Bartholomew & Mittwer, 1952). Briefly, single colonies were picked, mounted onto a microscopic slide, and heat-fixed. These were stained with crystal violet (Sigma-Aldrich, Buchs, Switzerland) for one minute and rinsed with water. Iodine (Sigma-Aldrich, Darmstadt, Germany) was added for one minute, rinsed with water, and flooded with Gram's decolouriser solution (acetone and ethanol) (Sigma-Aldrich, Buchs, Switzerland). Finally, slides were stained with safranin (Sigma-Aldrich, Buchs, Switzerland) for one minute and rinsed with water. The slides were dried and visualised under an Olympus CKX41 light microscope at 200 X magnification. Images were taken with an attached Moticam 3.0 MP.

3.4. Bacterial growth kinetics

The overall health and proliferation of the cells were tested using microdilution assays and spread plating techniques. First, 100 µL of the bacteria were standardised to 1 x 10^5 CFU/mL (~OD600=0.100 ±0.01) [recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)], added onto a flat bottom TPP® 96-well tissue culture plate (Sigma, Darmstadt, Germany) and incubated anaerobically at 37°C. The bacterial growth was measured at OD600 nm after 0-, 3-, 24- and 48 hours' incubation using a VersaMax microplate reader (Molecular Devices, San Jose, CA, USA), and the SoftMax Pro software version 7 was used to capture the growth dynamics of bacterial species in the absence of ARVs. Secondly, overnight standardised cultures were incubated anaerobically at 37 °C for 0-, 3-, 24-, 30-, 48-, 51- and 72 hours. Ten serial doubling dilutions were prepared and 100 µL of each dilution was spread plated onto MRS agar. The plates were incubated anaerobically for 48 hours at 37 °C. Thereafter, the number of colonies was counted and recorded. The weighted mean colony forming unit (CFU)/mL was calculated. Experiments were performed in triplicate.

3.5. The relationship between LABs and pH

3.5.1. The effect of pH on bacterial growth

The MRS and the BHI broths were adjusted to different pH levels, viz. pH 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, and 6 using the 1 M NaOH, 1 M HCL, and pH50 VioLab pH meter (Lasec ®, Cape Town, South Africa). Fifty microliters of these were each subsequently inoculated with 50 µL standardised overnight cultures in a flat bottom TPP® 96-well tissue culture plate. The plate was incubated anaerobically at 37°C and the bacterial growth was measured at OD600 nm after 0-, 3-, 24-, and 48 hours using the VersaMax microplate reader. All experiments were done in duplicate.

3.5.2. The influence of LABs on pH

The influence of LABs on pH was observed by cultivating standardised overnight cultures anaerobically at 37ºC. The bacterial growth of the bacteria at different pH was measured at OD600 nm after 0-, 3-, 24-, and 48 hours incubation using the Versa Max microplate reader. Experiments were done in duplicate analyses.

3.6. Lactic acid production by LABs

To determine the amount of lactic acid produced by the bacteria, D- and L-lactate was quantified using the D-lactate Colorimetric Assay (MAK0581KT) and L-Lactate Assay (MAK329-1kt) kits (Sigma-Aldrich, Darmstadt, Germany), respectively. Both assays were carried out according to the manufacturer's instructions as outlined below.

3.6.1 Determination of D-lactate production

The amount of D-lactate produced by cells was determined by first preparing five standard concentrations [0 mM (blank), 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM] by diluting the 100 mM D-

lactate standard solution with assay buffer. The samples were prepared by standardising overnight cultures to 2 x 10^6 CFU/mL and homogenising them in an ice-cold assay buffer. Samples were centrifuged for ten minutes at 10 000 x g and 50 µL of each sample was treated with 50 µL of the reaction mix in a 96-well plate, including the D-lactate standards. Both MRS and BHI broths were used as negative controls. The plate was incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 450nm (A_{450nm}) using the VersaMax microplate reader and analysed on the SoftMax Pro version 7 software. The D-lactate standards were used to generate the standard curve in Figure 3.1.A, which was used to calculate the concentration of D-lactate in the samples, using the equation: $C=S_a/S_v$. The amount of D-lactate extrapolated from the graph was denoted as S_a, S_v being the volume (µL) added to the wells and C was the concentration of D-lactate.

3.6.2. Determination of L-lactate production

The L-lactate assay was conducted by first preparing a 2 mM L-lactate premix from a 20 mM L-lactate standard, using ultrapure water. The premix was diluted in ultrapure water to make 1.6 mM, 1.2 mM, 0.8 mM, 0.6 mM, 0.4 mM, and 0.2 mM L-lactate solutions to generate the standard curve (assay buffer alone represented the 0 mM L-lactate sample), shown in Figure 3.1.B. A master reaction mix was prepared, consisting of assay buffer, enzyme A, enzyme B, nicotinamide adenine dinucleotide (NAD) solution, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution, which was used for the test samples. A negative control consisting of assay buffer, enzyme B, NAD solution, and MTT solution was prepared. Twenty microliters of the L-lactate standards and the standardised overnight cultures were added to separate well in the 96-well plates, followed by 80 µL of the respective reaction mixes. Before incubation in the dark at room temperature for 20 minutes, the initial absorbance at 565nm (A_{565nm}) was measured using the VersaMax microplate reader with SoftMax Pro version 7 software. The final absorbance was measured at 565nm after the incubation time had expired. The initial absorbance was subtracted from the final absorbance, the difference was inputted in the equation shown in Figure 3.1 B to quantify the amount of L-lactate produced by each species.

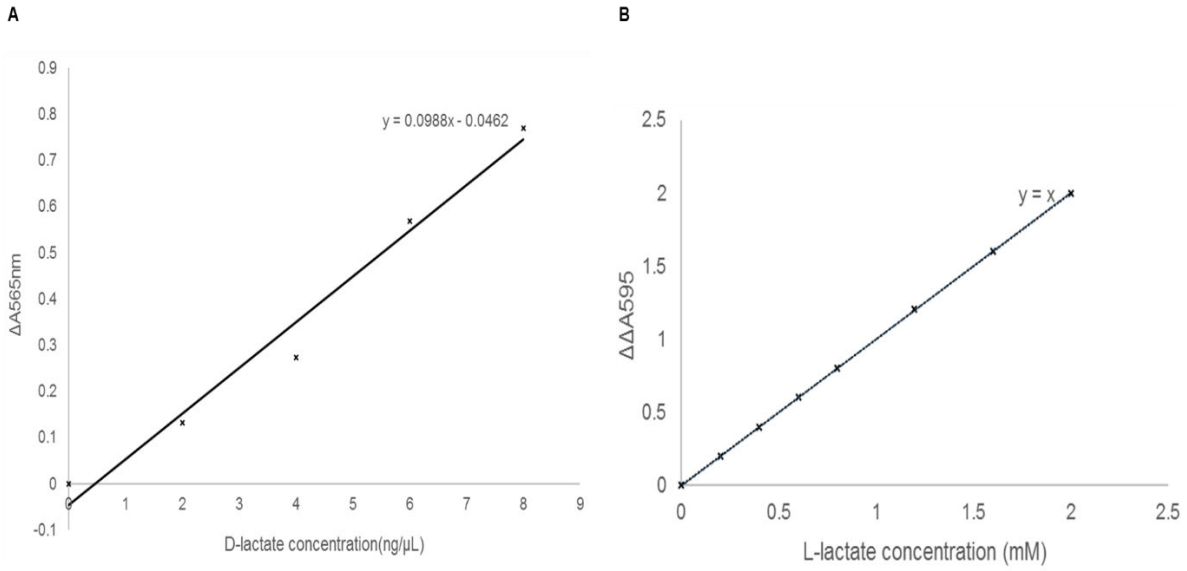

Figure 3.1: Lactic acid standard curve. The standard curve for D-lactate **(A)** was generated by diluting 100 mM of D-lactate standard with assay buffer to make 2, 4, 6, 8, and 10 mM. Fifty microliters of these were inoculated with 50 µL of reaction mix in a 96-well plate. The plate was incubated at room temperature for 30 minutes and the absorbance was measured at 450 nm. A 0 mM D-lactate standard (assay buffer alone) was used as a blank. The L-lactate curve (B) was generated by preparing different concentrations of L-lactate standard by diluting with ultrapure water. These were treated with a reaction mix on a 96-well plate. The plate was incubated for 30 minutes at room temperature in the dark and the absorbance was measured at 565 nm using the VersaMax microplate reader (Molecular Devices, San Jose, CA, USA). The D-lactate Calorimetric Assay (MAK0581KT) and L-lactate Assay (MAK329-1kt) kits (Sigma-Aldrich, Darmstadt, Germany) were used. Samples that had concentrations outside the range of the standards were diluted and re-analysed.

3.7. Hydrogen peroxide production by bacteria

The hydrogen peroxide (H_2O_2) produced by the isolated bacteria was measured using the Pierce™ Quantitative Peroxide Assay kit (Thermo Fisher Scientific™, Waltham, MA, US, catalogue no. 23280). The working reagent was made by diluting one µL of Pierce™ peroxide reagent A (Consisting of 25mM ammonium ferrous (II) sulfate and 2.5 M H_2SO_4) with 100 µL of Pierce™ peroxide reagent B (consisting of 100 mM sorbitol and 125 µM xylenol orange in water). Thirty percent H_2O_2 (8.8 mM) was serially diluted with ultrapure water to make nine dilutions and ultrapure water was used as a negative control. Twenty µL of standardised overnight cultures were mixed with 200 µL of working reagent. The plate was incubated at room temperature for 20 minutes. The absorbance at 595 nm (A_{595nm}) was measured using the VersaMax microplate reader with SoftMax Pro version 7 software. The L-lactate standards were used to construct the

standard curve shown in Figure 3.2, which was used to determine the concentration of H_2O_2 produced by each isolate, by extrapolation.

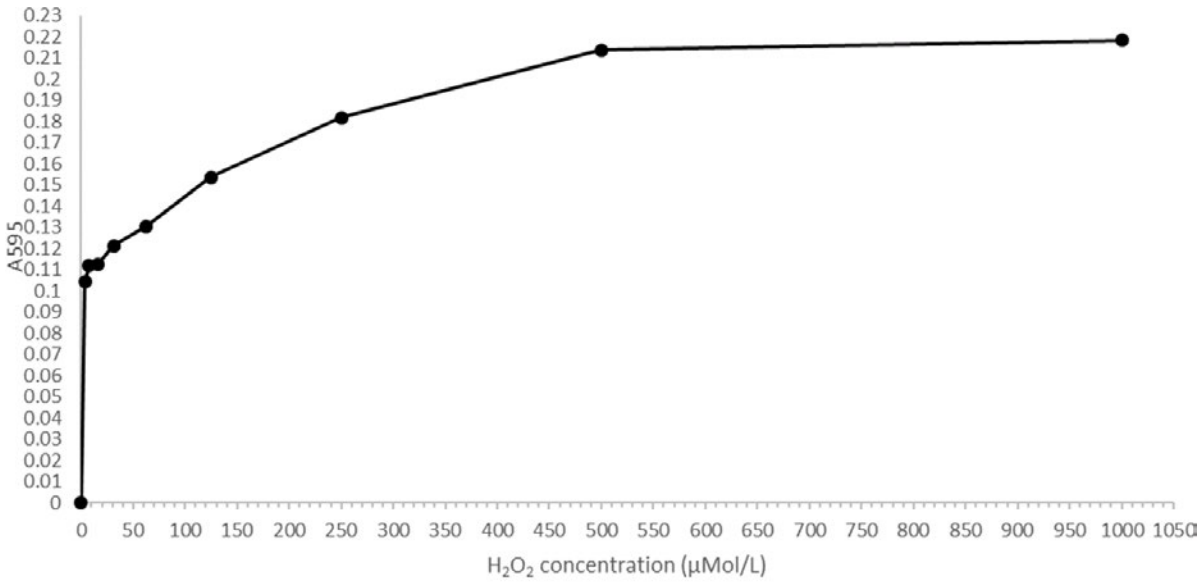

Figure 3.2: Hydrogen peroxide standard curve. Hydrogen peroxide (H_2O_2) production was measured using the Pierce™ Quantitative Peroxide Assay kit. Briefly, thirty percent H_2O_2 was serially diluted (1:2) to make nine dilutions with ultrapure water and treated with the H_2O_2 buffers, and the absorbance was measured at 595nm.

3.8. The antibacterial properties of antibiotics against bacteria of the mucosal microbiota

The first row of a 96-well plate was filled with 100 µL of 20 mg/mL amoxicillin, ampicillin, clindamycin, metronidazole, penicillin, rifampicin, or streptomycin in separate wells, while the remaining wells were filled with 50 µL MRS broth and BHI broth alone. Six serial dilutions (1:2) were performed in the wells containing 50 µL MRS broth or BHI broth to achieve a final concentration range of 5 mg/mL – 0.315 mg/mL. The last row was used as a no antibiotic control. Fifty microliters of the standardised bacteria were added to each of the wells and the plates were incubated anaerobically at 37°C. The bacterial growth was measured at OD600 nm and was measured on the VersaMax microplate reader. Data was acquired on the SOFTMax Pro version 7 software at different times (0-, 3-, 24-, and 48 hours). The experiments were done in duplicate.

3.9. Identification of optimal solvents for ARVs

The solubility of ARVs in different solvents was tested. Firstly, MRS broth, BHI broth, deionised water (dH_2O), phosphate-buffered saline (PBS) (Gibco™, Waltham, MA USA) absolute ethanol (Acechem, Johannesburg, South Africa), 70% ethanol (Acechem, Johannesburg, South Africa), 99.98% methanol (Acechem, Johannesburg, South Africa), and 99.99% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Darmstadt, Germany) were used to dissolve DTG (Aurobindo Pharma, Johannesburg, South Africa), TDF (Mylan, Kempton Park, South Africa), and TLD (Aspen, South Africa), by adding 1 mL of solvent at a rate of 1 mL per minute. This was done to determine the minimum volume sufficient to completely dissolve the drugs. The volume used to completely dissolve selected ARV drugs and final concentrations are reported in Table 3.1. Only one millilitre of MRS broth was required to completely dissolve DTG, resulting in the highest concentration of 310 mg/mL, while 3 mL, 2 mL, 2mL, and 4 mL of BHI broth, dH_2O, PBS, and DMSO were required, respectively. The MRS broth was also the most effective in dissolving TDF as only 1 mL was required, while 6 mL, 3 mL, 3 mL, and 3 mL of BHI broth, dH_2O, PBS, and DMSO were needed to completely dissolve this drug, respectively. Generally, TLD was less soluble. Five mL of MRS was required to dissolve TLD and 4 mL of the remaining solvents was required to dissolve TLD. Overall, MRS seemed like the most ideal solvent to be used for downstream assays compared to the other solvents.

Table 3.1: The solubility of different antiretroviral drugs in different solvents and their final concentrations.

Solvent	ARV name	ARV mass (mg)	The final volume (mL)	Concentration (mg/mL)[a]
MRS broth	DTG	310	1	310
	TDF	620	2	310
	TLD	1370	5	274
BHI broth	DTG	310	3	103.33
	TDF	620	6	103.33
	TLD	1370	4	342.5
dH_2O	DTG	310	2	155
	TDF	620	3	206.67
	TLD	1370	4	342.5
PBS	DTG	310	2	155
	TDF	620	3	206.67
	TLD	1370	3	456.67
DMSO	DTG	310	4	77.5
	TDF	620	3	206.67
	TLD	1370	4	342.5

[a] Concentration= mass/volume; MRS- De Man, Rogosa and Sharpe; dH_2O- distilled water; PBS- phosphate buffered saline; DMSO-Dimethyl sulfoxide; TLD- tenofovir/lamivudine/dolutegravir; DTG- dolutegravir; TDF- tenofovir disoproxil fumarate

3.10. The effect of ARV solvents on bacterial growth

The effect of solvent on bacterial viability was also determined. Overnight cultures of bacteria were standardised to 1 x 10^5 CFU/mL (~OD600=0.100 ±0.01), and 50 µL of solvent was inoculated with 50 µL of standardised cultures in a flat bottom TPP® 96-well tissue culture plate. The bacterial growth was measured at OD600 nm after 0, 3, 24, and 48 hours using the VersaMax microplate reader and analysed using the SoftMax Pro software version 7. Lastly, standardised cultures were inoculated separately with 50% (v/v) of BHI broth, dH_2O, PBS, absolute ethanol, 70% ethanol, 99.98% methanol, or 99.99% DMSO. One hundred µL of these were spread-plated onto MRS agar plates using sterile glass beads and incubated anaerobically at 37°C for 48 hours. All these experiments were done in duplicate.

3.11. The antibacterial properties of ARVs against bacteria of the mucosal microbiota

Overnight cultures of the purified bacteria were standardised to 1 x 10^5 CFU/mL (~OD600 = 0.100 ±0.01). The first row of the 96-well plate was filled with a final concentration of 5 mg/mL of each of the ARVs, DTG, TDF, TLD, TAF, or CAB in 100 µL broth. To the rest of the wells, 50 µL of the broth alone was added. Serial (1:2) dilutions of the ARVs were done in the wells containing 50 µL broth, excluding rows six and twelve, which were used as negative controls (no ARVs). A medium control (no bacteria or ARVs, to make sure the medium is not contaminated) was included. Fifty microliters of adjusted bacterial species were added to each well in triplicate, sealed, and anaerobically incubated at 37ºC. The bacterial growth was measured at OD600 nm after 0, 3, 24, and 48 hours using the VersaMAX microplate reader. Analysis was performed using the SoftMax Pro software version 7. The lowest concentrations of ARV inhibiting growth in wells were considered as the minimal inhibitory concentration (MIC) for a particular bacterial species. Experiments were repeated three times.

3.12. The effect of ARVs on the pH of the broth

The pH of the DTG, TDF and TLD dissolved in MRS at various concentrations (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.63 mg/mL, and 0.315 mg/mL) was measured after 0-, 3-, 24- and 48 hours

of anaerobic incubation at 37ºC using the pH50 VioLab pH meter. Duplicate analyses were performed.

3.13. Statistical Analysis

Generation of graphs and statistical analyses were performed in GraphPad Prism software version 8.2.0 (San Diego, CA, USA). The two-way ANOVA and subsequently Dunnett's multiple comparison test were used to analyse data. A repeated measure (RM) one-way ANOVA with Dunnett's multiple comparison test or ordinary ANOVA with Dunnett's was also conducted. A false-discovery rate step-down procedure was used to adjust p-values for multiple comparisons and adjusted $p<0.05$ was considered significant.

Chapter 4

Results

4.1. Gram staining

The isolated bacteria were identified and confirmed based on their colony morphology, Gram stain, colony PCR, 16S rRNA PCR, Sanger sequencing, and BLASTn analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Gram stain images of vaginal *L. crispatus, L. gasseri, L. jensenii, L. mucosae,* and *L. vaginalis* are depicted in Figure 4.1.A-E, while the gut-isolated species *E. faecium, L. fermentum, L. plantarum,* and *L. rhamnosus* are depicted in Figure 4.1.F-I. Gut-derived ATCC strains shown in Figure 4.1.J-O (*B. animalis, B. breve, E. faecalis, E. faecium, L. gasseri,* and *L. lactis*), were included for comparison. All these bacteria tested Gram-positive. The gut-derived species had the same morphology as the ATCC strains used for comparison. The morphology of *L. rhamnosus* differed in that it was a long rod-shaped bacterium that looked like spindles, and the other *Bifidobacteria* and *Lactobacilli* bacteria were short, slight rods (Figure 4.1.I). The *Enterococcal* bacteria on the other hand were cocci (Figure 4.1. F, L and M).

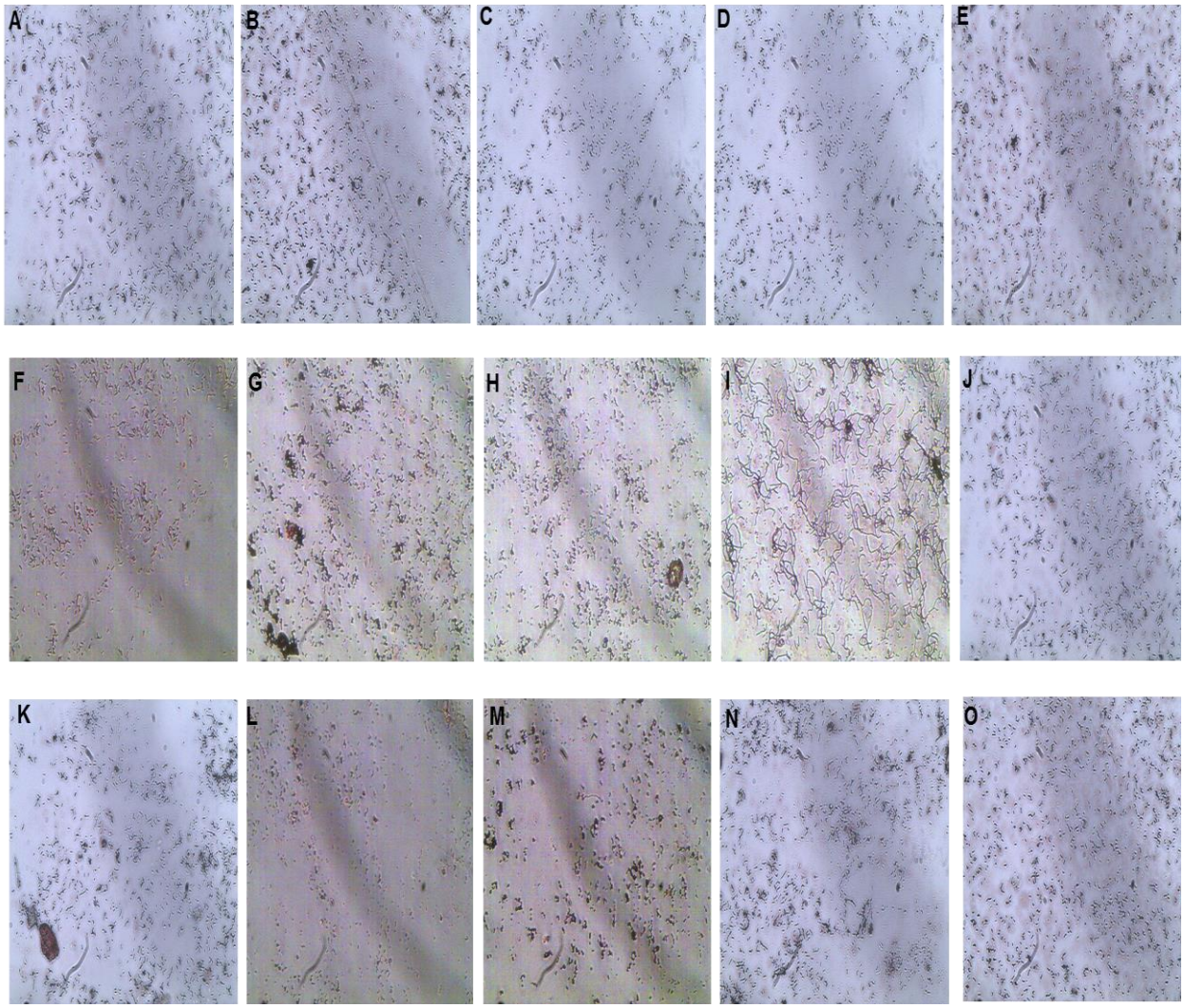

Figure 4.1: Gram stain of five vagina-derived Lactobacilli species, gut-derived LABs isolated from South African adolescent girls and young women, and gut-derived ATCC strains (200 X magnification). Gram-stained bacterial species associated with a healthy mucosal microbiota, isolated from adolescent girls and young women are shown in Panels A to I: five vaginal species namely *L. crispatus* (A), *L. gasseri* (B), *L. jensenii* (C), *L. mucosae* (D), and *L. vaginalis* (E) and four gut-derived species namely *E. faecium* (F), *L. fermentum* (G), *L. plantarum* (H), and *L. rhamnosus* (I). Six ATCC strains are shown: *B. animalis* (J), *B. breve* (K), *E. faecalis* (L), *E. faecium* (M), *L. gasseri* (N), and *L. lactis* (O). The slides were visualised under the Olympus CKX41 light microscope at 200 X magnification. The images were taken with a Moticam 3.0 MP camera attached to the microscope.

4.2. Identification and confirmation of gut-derived lactic acid-producing bacteria by PCR

The PCR amplicons of 16 putative LAB species isolated from stool samples of adolescent girls and young women are depicted in Figure 4.2. Lane 'M' is the standard size that was used to determine the sizes of the other amplicons. All the amplicons had the same size which was

approximately 500 bp which correlated with the size of the reference strains. The negative control lane showed no amplicon, indicating an absence of the lactate dehydrogenase gene, which is the gene responsible for lactic acid production by LABs.

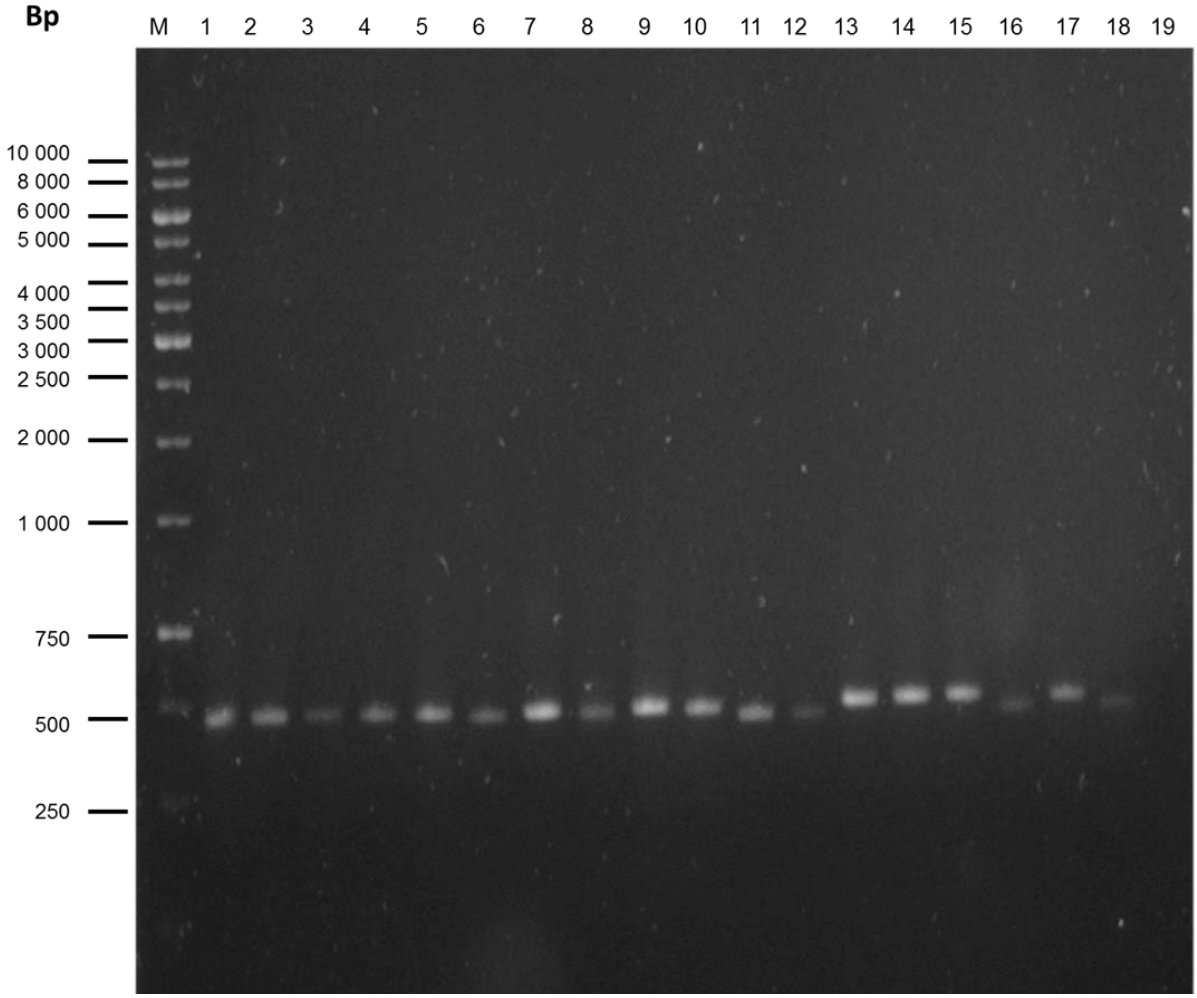

Figure 4.2: Confirmation of the lactate dehydrogenase gene of putative lactic acid-producing bacterial species isolated from the gut of South African adolescent girls and young women. The colony PCR results of the lactate dehydrogenase gene. Lane M: 1 Kb ladder molecular weight marker, lane 1: *L. gasseri* ATCC strain, lane 2: *L. gasseri* ATCC strain and *L. lactis*, lanes 3 – 18: gut isolates (L1 – L16), lane 19: negative control, containing only ultrapure water. The PCR was done using the 15F and 687R primers and the resultant amplicons were resolved on 1% (w/v) agarose gel with SafeView staining. The gel was viewed using the G: Box and the GeneSyne 2.0 software.

To identify bacteria at species level, the 16S rRNA amplification was done. The goal was to select at least 10 distinct species. Therefore, based on the morphology results, only 10 out of the 16 putative LABs were selected for further analysis (Figure 4.3). The results of the 16S rRNA amplification of 10 putative LABs are depicted in Figure 4.3.A. Lane 'M' is the standard size that

was used to determine the sizes of the other putative LABs. , while lanes 1-10 show the genomic sizes of the LABs. The genomic size of the LAB in lane 1 is the smallest, approximately 1 Kb, while all the others have genomic sizes of about 1.5 Kb. Following Sanger Sequencing and BLASTn analysis using the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 4.1), the majority of the bacterial isolates were identified as *L. fermentum* (lanes 4, 5, 6, 7, 9 and 10), two were identified as *E. faecium* (lanes 1 and 2), while there was only one isolate of *L. plantarum* (Lane 8) and *L. rhamnosus* (Lane 3). One isolate of each species was used for downstream analyses (impacts of pH on the growth of bacteria, lactic acid production by the bacteria, H_2O_2 production by the bacteria, susceptibility of bacteria to antibiotics, and the inhibitory effects of ARVs). The isolated *Enteroccoci* were used for downstream assays as they are also associated with a healty gut microiota.

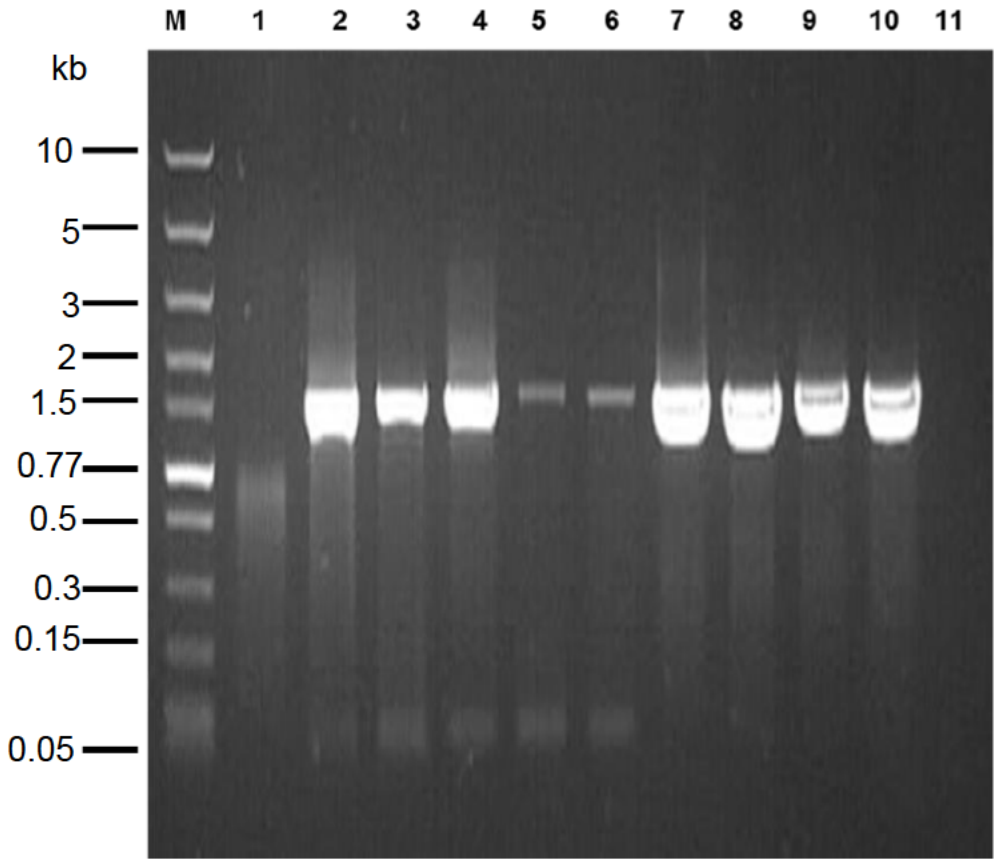

Figure 4.3: Agarose gel analysis of 16S subunit amplification. The 16S region was amplified using the 16S-27F and 16S-1492R primers post-total DNA isolation. Lane M: NEB N3228 fast ladder, lanes 1-10: putative LAB species, lane 11: negative control. The gel was viewed using the G: Box and the GeneSyne 2.0 software.

Table 4.1: Percentage similarity of the isolated gut bacteria compared to species available in the BLASTn NCBI website.

Species number	Predicted species name	Accession number	Percentage similarity (%)
1	*Enterococcus faecium*	MZ452347.1	99
2	*Lacticaseibacillus rhamnosus*	OM959233.1	92
3	*Lactobacillus fermentum*	CP094655.1	99
4	*Enterococcus faecium*	ON366690.1	99
5	*Lactobacillus fermentum*	ON005263.1	99
6	*Limosilactobacillus fermentum*	ON005263.1	99
7	*Lactiplantibacillus plantarum*	ON209902.1	99
8	*Lactobacillus fermentum*	CP094655.1	99
9	*Lactobacillus fermentum*	CP094655.1	99
10	*Limosilactobacillus fermentum*	MH771720.1	97

4.3. Bacterial Growth kinetics

The growth of the bacteria was monitored for the purpose of understanding growth kinetics and determining the ideal time to conduct downstream assays. The proliferation of the different bacterial species was also monitored by calculating the colony-forming units per millilitre (CFU/mL). The exponential growth phase of *L. crispatus, L. gasseri* and *L. vaginalis* was between 0 and 24 hours. The stationary phase was reached at 24 hours and the death phase was reached after 48 hours of incubation, except for *L. vaginalis* where the death phase was reached earlier,

after 30 hours of incubation. During the death phase, *L. crispatus* and *L. gasseri* declined much slower than *L. jensenii, L. mucosae* and *L. vaginalis,* whose growth declined rapidly (Figure 4.4. A). The growth of *L. jensenii* and *L. mucosae* was much slower during the exponential growth phase that was observed between 0 and 30 hours and the death phase started after 30 hours. The gut-isolated bacteria reached the exponential phase between 3 hours and 30 hours, the stationary phase after 30 hours, and the death phase after 48 hours of incubation (Figure 4.4.B). *Lactobacillus plantarum* had an exponential phase that lasted to 48 hours and the death phase started after 48 hours, while the death phase was not observed (Figure 4.4.B).The ATCC strains *B. animalis, B. breve, E. faecalis,* and *E. faecium (*g*E)* reached the exponential phase between zero and 24 hours. The stationary phase was observed between 24- and 48 hours of incubation and the death phase soon after. The ATCC strains of *L. gasseri,* and *L. lactis* reached the exponential phase at three hours, the stationary phase after 24 hours, and the death phase after 48 hours (Figure 4.4.C).

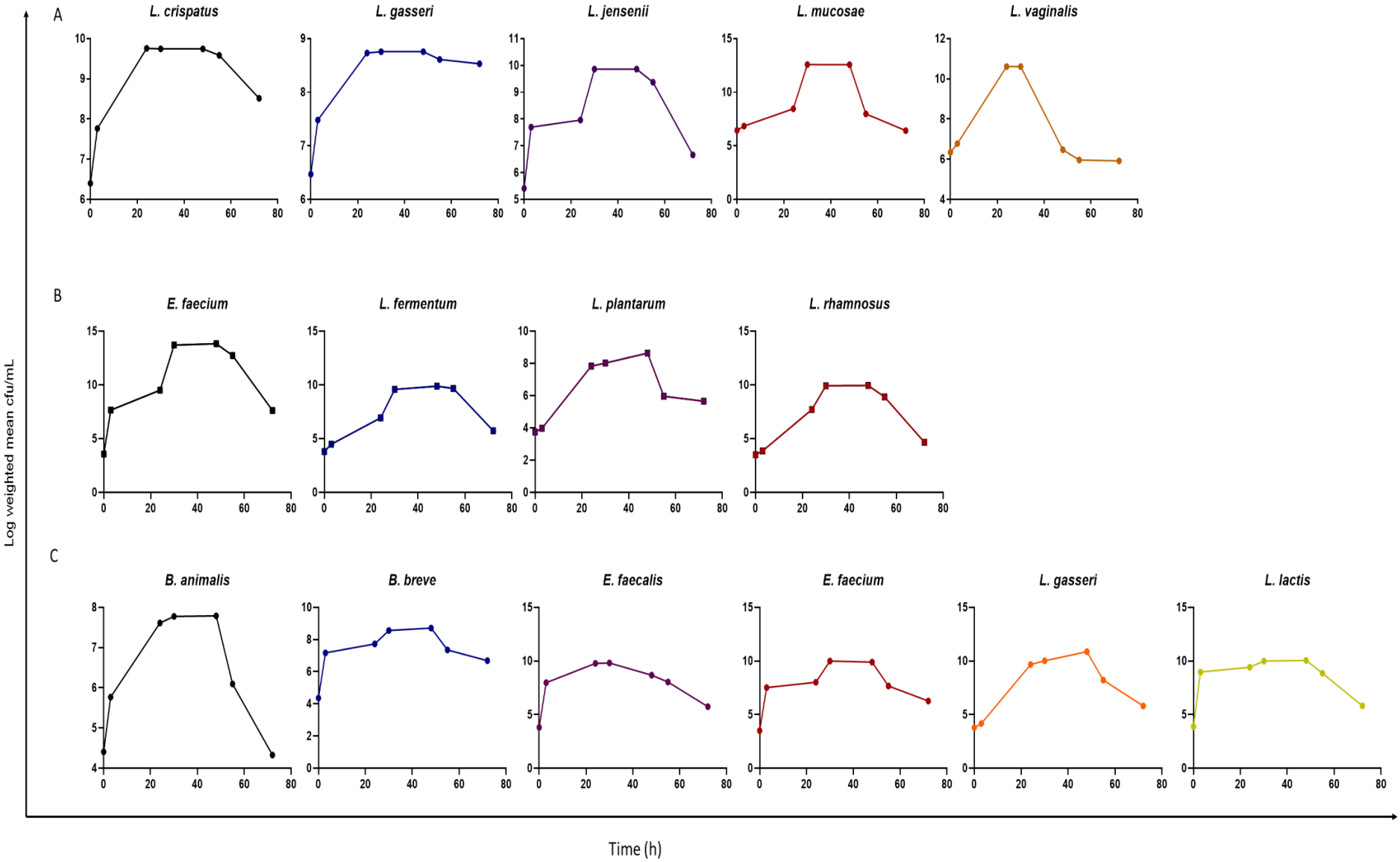

Figure 4.4: Growth curve of LABs isolated from the vagina and gut of South African adolescent girls and young women and gut-derived ATCC strains. The proliferation of the different species isolated from the vagina (A), gut (B), and gut-derived ATCC strains (C) was observed by duplicate calculating the colony-forming units per millilitre (CFU/mL). The CFU/ml for ten serial (1:2) dilutions were also calculated after 0-, 3-, 24-, 30-, 48-, 51-, and 72 hours of anaerobic incubation at 37°C. The weighted mean CFU/mL was calculated using duplicate experiments and the log CFU/mL was plotted.

The proliferation of the bacterial cells was monitored spectrophotometrically over time (Figure 4.5). The vaginal *Lactobacilli* and ATCC strains have a similar growth curve (Figure 4.5.A and Figure 4.5.C). The exponential phase was reached after three hours of incubation and continued until the stationary phase was reached at 24 hours and continued until 48 hours of incubation. On the other hand, the gut-isolated bacteria reached the exponential phase between zero- and three hours of incubation, and the stationary phase was reached after 24 hours (Figure 4.5.B).

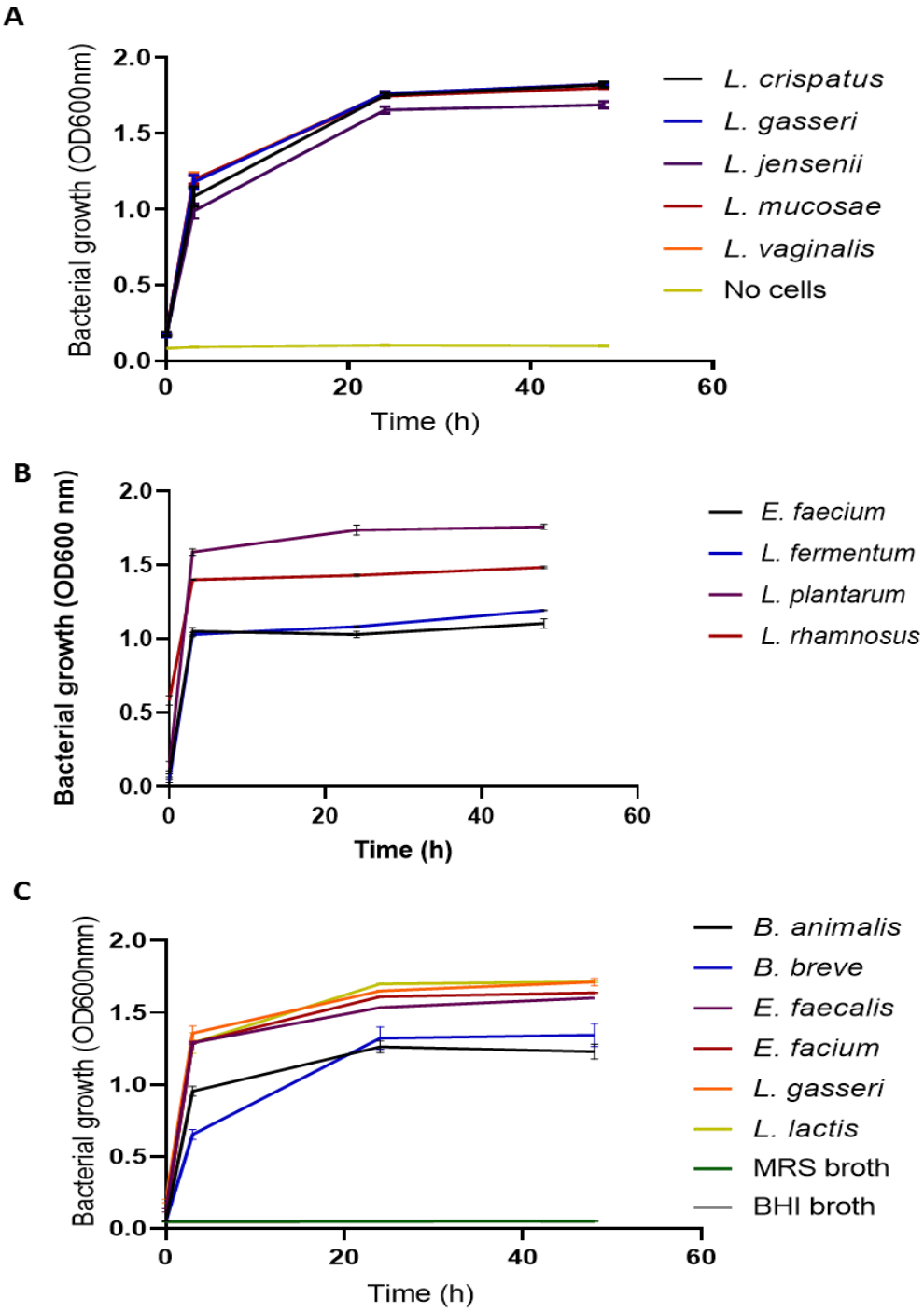

Figure 4.5: Growth curves of LABs isolated from the vagina and gut of South African adolescent girls and young women, compared to those of gut-derived ATCC strains. The proliferation of the different species isolated from the vagina (A), gut (B), and gut-derived ATCC strains (C) was monitored by spectrophotometry. Duplicate bacterial growth at OD600 nm was measured after 0-, 3-, 24-, and 48 hours of anaerobic incubation at 37°C using a microplate reader.

4.4. The effect of pH on the growth kinetics of bacteria

The effect of pH on the growth of vaginal species, gut species, and ATCC strains was assessed (Figure 4.6). The growth of all the vaginal *Lactobacillus* spp. was significantly inhibited at pH 2 and pH 2.5 compared to the unadjusted control grown at pH 5.86 (P= <0.0001). Additionally, the growth of *L. crispatus, L. gasseri,* and *L. mucosae* was significantly reduced at pH 3 (P= 0.0076, 0.0023, and 0.0255, respectively). Overall, the optimum pH for all the vaginal bacteria was between pH 3.5 and pH 5.5. (Figure 4.6.A). The impact of pH on the growth of gut-derived bacteria is shown in Figure 4.6.B. The growth of *E. faecium* was significantly inhibited by pH values from 2.5 to 5 (P= <0.0001, <0.0001, <0.0001, 0.0024, 0.0252, and <0.0001, respectively). However, at pH 2, 5.5, and 6, the growth of *E. faecium* was not inhibited. *Lactobacillus fermentum* growth was sensitive to a wider pH range, viz, pH values from 2 to 5.5 (P= 0.023, 0.007, 0.0258, 0.0205, 0.0046, and 0.0133, respectively). *Lactobacillus plantarum* was inhibited by pH 2, 2.5, and pH 3 (P= <0.0001, 0.0018, and 0.0241, respectively), while the optimal growth was observed at pH values between pH 3.5 to pH 6.85. The growth of *L. rhamnosus* was not affected by pH changes. All the gut-derived ATCC strains were sensitive to pH 2, except *E. faecium* and *L. gasseri* which were not sensitive to pH changes. In addition, *B. breve* and *E. faecalis* were sensitive to pH 2.5 (P= 0.009). *Enterococcus faecalis* was further inhibited by pH 3 and pH 5 (P= 0.0054 and 0.0172, respectively). Overall, most gut-derived bacteria (except *L. fermentum* and ATCC *E. faecalis*) were able to survive at a pH above 3.0 (Figure 4.6.B and Figure 4.6.C).

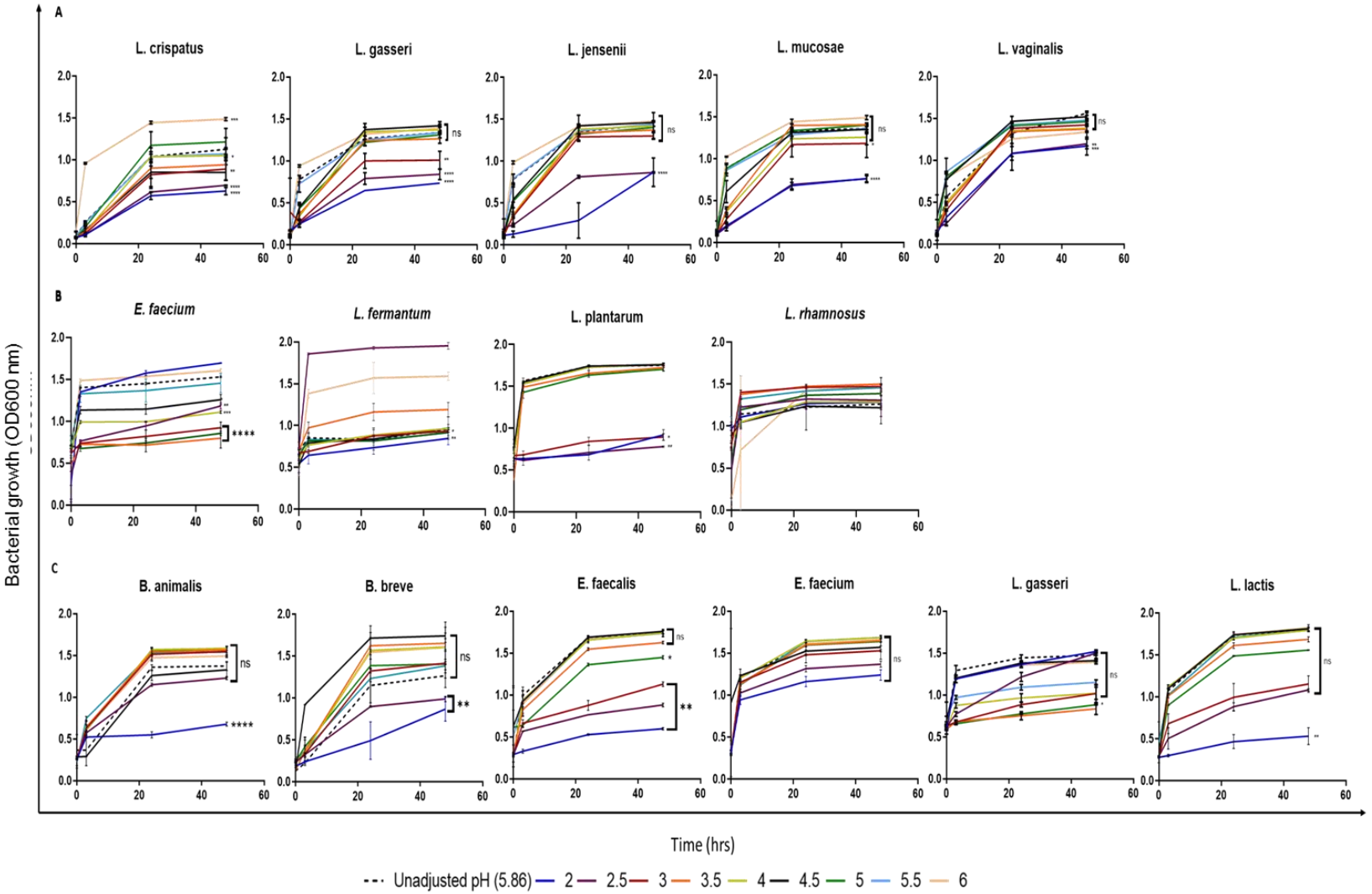

Figure 4.6: The impact of pH on bacterial growth. The effect of pH on the growth of species isolated from the vagina (A), gut (B), and ATCC strains (C) was determined by inoculating 50 µL of standardised overnight cultures of each isolate with MRS broth that had been adjusted to pH 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 with 1 M hydrochloric acid (HCL) or 1 M sodium hydroxide (NaOH). Statistical significance was tested using two-way ANOVA (parametric) and Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered significant. The level of significance was denoted as follows: ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). The OD600 nm reading represents duplicate experiments.

4.5. The ability of lactic acid-producing bacteria to reduce pH

In order to understand how LABs change the culture pH over time, the culture pH was measured after 3-, 24- and 48 hours of incubation. The changes in unadjusted MRS broth pH (pH 5.86) caused by the bacteria are depicted in Figure 4.7. All the bacteria reduced the pH of the media after three hours of incubation and the pH remained the same after 24 hours. The vaginal bacteria reduced the pH the most, from an average pH of 5.97 at 0 hours to a pH of 3.19 after 48 hours. The gut-derived bacteria reduced the pH slightly less compared to vaginal bacteria, i.e. from an average pH of 5.91 at 0 hours to a pH of 3.86 after 48 hours (Figure 4.7.A and Figure 7.4.B). Similarly, the gut-derived ATCC strains reduced the pH from the average pH of 7.22 to pH 4.03 (Figure 4.7. C).

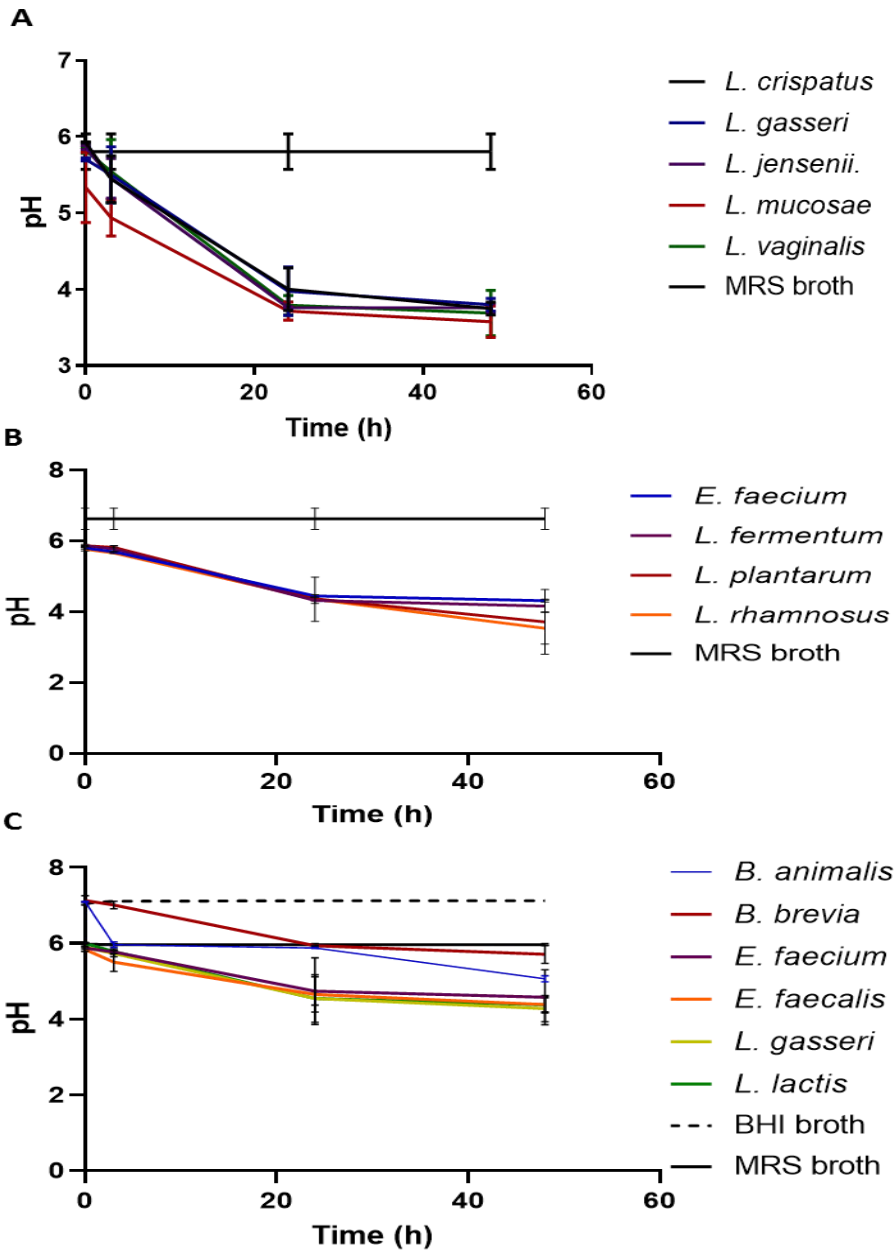

Figure 4.7: The effect of lactic acid-producing bacteria on the culture pH. The effect of the respective bacterial isolates on the pH of the growth medium was determined and the change in pH caused by vaginal-derived species (A) gut-derived species (B) and gut-derived ATCC strains (C) was measured. The cells were incubated anaerobically at 37°C and the OD600 nm was taken after 0, 3, 24, and 48 hours. This experiment was done in duplicate.

4.6. Lactic acid production by LABs

4.6.1. D-lactate

The amount of lactic acid produced by LABs was measured to determine which bacteria was most likely to confer protection against pathogens. The production of D-lactate by the lactic acid-producing bacteria was determined. Figure 4.8. A shows the production of D-lactate by vaginal *Lactobacillus* spp. compared to MRS broth which was used as a negative control. All the vaginal *Lactobacilli* produced significantly higher levels of D-lactate compared to the control (P= <0.0001). The vaginal *Lactobacilli* produced comparable amounts of D-lactate which ranged between 6.99 – 9.135 ng/µL. There was no detection of D-lactate for either the isolated gut species or the gut-derived ATCC strains and these were not significantly different from each other (Figure 4.8.B and Figure 4.8.C, respectively).

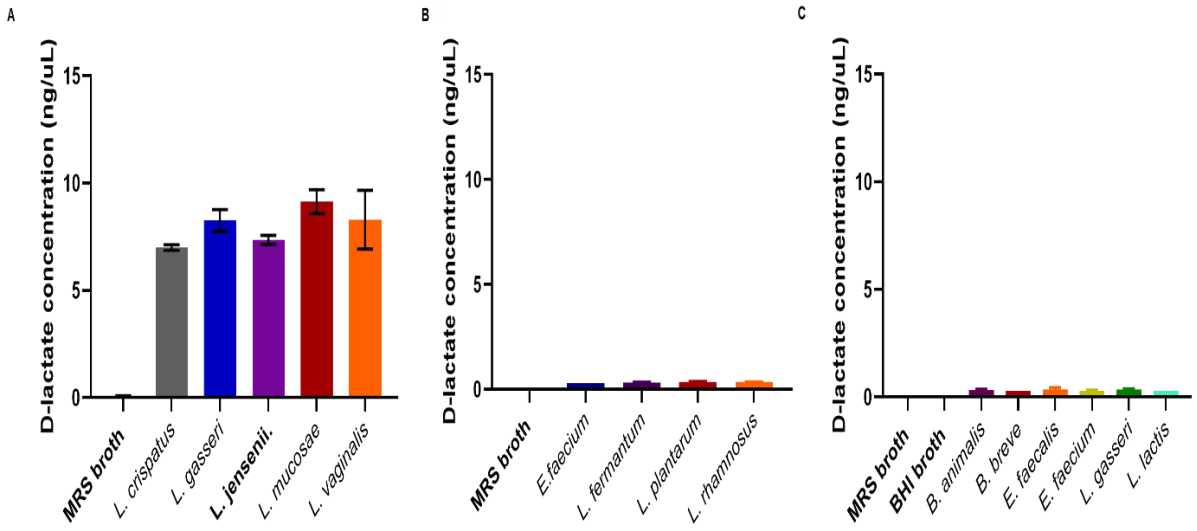

Figure 4.8: D-lactate production by species of the mucosal microbiota. The production of lactic acid by species isolated from the vagina (A), gut (B), and ATCC strains (C) was quantified using the D-lactate calorimetric assay kit. The concentration of D-lactate produced by each species was determined by extrapolating from the D-lactate standard curve. Statistical significance was tested using RM one-way ANOVA (nonparametric) and Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered significant. This experiment was done in duplicate.

4.6.2. L-lactate

The production of L-lactate by all the bacteria of interest was assessed and compared to the MRS and BHI broths which were used as the negative controls. Figure 4.9 shows that all the bacteria produced significant amounts of L-lactate when compared to the negative controls (P= <0.0001). The vaginal *L. vaginalis* produced significantly lower amounts of L-lactate compared to *L. crispatus, L. gasseri, L. jensenii,* and *L. mucosae* (P= <0.0001) while *L. crispatus, L. gasseri, L. jensenii,* and *L. mucosae* produced similar amounts of L-lactate. *Lactobacillus rhamnosus* produced significantly higher amounts of L-lactate, compared to *E. faecium* (P= 0.0172), *L. fermentum* (P= 0.0374), and *L. plantarum* (P= 0.009) (Figure 4.9.B). All the other gut-derived species produced similar amounts of L-lactate. The ATCC strains produced similar amounts of L-lactate (Figure 4.9.C).

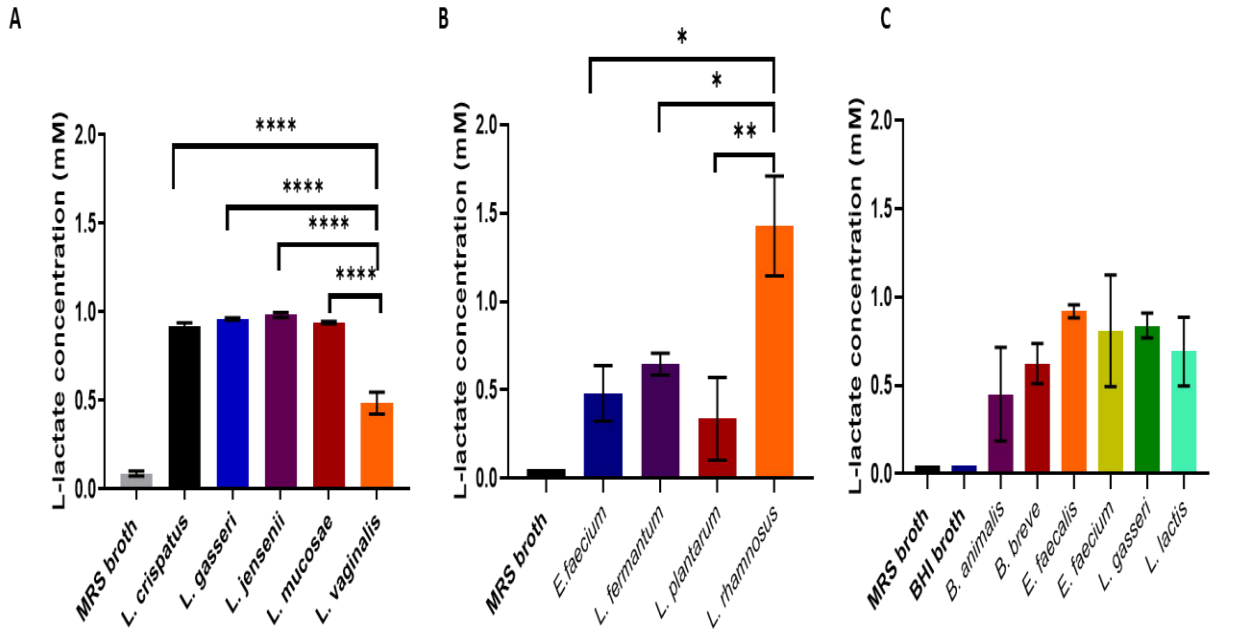

Figure 4.9: The production of L-lactate by species of the mucosal microbiota. The production of L-lactate by species isolated from the vagina (A), gut (B), and ATCC strains (C) was quantified using the L-lactate assay kit. The concentration of L-lactate produced by each species was determined using the L-lactate standard curve. Statistical significance was tested using RM one-way ANOVA (nonparametric) and Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered significant. The level of significance comparing the bacteria to each other was denoted as follows: ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). All the experiments were done in duplicate.

4.7. Hydrogen peroxide production

The amount of H_2O_2 produced by the bacteria was measured to determine which bacteria was most likely to confer protection against pathogens lacking catalase. The production of H_2O_2 by the vaginal, gut and ATCC bacteria was examined and compared to MRS and BHI broth which were negative controls. The amounts of H_2O_2 produced by the vaginal bacteria were higher compared to the MRS control (Figure 4.10. A). However, there was no significant statistical difference in the production of H_2O_2 when comparing different vaginal bacteria species to each other. Though not statistically significant, *L. gasseri* produced the most H_2O_2, followed by *L. vaginalis, L. crispatus, L. mucosae* and lastly *L. jensenii.* The gut bacteria produced lower amounts of H_2O_2 compared to the vaginal bacteria. All gut bacterial isolates produced significantly higher amounts of H_2O_2 compared to the broth controls but produced similar amounts of H_2O_2 (Figure 4.10.B). The ATCC strains also produced similar amounts of H_2O_2 and significantly higher amounts of H_2O_2 compared to the broth controls.

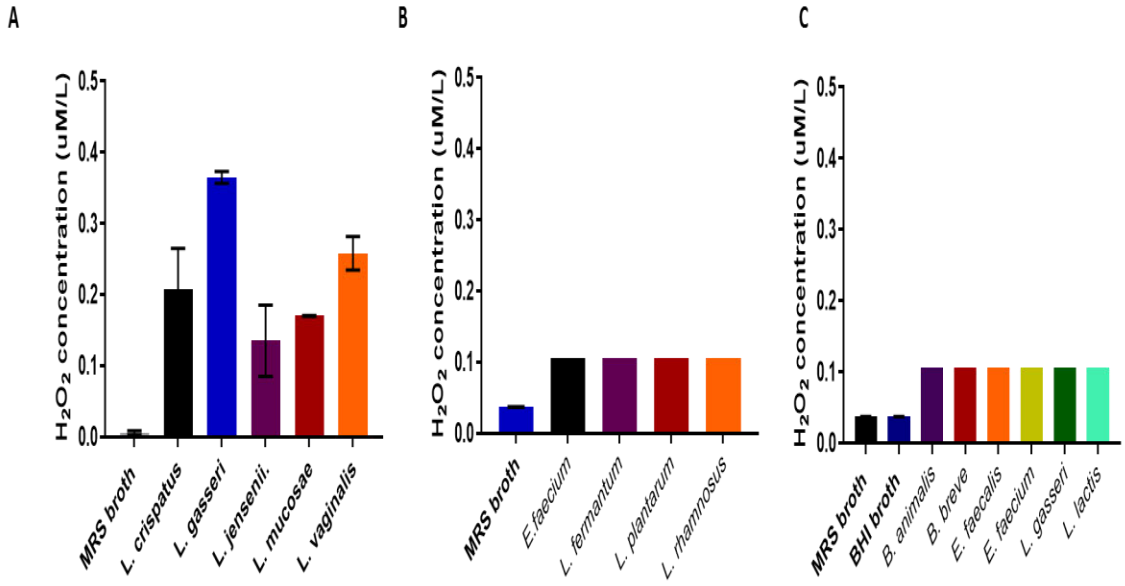

Figure 4.10: The production of hydrogen peroxide by species of the mucosal microbiota. The Pierce™ quantitative peroxide assay kit was used to quantify the amount of hydrogen peroxide produced by species isolated from the vagina (A), gut (B), and ATCC strains (C). The standard curve was used to determine the concentration of hydrogen peroxide produced by each species. Statistical significance was tested using ordinary one-way ANOVA (nonparametric) and Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered significant. The level of significance was denoted as: ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). This experiment was done in duplicate.

4.8. The susceptibility of species of mucosal microbiota to antibiotics

Antibiotic use is crucial for many HIV-coupled infections, such as TB, pneumonia and other STIs. However, the susceptibility of the "healthy" bacteria to the antibiotics is highly possible. For this reason, the susceptibility of the vagina, gut, and gut ATCC bacteria to commonly used antibiotics was also investigated. There was a trend toward hierarchical clustering of antibiotics as summarised in Figure 4.11. Overall, ampicillin exhibited the highest potency and the serial dilutions of this antibiotic tended to cluster together, followed by clindamycin, amoxicillin, penicillin, metronidazole, rifampicin, and lastly streptomycin. Moreover, the vaginal bacteria were most susceptible to all the antibiotics, compared to gut bacteria. Vaginal *L. jensenii* was the most susceptible to the antibiotics, followed by *L. crispatus, L. mucosae, L. vaginalis and L. gasseri. Lactobacillus rhamnosus* and *L. lactis,* on the other hand, were the least susceptible to the antibiotics. The vaginal bacterial isolates exhibited resistance against streptomycin. In addition, differences in antibiotics resistance patterns were noted between the isolated *E. faecium* and the American derived ATCC *E. faecium*. The results showed that the isolated *E. faecium* was more susceptible to the antibiotics than the ATCC *E. faecium*, suggesting that antibiotic susceptibility profiles of *E faecium* isolates may vary by geography.

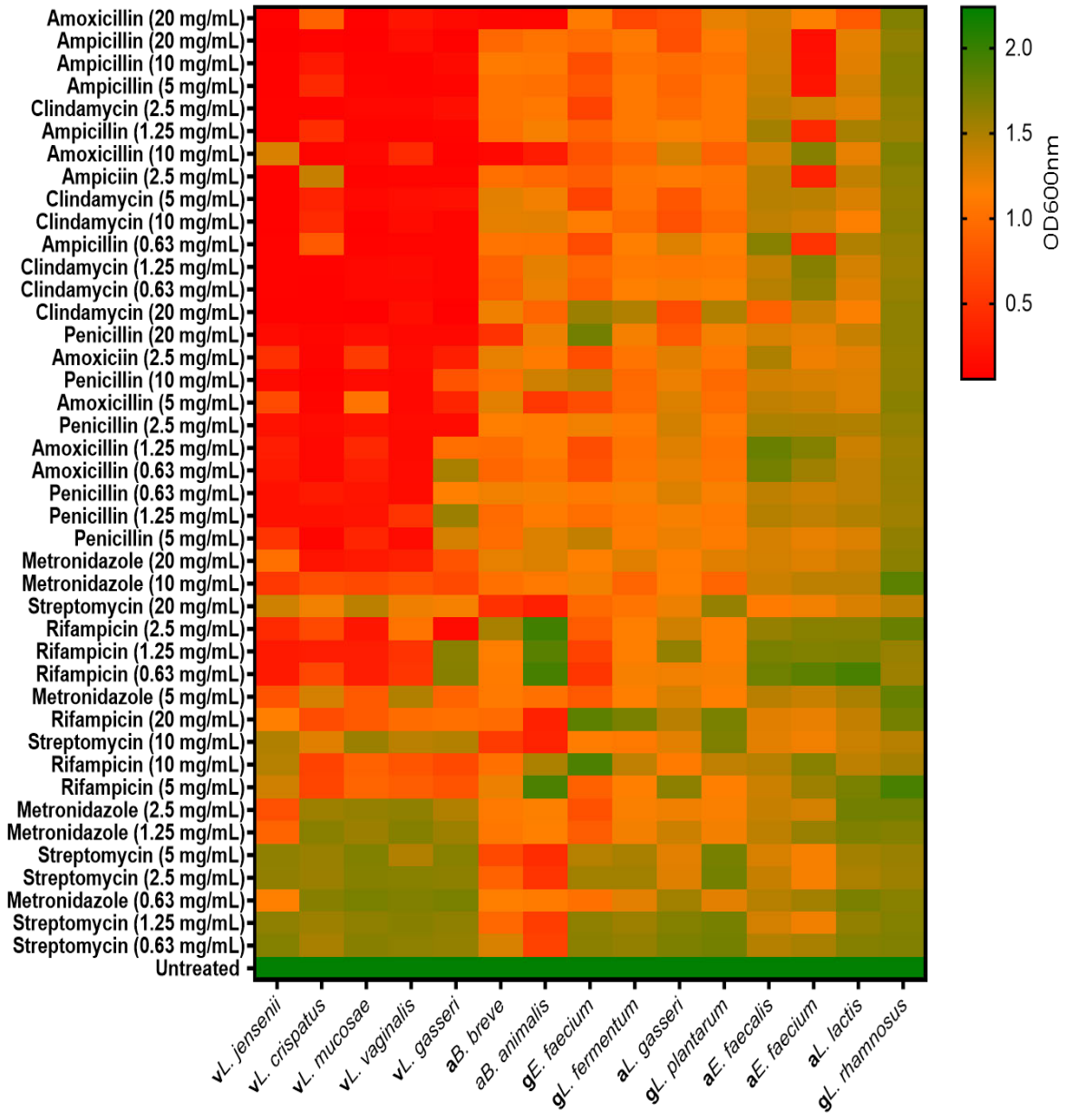

Figure 4.11: Hierarchical clustering of antibiotics on bacterial growth. The antimicrobial impact of seven antibiotics against strains isolated from the vagina and gut of HIV-negative women aged 14-24 years was tested. ATCC strains were also tested. The antibiotics amoxicillin, ampicillin, clindamycin, metronidazole, penicillin, rifampicin, and streptomycin were tested against five vaginal *Lactobacillus* (v*L*) species, two ATCC strains of *Bifidobacteria* (a*B*), two *Enterococcal* ATCC strains (a*E*), two ATCC strains of *Lactobacillus* (a*L*), gut-derived *E* (g*E*)*. Faecium,* and three gut-derived *Lactobacilli* (g*L*). The heatmap was generated in GraphPad Prism version 8.4.3 (686).

4.9. Effect of solvents used to dissolve ARVs on species of the mucosal microbiota growth

Various solvents were tested to determine which solvent would effectively dissolve the ARVs without killing the cells. To determine which solvent was ideal to dissolve ARVs, without killing the cells, the survival of bacteria in different solvents, namely, BHI broth, dH_2O, PBS, 100% (v/v) ethanol, 70%(v/v) ethanol, 99.99% methanol, and DMSO was examined (Figure 4.12). The solvents were prepared by dissolving in MRS broth (50% v/v) and were spread plated onto MRS agar plates. The cells grown in MRS broth were used as a no solvent control. In the absence of a solvent, all bacterial isolates formed a lawn culture, which was also observable in the presence of BHI broth, dH_2O, PBS, and 99.98% DMSO for all species. On the contrary, there was no bacterial growth in 100% ethanol. There was also no growth of *L. crispatus* and *L. jensenii* in the presence of 70% (v/v) ethanol and methanol. *Lactobacillus gasseri, L. mucosae,* and *L. vaginalis,* on the other hand, survived in the presence of these solvents, showing an overgrowth in the presence of 70% (v/v) ethanol and fewer colonies in the presence of methanol.

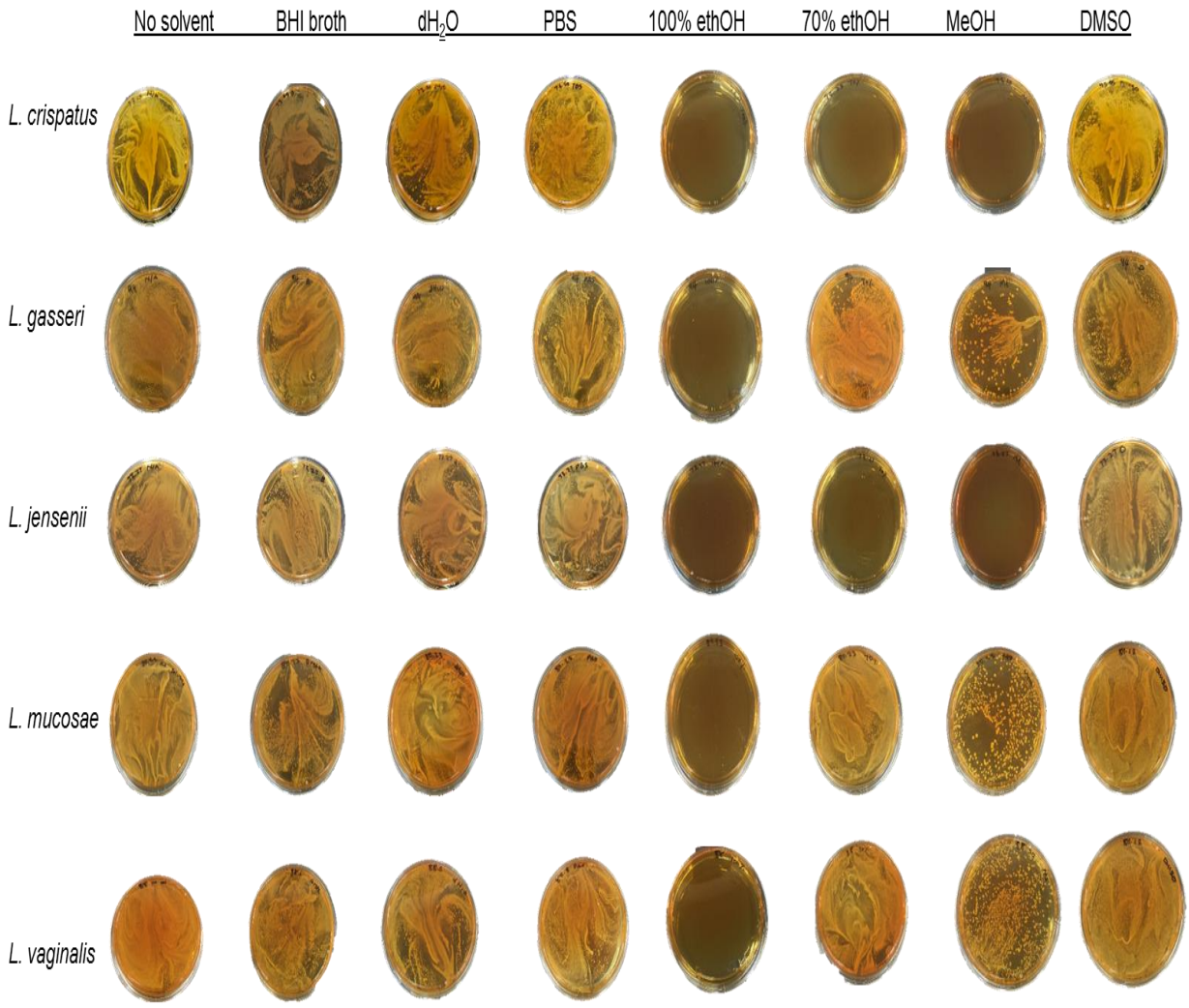

Figure 4.12: The effect of different solvents on Lactobacilli growth. The overnight cultures of *L. crispatus, L. gasseri*, L. *jensenii L. mucosae,* and *L. vaginalis* were standardised to 1 x 10^5 CFU/mL and inoculated with 50% (v/v) concentration of different solvents, brain heart infusion (BHI) broth, distilled water (d.H_2O), 99.99% methanol (MeOH),100% ethanol (EtOH), 70% EtOH, dimethyl sulfoxide (DMSO), and phosphate-buffered saline (PBS), spread plates were subsequently prepared and incubated anaerobically for 48 hours at 37°C. Cells that were grown in De Man, Rogosa, and Sharpe (MRS) broth were used as a no solvent control. This experiment was done in duplicate.

4.10. The growth inhibition of vaginal and gut bacterial species caused by ARVs

The antibacterial activity of ARVs against bacteria associated with a healthy microbiota was monitored to assess the impact of ARVs on the bacteria. The impact of ARVs on the growth of vaginal *Lactobacilli* was determined using the minimal inhibitory (MIC) assay by serially diluting different ARVs (5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.63 mg/mL, and 0.315 mg/mL) (Figure 4.13).

The MIC was regarded as the lowest concentration of ARV that was able to significantly inhibit the growth of cells when compared to the untreated control (0 mg/mL). Overall, most of the drugs, namely DTG, TDF, and TLD inhibited the growth of all the bacteria, while TAF only inhibited the growth of *L. mucosae* and CAB inhibited the growth of *L. gasseri, L. jensenii,* and *L. mucosae* (Figure 4.13). Cabotegravir exhibited the least inhibitory effects on all the vaginal bacteria. The highest antibacterial activity against *L. crispatus* was exhibited by TLD and had a MIC of 1.25 mg/mL (P= 0.0494), followed by TDF and DTG which had a MIC of 5 mg/mL (P= 0.0032 and 0.0052, respectively) (Figure 4.13.A), while TAF and CAB had no impact on the growth of *L. crispatus*. *Lactobacillus gasseri* was most susceptible to DTG, which had a MIC of 0.315 mg/mL (P= <0.0001), followed by TLD, while CAB and TDF were less potent and had MICs of 1.25, 2.5, and 5 mg/mL, respectively (P= 0.0058, 0.0005 and <0.0001) (Figure 4.13.B). Tenfovir alafenamide on the other hand, exhibited no antibacterial activity against *L. gasseri.* Dolutegravir, followed by TLD exhibited the highest antibacterial activity against *L. jensenii*, which had MICs of 0.315 (P= 0.0220 and <0.0001) followed by CAB and lastly TDF, which had MICs of 2.5 mg/mL (P= 0.0007) and 5 mg/mL (P= <0.0001) (Figure 4.13.C). Dolutegravir was most lethal to *L. mucosae* and had a MIC of 0.315 mg/mL (P= 0.0004), followed by TLD, TAF, TDF, and CAB, which had MICs of 1.25, 2.5, and 5 mg/mL, respectively (P= 0.0131, <0.0001, <0.0001 and 0.0443) (Figure 4.13.D). *Lactobacillus vaginalis* was inhibited the most by DTG, followed by TLD and TDF, which had MICs of 0.63, 1.25, and 5 mg/mL, respectively (P=0.0159. 0.0085 and <0.0001) (Figure 4.13.E).

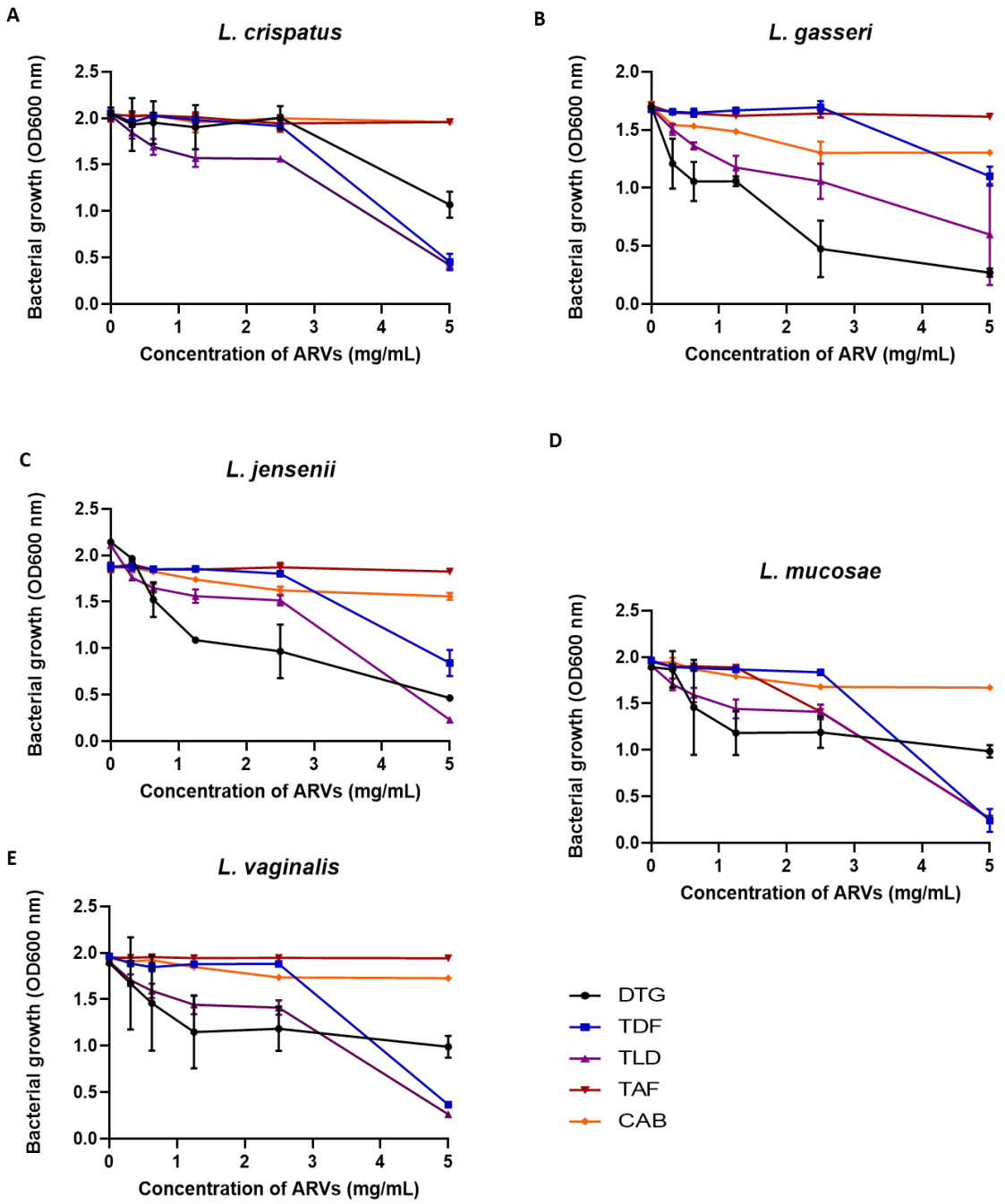

Figure 4.13: The minimal inhibitory concentration (MIC) of antiretroviral drugs against vaginal *Lactobacillus* species South African adolescent girls and young women. The antibacterial activity of different antiretroviral drugs (ARVs)), namely dolutegravir (DTG) (black), tenofovir disoproxil fumarate (TDF) (blue), tenofovir/lamivudine/dolutegravir (TLD) (purple), tenofovir alafenamide (TAF) (red) and cabotegravir (CAB) (orange), against five vaginal bacteria, namely *L. crispatus* (A), *L. gasseri* (B), *L. jensenii* (C), *L. mucosae* (D) and *L. vaginalis* (E), isolated from HIV-negative adolescent girls and young women was observed. The bacterial growth at OD600 nm was measured in a microplate reader after 48 hours of incubation at 37°C. The lowest concentration needed to inhibit the growth of bacteria was considered the MIC. Statistical analysis was performed on GraphPad Prism version 8.4.3.

The antibacterial impact of ARVs against gut-derived microbial species was evaluated and the results are depicted in Figure 4.14. Generally, 5 and 2.5 mg/mL of DTG, TDF, and TLD

suppressed the growth of all the gut bacteria, except *L. fermentum* which was not inhibited by any of the ARVs. The growth of *E. faecium* was inhibited by DTG which had a MIC of 1.25 mg/mL (P=<0.0001) while TDF and TLD, exhibited a MIC of 5 mg/mL (P= <0.0001 and 0.0001, respectively) (Figure 4.14.A). Dolutegravir, TDF, and TLD had MICs of 2.5 and 5 mg/mL against *L. plantarum,* respectively (P= 0.0005, <0.0001, and <0.0001), while TAF and CAB had no impact on the growth of *L. plantarum.* (Figure 4.14. C). Tenofovir/lamivudine/dolutegravir and TAF were highly potent against *L. rhamnosus,* exhibiting MICs of 0.315 mg/mL (P= <0.0001), followed by TDF and DTG which had MICs of 0.63 and 2.5 mg/mL, respectively (P= 0.0129 and <0.0001). However, at higher concentrations (5 mg/mL) of TLD and CAB, the growth of *L. rhamnosus* was not inhibited, possibly as a result of steric hindrance, preventing drug-bacteria interaction due to saturation of the drug (Figure 4.14.D).

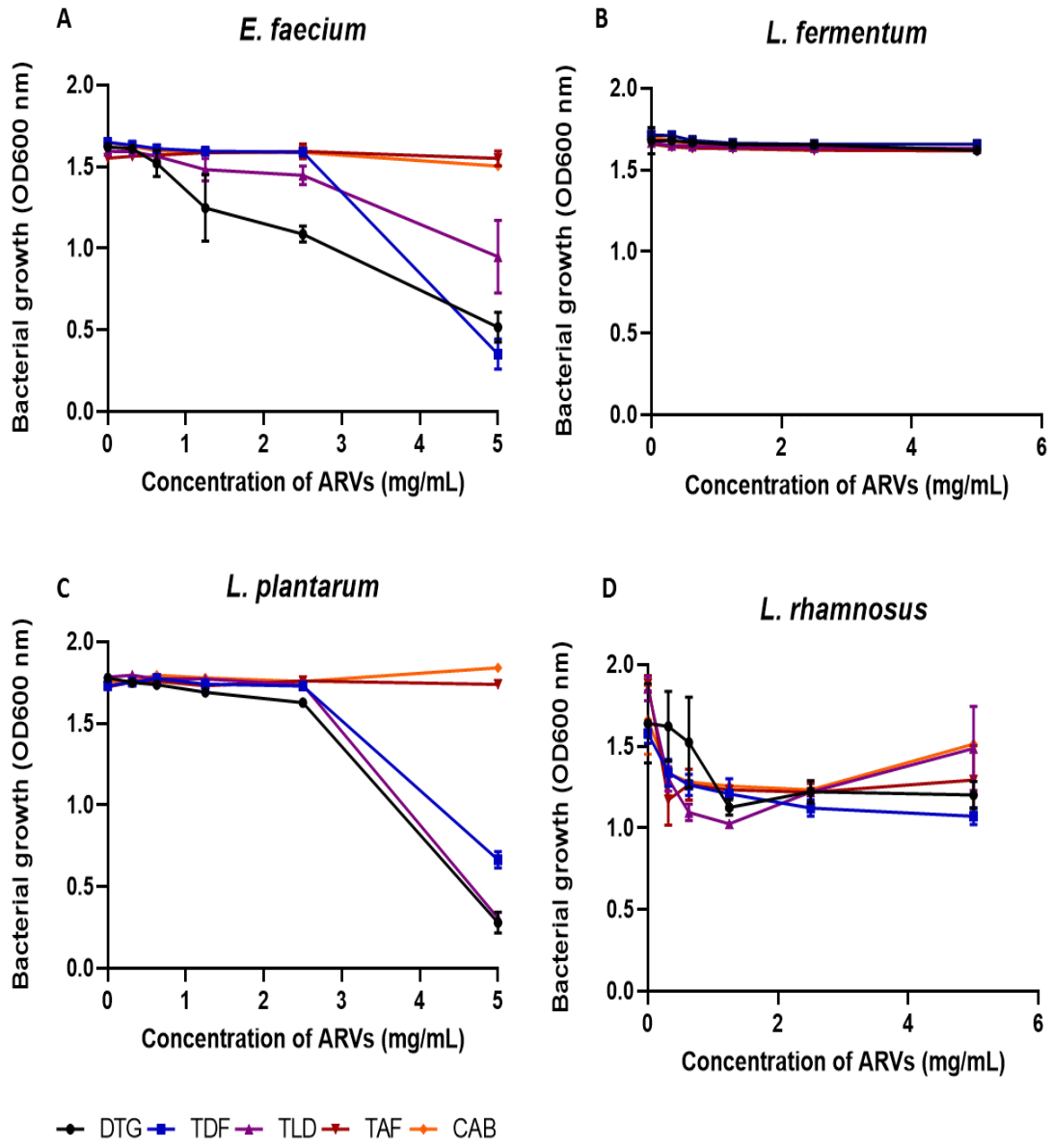

Figure 4.14: The minimal inhibitory concentration (MIC) of antiretroviral drugs against gut-isolated bacterial species isolated from South African adolescent girls and young women. The antibacterial activity of different antiretroviral drugs (ARVs), namely dolutegravir (DTG) (black), tenofovir disoproxil fumarate (TDF) (blue), tenofovir/lamivudine/dolutegravir (TLD) (purple), tenofovir alafenamide (TAF) (red) and cabotegravir (CAB) (orange), against four gut bacteria, namely *E. faecium* (A), *L. fermentum* (B), *L. plantarum* (C), and *L. rhamnosus* (D), isolated from HIV-negative adolescent girls and young women was observed. The bacterial growth at OD600 nm was measured in a microplate reader after 48 hours of incubation at 37°C. The lowest concentration needed to inhibit the growth of bacteria was considered the MIC. The graphs were constructed on GraphPad Prism version 8.4.3.

The inhibitory effect of ARVs on the growth of gut ATCC strains is depicted in Figure 4.15. Generally, the growth of all the bacteria was suppressed by DTG, TDF, TLD, and TAF, except the *Bifidobacteria* which were not susceptible to TAF (Figure 4.15). *Bifidobacterium animalis* was sensitive to DTG and had a MIC of 1.25 mg/mL (P= 0.0307), followed by TDF and TLD, exhibiting a MIC of 5 mg/mL (P= <0.0001) (Figure 4.15A). Additionally, 5 mg/mL CAB

increased the bacterial growth observed for *B. animalis*, which could be a result of steric hindrance, which prevents drug-bacteria interaction due to saturation of the drug. *Bifidbacterium breve* had MICs of 5 mg/mL post-stimulation with DTG, TDF, and TLD (P= <0.0001), as shown in Figure 4.15.B. Dolutegravir was the most potent ARV against *E. faecalis,* exhibiting a MIC of 1.25 mg/mL (P= <0.0001), followed by TLD and TAF with MICs of 2.5 mg/mL (P= 0.0450 and <0.0001), while TDF exhibited the least antibacterial activity against *E. faecalis,* (MIC of 5 mg/ml; P= <0.0001) (Figure 4.15.C). Of all the gut ATCC strains, *E. faecium* was the most sensitive to all the drugs. Dolutegravir exhibited the highest antibacterial activity against *E. faecium* and had a MIC of 0.63 mg/mL (P= 0.0415), followed by TAF and TLD, with MICs of 2.5 mg/ml (P=<0.0001 and 0.0082). Tenofovir disoproxil fumarate and CAB exhibited the least potency against *E. faecium* and had MICs of 5 mg/mL (P=<0.0001 and 0.0387) (Figure 4.15.D). The growth of *L. gasseri* was suppressed by DGT, TDF, TLD, and TAF, showing MICs of 1.25, 5, 5, and 2.5 mg/mL respectively (P= <0.0001) (Figure 4.15.E). *Lactobacillus lactis* had similar sensitivity to ARVs as *L. gasseri.* Dolutegravir exhibited the highest growth inhibition against *L. lactis* and had a MIC of 1.25mg/mL (P= 0.0349), followed by TLD and TAF which had MICs of 2.5 mg/mL (P= 0.0172 and 0.0329). Tenofovir disoproxil fumarate exhibited the least potency against *L. lactis* with a MIC of 5 mg/mL (P= <0.0001) (Figure 4.15.F).

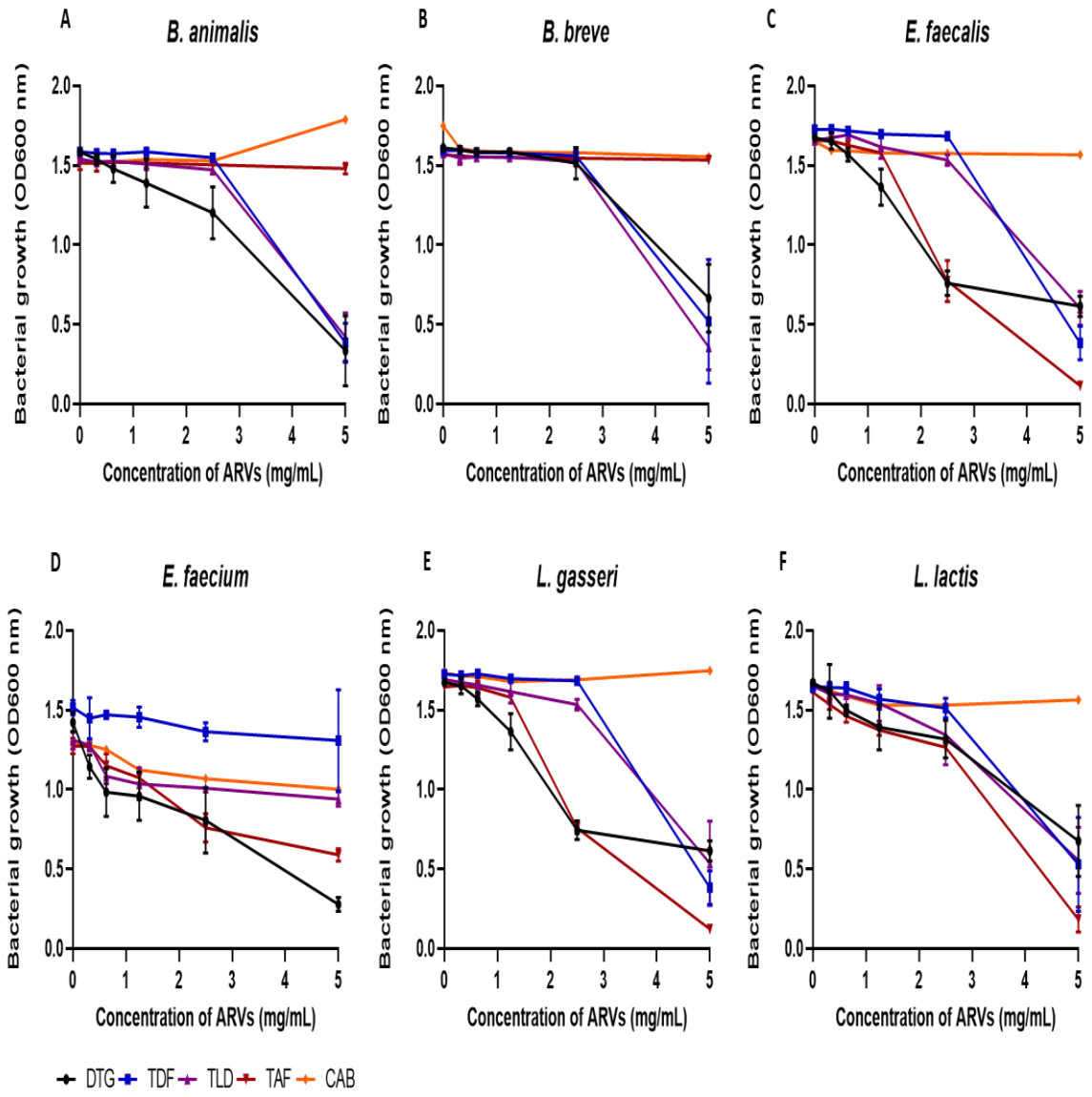

Figure 4.15: The minimal inhibitory concentration (MIC) of antiretroviral drugs against gut ATCC bacterial species. The antibacterial activity of different antiretroviral drugs (ARVs), namely dolutegravir (DTG) (black), tenofovir disoproxil fumarate (TDF) (blue), tenofovir/lamivudine/dolutegravir (TLD) (purple), tenofovir alafenamide (TAF) (red) and cabotegravir (CAB) (orange), against four gut ATCC strains, namely, *Bifidobacterium animalis* (A), *B. breve* (B) *E. faecalis* (C), *E. faecium* (D), *L. gasseri* (E), and *L. lactis* (F), ATCC species was observed. The bacterial growth at OD600 nm was measured in a microplate reader after 48 hours incubation at 37°C. The lowest concentration needed to inhibit the growth of bacteria was considered the MIC. The graphs were constructed on GraphPad Prism version 8.4.3 (686).

Figures 4.16, 4.17, and 4.18 highlight all the drug concentrations that resulted in significant bacterial inhibition of 50% and above. The *in vitro* percentage inhibition of vaginal bacterial growth by different ARVs was evaluated and shown in Figure 4.16. Dolutegravir, TDF, and TLD significantly reduced the growth of *L. crispatus.* Additionally, 5 mg/mL TLD and 5 mg/mL TDF exhibited the greatest inhibition of 79.4 and 77.8%, respectively (P= <0.0001 and 0.0032)

(Figure 4.16. A). The growth of *L. gasseri* was significantly suppressed by all the ARVs except TAF, which had no significant impact on the growth of this bacterium. Additionally, 5 mg/mL TLD and 5 mg/mL TDF exhibited the greatest inhibition of 79.4% and 77.8%, respectively (P= <0.0001 and 0.0032) (Figure 4.16. A). The growth of *L. gasseri* was significantly suppressed by all the ARVs except TAF, which had no significant impact on the growth of this bacterium. The highest concentrations of DTG (5 mg/mL and 2.5 mg/mL) exhibited the highest percentage inhibition of *L. gasseri* that was 84% and 71.9%, respectively, followed by 5 mg/mL of TLD (64.7%) (P= <0.0001) (Figure 4.16. B). Likewise, *L. jensenii* was inhibited by all the ARVs except TAF, which had no significant impact on the growth of *L. jensenii.* The highest concentration (5 mg/mL) of TLD, DTG, and TDF were most potent against *L. jensenii* (88.2% P= <0.0001, 76.4% P= <0.0001, and 57.4% P= <0.0001 inhibition, respectively). Additionally, 2.5 mg/mL DTG also exhibited growth inhibition of 51% against *L. jensenii* (P= <0.0001) (Figure 4.16.C). *Lactobacillus mucosae* was also suppressed by all the ARVs except TAF. The highest concentration of TDF and TLD (5 mg/mL) exhibited the highest inhibitory effects against *L. mucosae* (87.5%, and 86.3%, respectively) (P=<0.00001) (Figure 4.16. D). *Lactobacillus vaginalis* was significantly susceptible to DTG, TDF, and TLD, with the highest concentration (5 mg/mL) of TLD and TDF exhibiting the greatest potency, of 86.4% and 81% inhibition , respectively (P= <0.0001) (Figure 4.16. E).

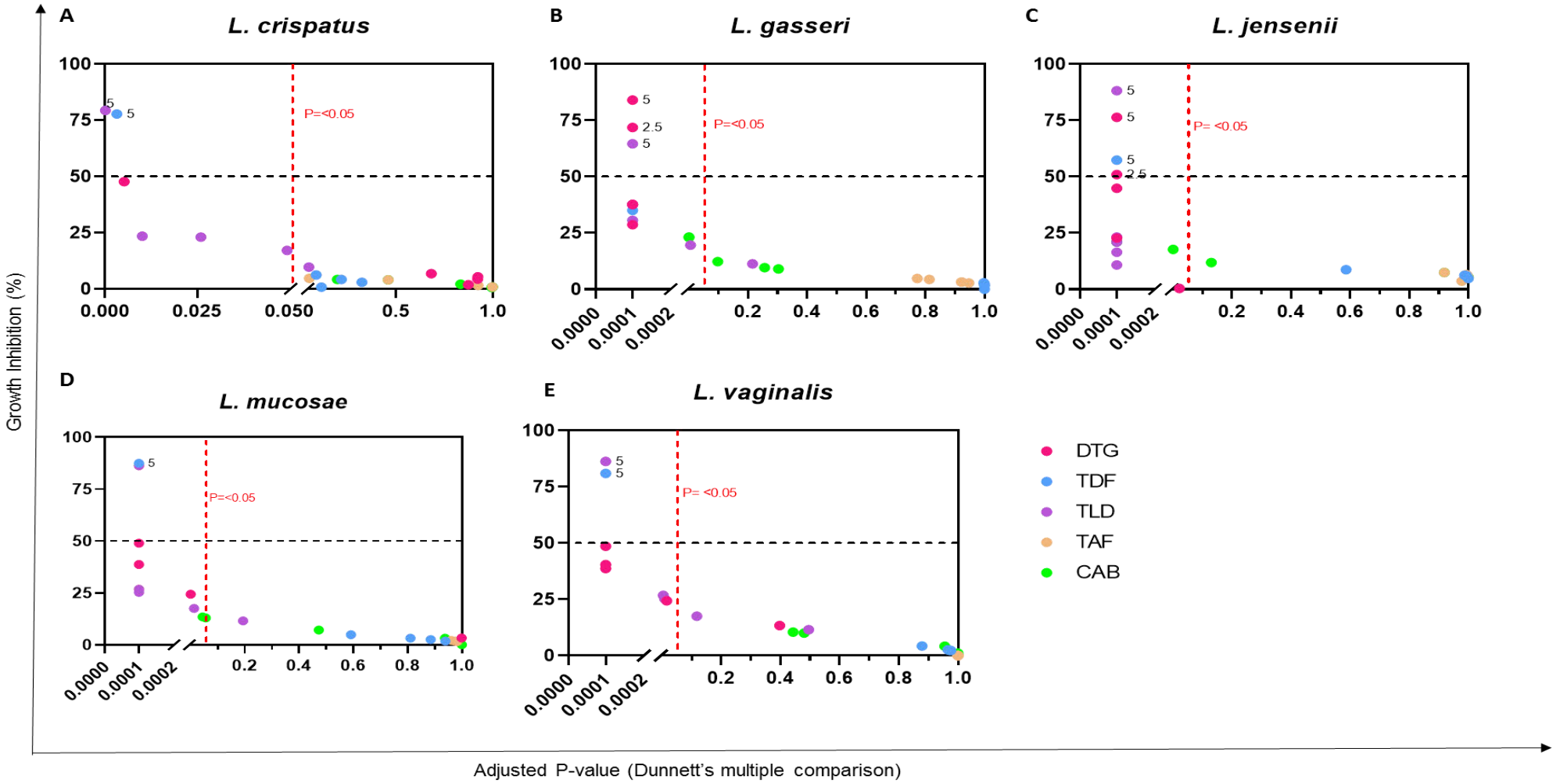

Figure 4.16: The percentage growth inhibitory effect of ARVs against vaginal bacteria. The inhibitory effect of dolutegravir (DTG, pink), tenofovir disoproxil fumarate (TDF, blue), tenofovir/lamivudine/dolutegravir (TLD, purple), tenofovir alafenamide (TAF, brown) and cabotegravir (CAB, green) against vaginal bacteria, namely, *L. crispatus* (A), *L. gasseri* (B), *L. jensenii* (C), *L. mucosae* (D) and *L. vaginalis* (E). The two-way ANOVA and subsequently Dunnett's multiple comparison tests were used to calculate statistical significance compared to the untreated cells. An adjusted P-value of ≤0.05 was considered significant. The red vertical line represents the statistically significant adjusted p-values that are ≤0.05, while the black horizontal line represents the 50% growth inhibition of the bacteria by the different drugs. The numbers next to the dots represent the drug concentrations that exhibited ≥50% inhibition. Some data points are not visible due to clustering.

The growth inhibitory impact of ARVs against gut bacteria was measured and depicted in Figure 4.17. The growth of *E. faecium* was significantly inhibited by DTG, TDF, and TLD. Additionally, 5 mg/mL of TDF and 5 mg/mL DTG exhibited inhibitory effects of 78.1% and 67.9% (P= <0.0001) (Figure 4.17. A). The growth of *L. plantarum* was reduced by DTG, TDF, and TLD, where the highest concentration of TDF, TLD, and DTG exhibited the highest inhibition of 84%, 82.1%, and 62%, respectively (P= <0.0001) (Figure 4.17. C). *Lactobacillus rhamnosus* growth was suppressed by all the ARVs, however, none of these exhibited growth inhibitions that were greater than 50% (Figure 4.17. D). The growth of *L. fermentum*, on the other hand, was not inhibited by any of the ARVs (Figure 4.17. B).

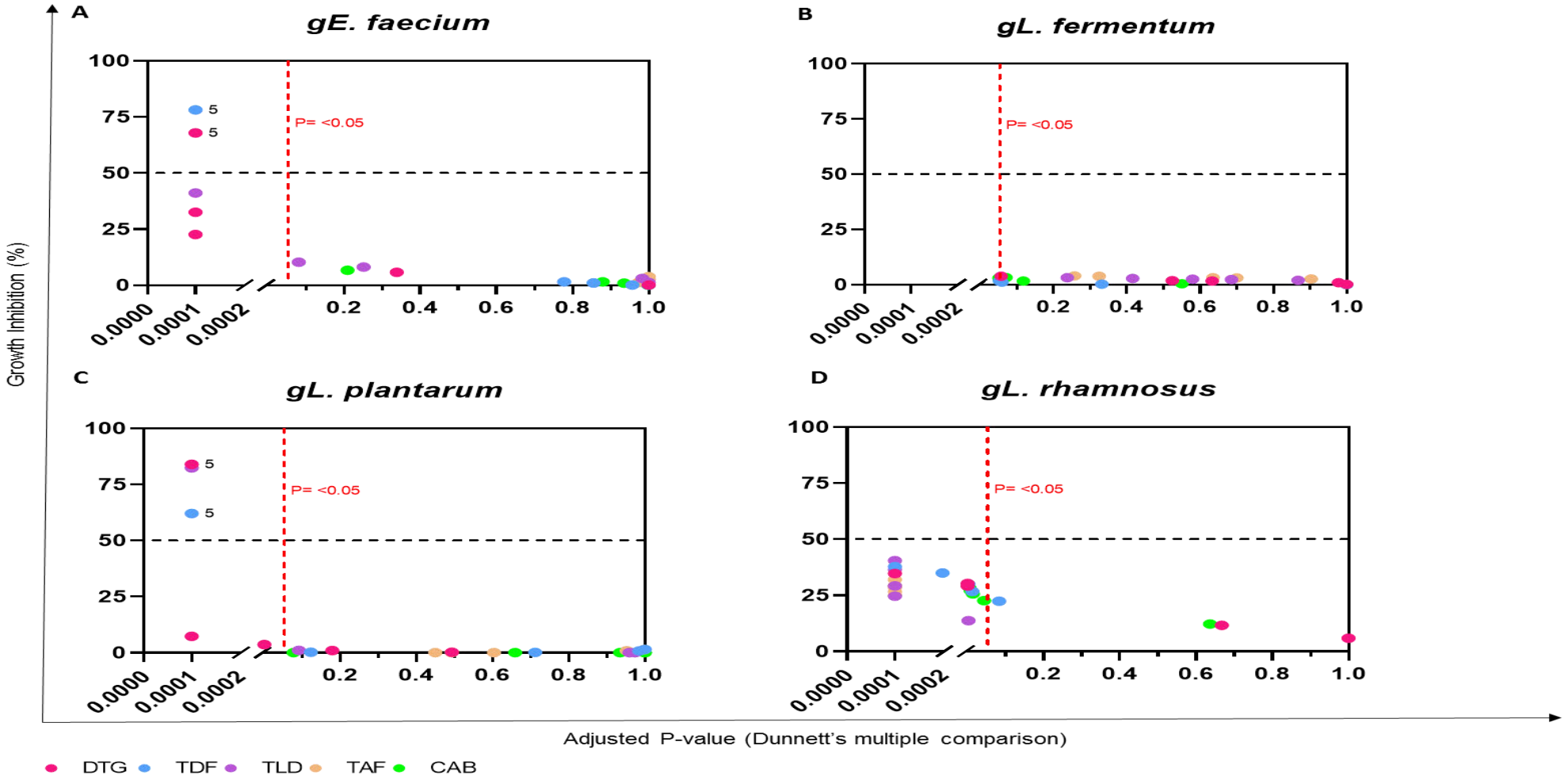

Figure 4.17: The percentage growth inhibitory effect of ARVs against gut bacteria. The inhibitory effect of dolutegravir (DTG, pink), tenofovir disoproxil fumarate (TDF, blue), tenofovir/lamivudine/dolutegravir (TLD, purple), tenofovir alafenamide (TAF, brown) and cabotegravir (CAB, green) against gut bacteria, namely, *E. faecium* (A), *L. fermentum* (B), *L. plantarum*(C), and *L. rhamnosus* (D). The two-way ANOVA and subsequently Dunnett's multiple comparison tests were used to calculate statistical significance compared to the untreated cells. An adjusted P-value of ≤0.05 was considered significant. The red vertical line represents the statistically significant adjusted p-values that are ≤0.05, while the black horizontal line represents the 50% growth inhibition of the bacteria by the different drugs. The numbers next to the dots represent the drug concentrations that exhibited ≥50% inhibition. Some data points are not visible due to clustering.

The percentage growth inhibition of gut-derived ATCC strain by ARVs was evaluated (Figure 4.18). The growth of *B. animalis* was inhibited by DTG, TDF, and TLD (Figure 4.18. A), with the highest concentration (5 mg/m) of DTG, TDF, and TLD exhibiting growth inhibition of 80.5%, 77.7%, and 75.5% respectively, (P= <0.0001). The same results were observed where the growth of *B. breve* was inhibited by 5 mg/mL of DTG, TDF, and TLD by 79.2, 69.8 and 61.4%, respectively (P= <0.0001) (Figure 4.18 B). The growth of *E. faecalis* was inhibited by all the ARVs except CAB. The highest concentration (5 mg/mL) of TAF was most potent against *E. faecalis*, followed by 5 mg/mL TDF, 5 mg/mL TLD, 5 mg/mL DTG, and 2.5 mg/mL DTG, exhibiting growth inhibition of 93.1, 77.1, 64 and 63.2%, respectively (P= <0.0001) (Figure 4.18. C). The growth of *E. faecium* was significantly reduced by all the ARVs, while 5 mg/mL DTG and 5 mg/mL TAF exhibited the greatest inhibitory effects that were 79.5% and 56.6%, respectively (P= <0.0001) (Figure 4.18. D). All the ARVs, except CAB, significantly inhibited the growth of *L. gasseri.* The highest concentration (5 mg/mL) of TAF, TDF, TLD, DTG, and 2.5 mg/mL DTG inhibition growth by 92.7, 77.3, 68.3, 63.6, and 55.2%, respectively (P= <0.0001) (Figure 4.18. E). Similarly, the growth of *L. lactis* was also inhibited by all the ARVs except CAB. The highest concentration (5 mg/mL) of TAF, TDF, TLD, and DTG exhibited inhibitory effects that were 88.8%, 67.8%, 66.3%, and 58.9%, respectively (P= <0.0001) (Figure 4.18. F).

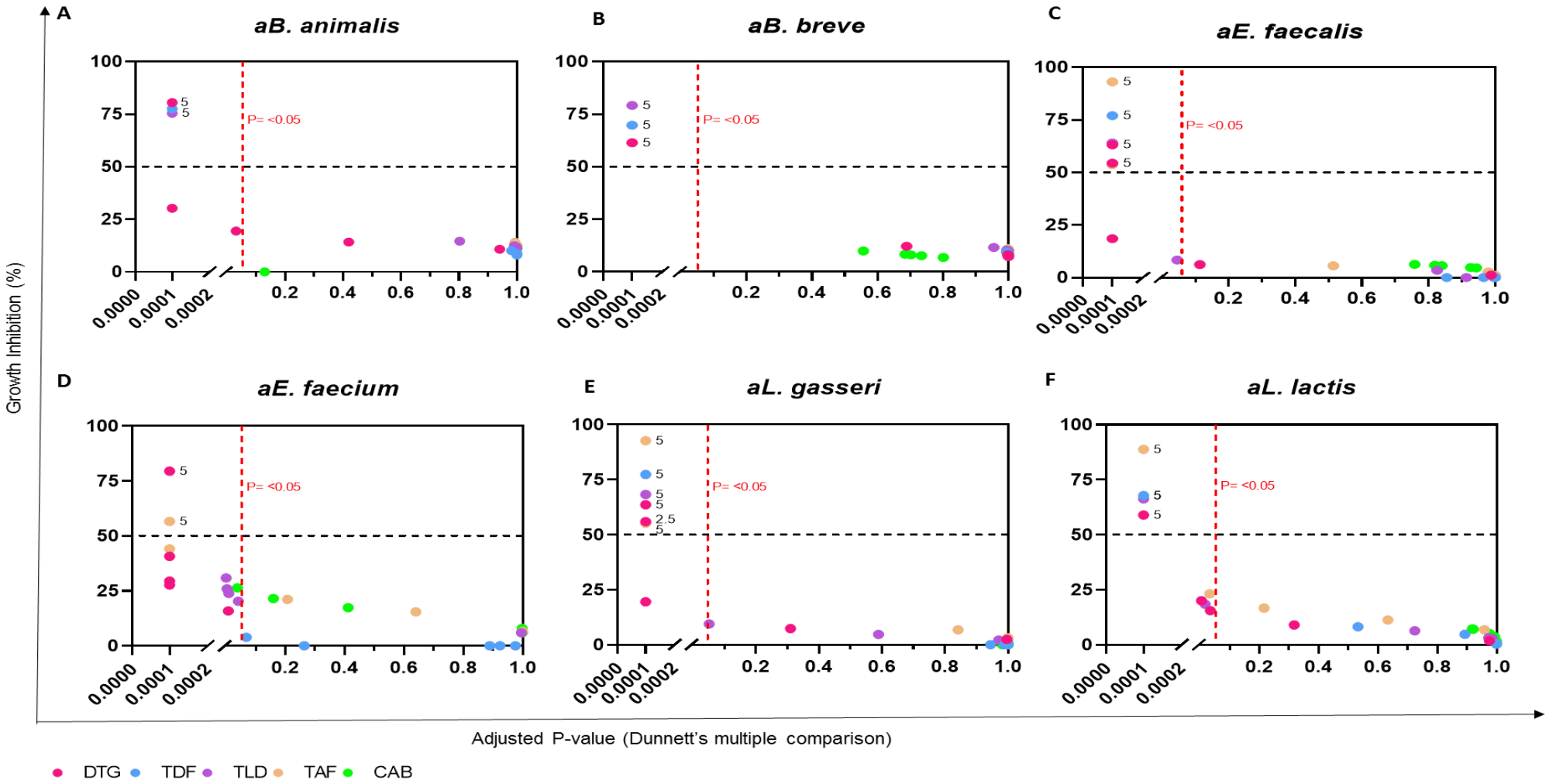

Figure 4.18: The percentage growth inhibitory effect of ARVs against gut-derived ATCC strains. The inhibitory effect of dolutegravir (DTG, pink), tenofovir disoproxil fumarate (TDF, blue), tenofovir/lamivudine/dolutegravir (TLD, purple), tenofovir alafenamide (TAF, brown) and cabotegravir (CAB, green) against gut ATCC strains, namely, *B. animalis* (A), *B. breve* (B), *E. faecalis* (C), *E. faecium* (D), *L.gasseri* (E), and *L. lactis* (F). The two-way ANOVA and subsequently Dunnett's multiple comparison tests were used to calculate statistical significance compared to the untreated cells. An adjusted P-value of ≤0.05 was considered significant. The red vertical line represents the statistically significant adjusted p-values that are ≤0.05, while the black horizontal line represents the 50% growth inhibition of the bacteria by the different drugs. The numbers next to the dots represent the drug concentrations that exhibited ≥50% inhibition. Some data points are not visible due to clustering.

The overall antibacterial impact of ARVs against vaginal, gut, and ATCC bacterial strains is summarised in Figure 4.19. The highest concentrations (5 mg/mL) of TLD, TDF, and DTG were the most inhibitory. The ARVs, TLD, and DTG tended to cluster together as the most potent drugs even at their lower concentrations. These were followed by TAF and TDF. Cabotegravir (CAB) exhibited the least antibacterial effect. The ATCC strain of *E. faecium* was the most susceptible to all the drugs and *L. crispatus* was the least susceptible.

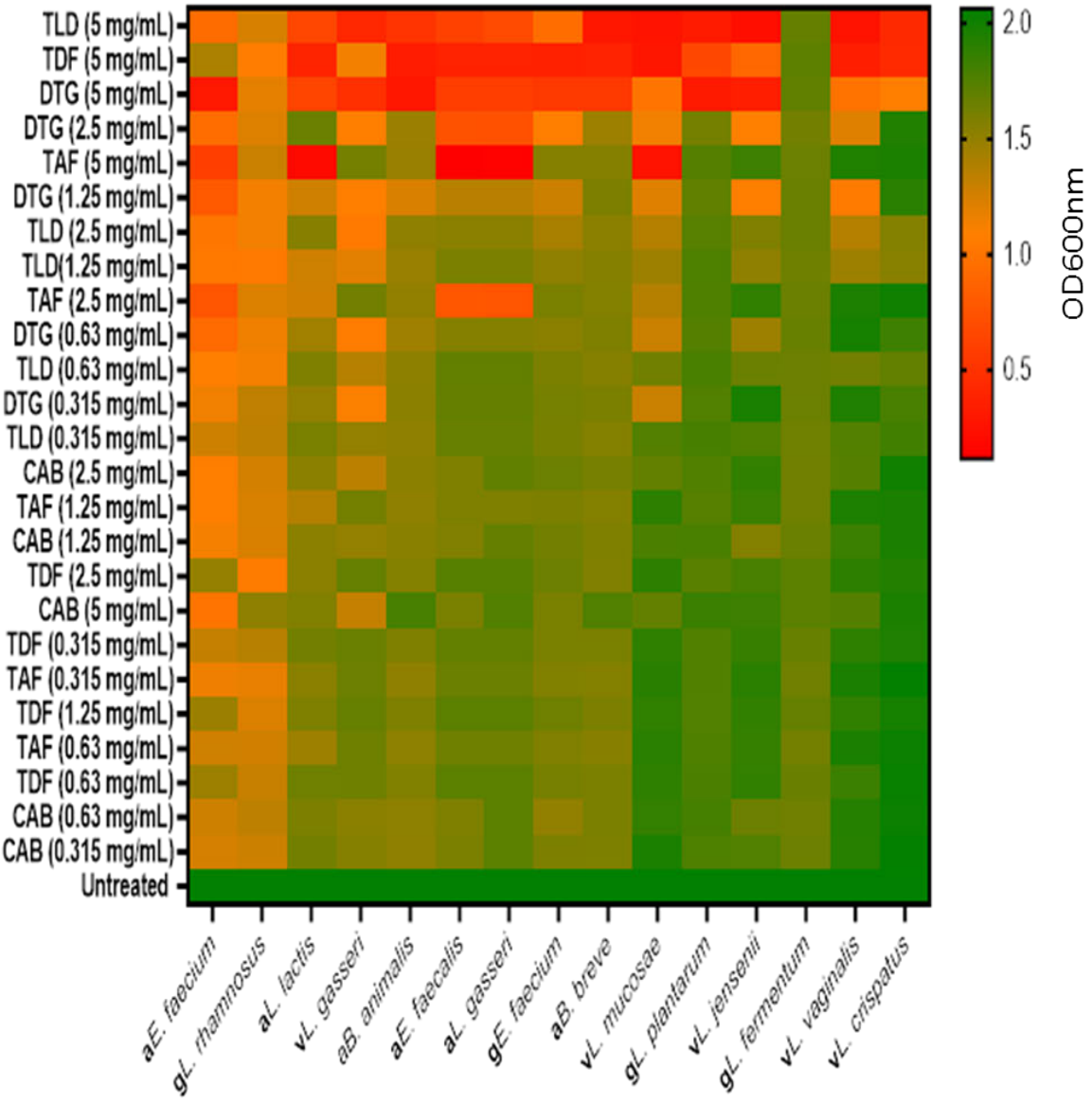

Figure 4.19: Summary of the impact of ARVs on bacterial growth. The antibacterial activity of the ARVs dolutegravir (DTG), tenofovir disoproxil fumarate (TDF), tenofovir/lamivudine/dolutegravir (TLD), tenofovir alafenamide (TAF) and cabotgravir (CAB) was tested against strains isolated from the vagina and gut of HIV-negative women between the ages of 14 and 24 years. Additionally, ATCC strains were also tested. In total there were five vaginal *Lactobacillus* (v*L*) species, two ATCC strains of *Bifidobacteria* (a*B*), two *Enterococcal* ATCC strains (a*E*), two ATCC strains of *Lactobacillus* (a*L*), gut-derived *E* (g*E*). *Faecium,* and three gut-derived *Lactobacilli* (g*L*). The heatmap was generated in GraphPad Prism 8.4.3.

4.11. The effect of ARVs on the pH of the broth

To investigate the mechanism by which ARVs were able to inhibit the growth of some bacteria, the pH of the ARVs was measured over 0-, 3-, 24- and 48- hours. The pH of the drugs that exhibited the highest potency against the bacteria (DTG TLD and TDF), was measured over time. All the drugs are acidic and fall within the optimal bacterial growth (Figure 4.20). The highest concentration of DTG, TDF, and TLD had pH values that were 5.83, 5.68, and 5.74, respectively. The pH for DTG was maintained through all the concentrations while the pH of TDF and TLD was reduced slightly with increasing concentrations, however, even at high concentrations, the pH was maintained in the optimal bacterial growth range (pH 4.5 – 6) (Figure 4.20A, Figure 4.20B and Figure 4.20C). Time had no impact on the change in broth pH.

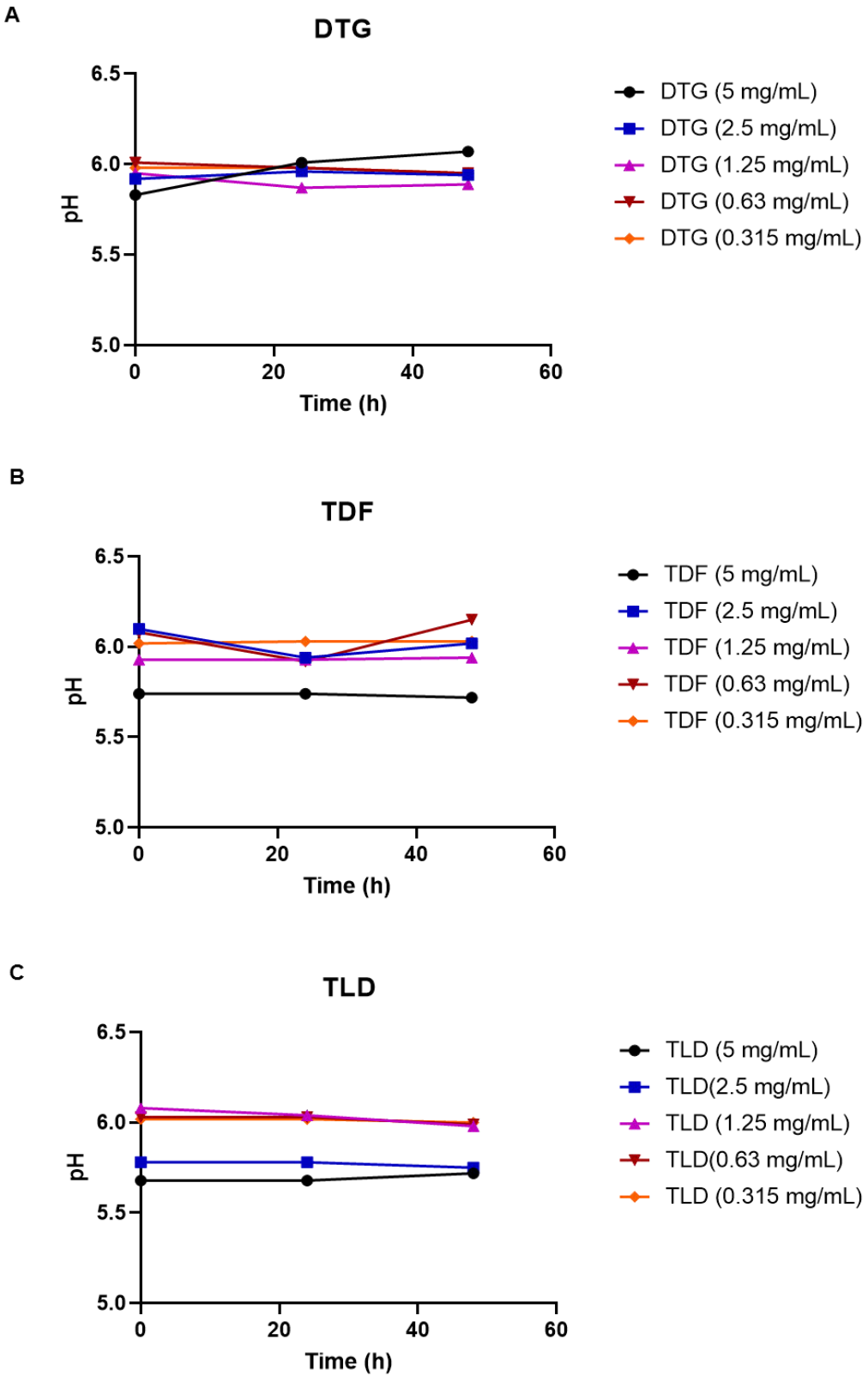

Figure 4.20: The effect of different antiretroviral drugs on the pH of selected bacterial cultures. The change in culture pH caused by different drug concentrations of dolutegravir (DTG), tenofovir disoproxil fumarate (TDF), tenofovir/lamivudine/dolutegravir (TLD), tenofovir alafenamide (TAF) and cabotegravir (CAB) was measured and compared to the change in the pH of the MRS broth. The graph was plotted in GraphPad Prism 8.4.3.

4.12. Summary of data

Table 4.2 shows a summary of all the data obtained for all the bacteria. The growth of *L. rhamnosus* and *ATCC E. faecium* was not affected by pH changes, while *L. plantarum* was susceptible to most pH changes. The greatest producer of D-lactate, L- lactate and H_2O_2 was *L. mucosae, L. rhamnosus* and *L. gasseri,* respectively. The ATCC strain of *L. lactis* and *L. rhamnosus* were resistant to all the antibiotics, while gut isolated *L. fermentum* and *L. rhamnosus* were resistant to all the ARVs.

Table 4.2: The summary of data obtained from the effect of pH on bacterial growth, lactic acid production, H_2O_2 production, growth inhibition assays by antibiotics and ARVs

Species name	Optimal pH	D-lactate concentration (mM)	L-lactate concentration (mM)	H_2O_2 concentration (µM/L)	Number of antibiotics resistant to (MIC ≥5 mg/mL)	Number of ARVs that exhibited ≥50% inhibition
L. crispatus	3.5 – 5.5	4.33	0.91685	0.2567	2	2
L. gasseri	.5 – 5.5	4.49	0.960715	0.3648	2	2
L. jensenii	.5 – 5.5	9.075	0.9835	0.18516	2	2
L. mucosae	.5 – 5.5	13.605	0.94065	0.170125	2	3
L. vaginalis	.5 – 5.5	3.16	0.485875	0.258255	2	2
gE. Faecium	2 and 6	0.3106265	0.481	0.1058	1	2
gL. fermentum	6	0.330035	0.64615	0.1058	1	0
gL. plantarum	3.5 – 6	0.338737	0.337	0.1058	1	2
gL. rhamnosus	2 - 6*	0.340915	1.42815	0.1058	7	0
aB. animalis	2.5 - 6	0.323093	0.4514	0.105852	1	3
aB. breve	3 - 6	0.297073	0.6242	0.10585	3	3
aE. faecalis	5 - 6	0.3620245	0.9204	0.1058	1	3
aE. faecium	2-6*	0.3096545	0.81065	0.1058	1	2
aL. gasseri	5 - 6	0.337623	0.8395	0.10585	2	4
aL. lactis	2.5 - 6	0.29888	0.6924	0.1058	7	4

*The growth was not affected by pH changes

Chapter 5

Discussion

The human microbiota plays vital roles in immunomodulation and protection against pathogenic invasion including HIV (Petrova *et al.*, 2013; Tachedjian *et al.*, 2017; Jang *et al.*, 2019; Armstrong & Kaul, 2021). Therefore, it is of utmost importance to understand how the microbiota may interact with the currently available and widely used ARV HIV treatment. For this reason, this study aimed to characterise species associated with a healthy vaginal and gut microbiota and to measure the antibacterial effects of both antibiotics and ARVs on these species. First, gut bacterial isolates were isolated from adolescent girls and young women and the purity was confirmed by Gram staining. The bacteria were identified to the species level using colony PCR and 16S rRNA PCR. Previous studies identified *L. gasseri, L. reuteri, L. salivarius, L. casei, L. plantarum*, *L. buchneri L. acidophilus, L. crispatus, L. johnsonii, L. ruminis, L. casei/paracasei, L. rhamnosus, L. plantarum, L. fermentum, L. brevis, L. delbrueckii, L. sakei, L. vaginalis*, and *L. curvatus* as the most common gut lactobacillus species (Walter, 2008; Ghosh *et al.*, 2020; Reuter, 2022).Therefore, this study aimed to isolate these common lactobacillus species. However, from the cohort of this study the only common *Lactobacillus* species isolated was *L. rhamnosus* from one participant*.* Other bacterial species which were isolated were *L*. *fermentum* from six participants*, L. plantarum* from one participant, and *E. faecium* from two participants. Each one of these distinct species was used for downstream assays. Even though *E. faecium* it is not a *Lactobacillus* species it is still associated with a healthy gut microbiota (Dubin & Pamer, 2014). The overall composition of the gut microbiome in the current study cohort could have been influenced by other factors such as diet, ethnicity, age and geographical region (Ghosh et al., 2020). How these factors modulate the abundant *Lactobacillus* species still remains to be elucidated. Overall, from the gut and vaginal species tested, gut isolated *L. rhamnosus* is predicted to be the most ideal potential probiotic candidate for individuals taking antibiotics and ARVs, since its growth was not affected by pH changes. In addition, *L. rhamnosus* was also one of the greatest producers of L- lactate, and was resistant to all the antibiotics, and all the ARVs tested.

It was hypothesized that the bacterial species that produce the most lactic acid and H_2O_2 were the most effective in preventing infection, including HIV (Boskey *et al.*, 2001; Petrova *et al.*, 2013; Tachedjian *et al.*, 2017; Jang *et al.*, 2019). The production of lactic acid, H_2O_2, and the consequent pH acidification were therefore evaluated. Overall, the vaginal *Lactobacilli*

produced about 86% more D-lactate compared to L-lactate. This was concordant with previous work that showed that probiotics and vaginal *Lactobacillus* spp. from South African adolescent girls and young women produced more D-lactate compared to L-lactate *in vitro* and *in vivo* when using the calorimetric assays (Chetwin *et al.*, 2019; Happel *et al.*, 2020). Additionally, cervicovaginal secretions from *L. crispatus* dominant vaginal microbiota (defined as healthy) produced more D-lactate than L-lactate, while *L. iners* produced more L- than D-lactate in young South African women (Delgado-Diaz *et al.*, 2022). A recent study reported that the D/L-lactic acid ratio was due to the degradation of L-lactate by l-lactate oxidase (Okano *et al.*, 2022)

In the present study, though not significant, the greatest D-lactate producer was *L. mucosae,* followed by *L. gasseri, L. vaginalis, L. jensenii,* and lastly *L. crispatus*. The production of L-lactate on the other hand was the same for these bacteria, while *L. vaginalis* produced the least. A similar prior *in vitro* research, however, showed varying amounts of both D-and L-lactate production by the same species. These differences were dependent on the participant, ATCC strain, and/or probiotic capsule when the calorimetric assay was used (Chetwin *et al.*, 2019; Happel *et al.*, 2020). The varying lactic acid production by a species when isolated from different participants could be a result of several factors that influence the vaginal microbiota, such as depression, menstrual cycle, vaginal product use, BV, and diet (Ravel *et al.*, 2011; Humphries *et al.*, 2019).

The lactic acid production is coupled with subsequent pH acidification to pH between 3.5 and 4.5, in which most microorganisms cannot grow. This, therefore, ensures that the *Lactobacilli* can inhibit the growth of invasive pathogens (Amabebe & Anumba, 2018). Although not significant, *L. mucosae* was more successful at reducing culture pH compared to the other species, coinciding with the D-lactate production. Other studies found varying results both aerobically and anaerobically using the calorimetric assays (Georgieva *et al.*, 2015; Chetwin *et al.*, 2019; Happel *et al.*, 2020). The findings of this current study are contrary to the findings of previous studies that revealed that *L. crispatus*-dominant women were most successful in lactic acid production, pH acidification, and inhibiting the growth of anaerobic pathogens (Amabebe & Anumba, 2018). Perhaps these species confer better protection in a mixed culture rather than individually (Ravel *et al.*, 2011; Amabebe & Anumba, 2018).

Hydrogen peroxide is essential for human cells and can provide antimicrobial effects, (McDonnell, 2014) therefore the H_2O_2 production by vaginal *Lactobacilli* was assessed. In addition to lactic acid production and pH acidification, hydrogen peroxide production is also one of the mechanisms anti-pathogenic bacteria use to prevent and limit microbial invasion (McDonnell, 2014). The production of H_2O_2 was not significantly different between the bacteria, while independent studies found that H_2O_2 production varied between species, with *L. jensenii* followed by *L. mucosae* producing the most (Chetwin *et al.*, 2019; Happel *et al.*, 2020). Additionally, the amount of H_2O_2 observed in the present study was within the toxic range (0.1 mM - 1 mM), meaning that these bacteria can effectively eliminate pathogens and aid in maintaining human health, thus killing catalase negative cells (Pericone *et al.*, 2003; Strus *et al.*, 2006; Mahaseth & Kuzminov, 2017). The efficiency of vaginal *Lactobacillus* in producing lactic acid, subsequent pH acidification, and H_2O_2 production is an indication that these species can indeed inhibit the growth of pathogens that cannot tolerate acidic environments (Amabebe & Anumba, 2018). Noteworthy, the gut isolated *E. faecium* and its ATCC strain produced similar amounts of D-/L lactic acid and hydrogen peroxide.

Although the vaginal *Lactobacilli* have these antimicrobial systems in place, they sometimes fail when they cannot produce sufficient lactic acid or H_2O_2. In this case, pathogens outcompete the *Lactobacilli* and accumulate in the vaginal tract, increasing pH (Boskey *et al.*, 2001). Therefore, the growth of vaginal *Lactobacilli* at different pH levels was evaluated. Vagina-derived *Lactobacillus* spp. were sensitive to conditions that were too acidic with *L. crispatus*, *L. gasseri*, and *L. mucosae* inhibited at pH 3 and below, while *L. jensenii* and *L. vaginalis* were inhibited at pH-values below 2.5. On the other hand, most *Lactobacillus* spp. resumed growth when the medium was neutralised to a higher pH. This was in particular more evident for *L. crispatus* and *L. gasseri.* These observations indicate that acidity is one of the main limiting factors for the growth of vagina *Lactobacillus* species. These findings also confirm that *Lactobacillus* species, particularly *L. crispatus and L. gasseri,* are likely to grow in less acidic environments associated with most infections and dysbiotic conditions, such as BV, and may therefore be major contributing factors in inhibiting such pathogens, explaining why many infections can clear without treatment when these bacterial species are dominant (Boskey *et al.*, 2001; Redelinghuys *et al.*, 2020). Similar to the findings of the present study *L. crispatus* showed increased growth at pH 6 (Happel *et al.*, 2020).

The gut LABs are also involved in immunomodulation and fermentation. The production of lactic acid by gut LABs is an indication of the digestion of complex plant materials that humans cannot digest via heterofermentation (Valdes *et al.*, 2018; Rossi *et al.*, 2019). Therefore, it is hypothesised that LABs that produce the highest amount of lactic acid are the better fermenters. Overall, the gut bacteria produced more L- compared to D-lactic acid *in vitro* which was predictable since L-lactate is the most common isomer in humans. Moreover, D-lactate poses a health threat to humans as it has been shown that excessive D-lactate production can result in acidosis in the physiologically acidic gut (Ewaschuk *et al.*, 2005; Pohanka, 2020). The gut bacteria produced similar amounts of D-lactate, while *L. rhamnosus* produced the most L-lactic acid compared to the other gut bacteria, which produced similar amounts of L-lactic acid, suggesting that *L. rhamnosus* is the greatest fermenter of all the gut bacteria. Additionally, the amount of H_2O_2 observed in this study was within the toxic range (0.1 mM – 1 mM), meaning that these bacteria can effectively eliminate pathogens and aid in maintaining human health, thus killing catalase-negative cells (Pericone *et al.*, 2003).

The lactic acid produced by gut LABs and subsequent pH acidification is thought to protect the gut mucosal barrier by inhibiting pathogen invasion. This was demonstrated by previous studies that showed that gut *Bifidobacteria, Lactobacilli,* and *Enterococci* all inhibit the growth of pathogens *in vitro* and *in vivo* (Campana *et al.*, 2017; Ren *et al.*, 2021; Anjana & Tiwari, 2022). *Lactobacillus rhamnosus* produced the highest amount of L-lactate, which coincided with the pH acidification caused by *L. rhamnosus.* This suggests that *L. rhamnosus* is more successful at antimicrobial activities and subsequent immunomodulation. This result agreed with a previous study that observed that *L. rhamnosus* mounted the most effective antimicrobial activities *in vitro* (Campana *et al.*, 2017). Additionally, lactic acid can modulate inflammation, thereby reducing the chances of developing inflammatory bowel syndrome (Manosalva *et al.*, 2022). These results imply that *L. rhamnosus* might be one of the best gut probiotics. Hydrogen peroxide is a further component that LABs use to protect from pathogenic invasion In this study, all the gut bacteria produced similar amounts of H_2O_2, therefore, these gut bacteria contribute equally to gut health in this regard.

Individuals that are HIV positive are immunocompromised and are vulnerable to opportunistic diseases such as other STIs, tuberculosis, and pneumonia (Akhavan *et al.*, 2022; Peechakara *et al.*, 2022; Yip & Gerriets, 2022). These conditions are vigorous and are mainly treated with antibiotics (Grossman *et al.*, 2014). For this reason, the impact of antibiotics was tested on the

health-associated bacteria. Overall, ampicillin was most potent against all the bacteria, except *B. animalis*, followed by clindamycin, amoxicillin, and penicillin. This suggests that antibiotic use can eliminate the “good” bacteria, given that the currently administrated dosage of these antibiotics is higher than the concentration tested (15 mg/mL - 1750 mg/day) (Akhavan *et al.*, 2022; Peechakara *et al.*, 2022). The depletion of these vaginal *Lactobacilli* has been associated with BV and STIs both *in vivo* and *in vitro* (Aroutcheva *et al.*, 2001; Larsson *et al.*, 2008; Georgieva *et al.*, 2015; Sharma, 2017). Additionally, the depletion of gut bacteria and a shift in microbiota richness may reduce the production of certain vitamins and minerals during microbial-assisted digestion and may lead to diseases such as diabetes, inflammatory bowel syndrome and *C. difficle* infection (Langdon *et al.*, 2016; Valdes *et al.*, 2018; Ramirez *et al.*, 2020).The activity of these antibiotics against all the bacteria is expected since they are all broad-spectrum antibiotics (Akhavan *et al.*, 2022; Peechakara *et al.*, 2022). These findings agreed with those of previous studies that showed that vaginal *Lactobacilli,* gut *Bifidobacterium* and *Lactobacilli* were highly susceptible to ampicillin, amoxicillin, clindamycin, and penicillin (Georgieva *et al.*, 2015; Happel *et al.*, 2020). Although the aforementioned antibiotics were highly potent against all bacteria, including *E. faecalis* and *E. faecium,* previous work revealed that *Enterococci* spp. were resistant to these antibiotics (Kristich *et al.*, 2014; Miller *et al.*, 2014). Metronidazole, rifampicin, and streptomycin were less lethal to the bacteria, in agreement with previous work that showed that these antibiotics exhibited moderate antibacterial activity (Ocaña *et al.*, 2006; Petrina *et al.*, 2017; Valdes *et al.*, 2018; Chetwin *et al.*, 2019; Happel *et al.*, 2020). Moreover, metronidazole was previously shown to have no antibacterial activity against *L. iners* and *L. crispatus* and in addition, there was increased growth of *L. gasseri* and *L. jensenii* (Armstrong *et al.*, 2022). This suggests that metronidazole is a relatively safe antibiotic to use for the treatment of BV and other STIs, although in many African women BV recurrence is problematic. Recurrence of BV and STIs may be linked to *L. iners* dominant microbiota since *L. iners* offers the least protection against infection (Payne *et al.*, 2010; Li & Ma, 2020; Mtshali *et al.*, 2021).

The present study also examined the relationship between the mucosal microbiota and ARVs that are currently being used or in the research pipeline for the treatment and prevention of HIV. To achieve this, the antimicrobial activity of ARVs was evaluated. The elimination of important species of the mucosal microbiota is likely to worsen the already HIV-compromised microbiota in HIV+ individuals and may result in STI and inflammatory bowel syndrome, which has been associated with an increased HIV acquisition risk (Cohen *et al.*, 2019; Crakes & Jiang, 2019; Ray & Mukherjee, 2021). Furthermore, the ARVs may perpetuate the dysbiotic

conditions caused by HIV infection (Zilberman-Schapira *et al.*, 2016). To the best of our knowledge, this was the first study to demonstrate the differential inhibitory effects of mainly DTG and TLD as well as other ARVS against health-associated bacteria. Overall, TLD, followed by DTG, TDF, and TAF was the most potent against all the bacteria, while CAB exhibited almost no inhibitory effect. The inhibitory effects of ARVs on vaginal and gut bacteria might perpetuate the dysbiotic condition caused by HIV infection, thereby increasing the risk of adverse health outcomes, including the observed excessive weight gain (Cohen *et al.*, 2019; Crakes & Jiang, 2019; Ray & Mukherjee, 2021; Ricky K. Hsu, 2022). The heightened potency of the ARV combination compared to the individual ARVs could be ascribed to the three different components that make up this cocktail acting individually on their targets, therefore TLD attacks pathogens using three mechanisms of attack instead of one. Tenofovir and 3TC inhibit the viral reverse transcriptase, while DTG inhibits integrase, therefore allowing the drug to exhibit heightened antibacterial activity (Arts & Hazuda, 2012; Sierra-Aragón & Walter, 2012). Previous research showed that the abundance of gut *E. faecium* in patients using TFV was reduced, while 3TC had no impact on the growth of bacteria (Fulcher *et al.*, 2019; Ray *et al.*, 2021). These findings and those of the present study suggest that TFV and DTG, but not 3TC, contribute towards the inhibitory effects of TLD. Moreover, of all the TFV-based drugs, TLD exhibited the greatest inhibitory effects, followed by TDF and lastly TAF, implying that TAF is a much safer drug to use, given that TAF reduced renal and bone defects when compared to TDF (Sax *et al.*, 2015; Antela *et al.*, 2016).

The inhibitory effects of the ARVs on the bacteria associated with the healthy gut microbiota are concerning and require further investigation, especially since the MICs observed in this study for DTG, TDF, and TAF were smaller than the currently administered doses (50 mg, 300 mg, and 25 mg, respectively) (LiverTox, 2012; WHO, 2021). Vaginal *L. crispatus* was the least susceptible to the ARVs, making this *Lactobacillus* species the ideal species for people using ARVs. However, women in Africa where the majority of ARVs are being used, do not have *L. crispatus*-dominated microbiota. Overall, *L. rhamnosus* isolated from the gut presented as the ideal species to be used as a probiotic due to its desirable traits, such as, its ability to produce the most L-lactate, resistance to pH changes, antibiotics and ARVs. Furthermore, the present study suggested that the ARVs do not inhibit the growth of bacteria by changing the pH, rather there might be a different mechanism that ARVs use to inhibit bacteria. However, further investigation is required to clearly define the mode of inhibition, as the significance of pH change *in vivo* is unknown, including several other factors possibly at play.

Limitations

The following are some of the limitations of this study: the concentration of the drugs ingested may not necessarily reflect physiological concentrations in both the gut and vagina. In addition, the *in vitro* experimental setup may not accurately represent microenvironmental factors *in vivo*. ARVs are a daily lifelong treatment, therefore repeated doses at regular intervals can achieve a steady-state level of drug. However, it is acknowledged that *in vivo* system is a non-equilibrium system, and the steady state depends on adherence to drugs and upon both the rate of entry and the rate of exit of the drug. Nevertheless, *in vitro* data sets are useful as a starting point in different studies to predict adverse effects.

Conclusion and recommendations

The present work revealed the antibacterial activity *in vitro* of ARVs against health-associated bacteria isolated from the vagina and gut of young South African women. This study also showed that the ARVs exhibit inhibitory effects against bacteria by lowering the prevailing pH. The results of this study provided insights as to why women of African descent, who use ARVs for HIV prevention and treatment, are at a higher risk of microbial dysbiosis that may lead to adverse health outcomes. Although this study demonstrated the antibacterial properties of ARVs against bacteria *in vitro,* more research needs to be conducted *in vivo* to further explore this relationship. Also, other factors that influence the microbiota may be involved in the antibacterial activity of ARVs. For this reason, future studies should monitor the microbiota of women who are initiated on current ARVs either for the prevention or treatment of HIV. The drug pharmacokinetics and metabolomics of the ARVs both *in vitro* and directly *ex vivo* should be conducted to understand how the ARVs exhibit this inhibitory activity.

Chapter 6

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Appendix A

Ethics approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

04 January 2022

Miss Nomacusi Sibonganjalo Lindiwe Sibeko (216015827)
School of Life Sciences
Pietermaritzburg

Dear Miss Sibeko,

Protocol reference number: BREC/00003679/2021
Project title: Investigation of the interaction between antiretroviral drugs and microbiota in African women
Degree: MSc

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 04 January 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Amended_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 04 January 2022. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 08 February 2022.

Yours sincerely,

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Appendix B

Preparation of media

De Man, Rogosa and Sharpe Broth and Agar

Broth

1. Dissolve 50 g of MRS powder, 0,0 g L-cysteine and 1 mL Tween 80 (0.1%) in 1 L warm deionized water
2. Stir until completely dissolved
3. Autoclave at 121°C for 15 minutes
4. Store at 4°C

Agar

1. Repeat steps 1 and 2 of broth preparation
2. Add 62 g MRS agar powder
3. Stir until completely dissolved
4. Autoclave at 121°C for 15 minutes
5. Once agar has cooled, pour into petrie dishes (15 mL) or 6-well plates (3 mL)
6. Store at 4°C and heat when needed

Storage protocol

1. Prepare 60% (v/v) glycerol
2. Mix 500 µL of cell suspension with 500 µL 60% (v/v) glycerol
3. Store at -80°C

Appendix C

Sequencing alignments

>Enterococcus faecium strain GXNN20201226-1 16S ribosomal RNA gene, partial sequence

Sequence ID: MZ452347.1 Length: 1523

Range 1: 10 to 902

Score:1441 bits(780), Expect:0.0,

Identities:843/896(94%), Gaps:5/896(0%), Strand: Plus/Minus

```
Query  1    TCCCMAGGGCCGGAGTGCTTAATGCGTTAGCTGCRGCACTGAAGGGCGGAAACCCTCCAA  60
            |||| | || |||||||||||||||||||||||| ||||||||||||||||||||||||||
Subject  902  TCCCCA-GG-CGGAGTGCTTAATGCGTTAGCTGCAGCACTGAAGGGCGGAAACCCTCCAA  845

Query  61   CACYTASCWYTCATCGTTTACGGCRTGGACTACCAGGGTATCTAATCCTGTTYGCTMCCC  120
            ||| || |  |||||||||||||| ||||||||||||||||||||||||||| ||| |||
Subject  844  CACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC  785

Query  121  AYGCTTTCGAGYCTCAGCGTCAGTTRCAGACCAGRGAGCCGCCTTCGCCACTGGTGTTCY  180
            | |||||||||| |||||||||||||| |||||||| ||||||||||||||||||||||||
Subject  784  ACGCTTTCGAGCCTCAGCGTCAGTTACAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCC  725

Query  181  TCCATATATCTACGCATTYCACCGCTACACATGGARTTCCACTMYCCTCTTCTGCACTCA  240
            |||||||||||||||||| |||||||||||||||| |||||||  ||||||||||||||||
Subject  724  TCCATATATCTACGCATTTCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCA  665
```

>gb|OM959233.1| Lacticaseibacillus rhamnosus strain Cupick2 16S ribosomal RNA gene, partial sequence

Length=1412

Score = 1059.0 bits (1173), Expect = 0E00
Identities = 613/666 (92%), Gaps = 0/666 (0%)
Strand = Plus/Minus

```
Query  1    CTTAATGCGTTAGCTSCGGCACTGAAGGGCGGAAACCCTCCAACACCTAGCAYTCATCGT  60
            ||||||||||||||| |||||||||||||||||||||||||||||||||| |||||||
Sbjct  837  CTTAATGCGTTAGCTGCGGCACTGAAGGGCGGAAACCCTCCAACACCTAGCATTCATCGT  778

Query  61   TTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTACCCATGCTTTCGAGYCTCAG  120
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  777  TTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTACCCATGCTTTCGAGCCTCAG  718

Query  121  CGTCAGTTRCAGACCAGRYAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCAT  180
            |||||||| ||||||||  ||||||||||||||||||||||||||||||||||||||||
Sbjct  717  CGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCAT  658

Query  181  TYCACCGCTACACATGGAGTTCCACTRYCCTCTTCTGCACTCAAGTTWYCCAGTTTCCGA  240
            | ||||||||||||||||||||||  ||||||||||||||||||  ||||||||||||
Sbjct  657  TTCACCGCTACACATGGAGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGA  598

Query  241  TGCACTTCYYCGGTTAAGCCGARGGCTTTCACATCAGACTTARAAAACCGCCTGCRCTCK  300
            ||||||||  |||||||||||| |||||||||||||||||| |||||||||||| |||
Sbjct  597  TGCACTTCCTCGGTTAAGCCGAGGGCTTTCACATCAGACTTAAAAAACCGCCTGCGCTCG  538

Query  301  CTTTACGCCCAATAAATCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  537  CTTTACGCCCAATAAATCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCA  478

Query  361  CGTAGTTAGCCGTGRCTTTCTGGTTRRATACCGTCAMSSCRACAACAGTTACTCTGMYRA  420
            ||||||||||||||| ||||||||||  |||||||||   | ||||||||||||||   |
Sbjct  477  CGTAGTTAGCCGTGGCTTTCTGGTTGGATACCGTCACGCCGACAACAGTTACTCTGCCGA  418
```

```
>gb|CP094655.1| Limosilactobacillus fermentum strain SCB0035 chromosome, complete genome
          Length=2016236

 Score = 2437.6 bits (2702),  Expect = 0E00
 Identities = 1352/1354 (99%), Gaps = 0/1354 (0%)
 Strand = Plus/Minus

Query  1       CGACTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162100  CGACTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC  162041

Query  61      GTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGCAGGCGAGT  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162040  GTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGCAGGCGAGT  161981

Query  121     TGCAGCCTGCAGTCCGAACTGAGAACGGTTTTAAGAGATTTGCTTGCCCTCGCGAGTTCG  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161980  TGCAGCCTGCAGTCCGAACTGAGAACGGTTTTAAGAGATTTGCTTGCCCTCGCGAGTTCG  161921

Query  181     CGACTCGTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGA  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161920  CGACTCGTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGA  161861

Query  241     TCTGACGTCGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAAC  300
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161860  TCTGACGTCGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAAC  161801

Query  301     TTAATGCTGGCAACTAGTAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCA  360
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161800  TTAATGCTGGCAACTAGTAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCA  161741

Query  361     CGACACGAGCTGACGACGACCATGCACCACCTGTCATTGCGTTCCCGAAGGAAACGCCCT  420
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161740  CGACACGAGCTGACGACGACCATGCACCACCTGTCATTGCGTTCCCGAAGGAAACGCCCT  161681

Query  421     ATCTCTAGGGTTGGCGCAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATT  480
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161680  ATCTCTAGGGTTGGCGCAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATT  161621

Query  481     AAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGC  540
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  161620  AAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGC  161561

Query  541     GGTCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTCCGGCACTGAAGGGCGGAAACC  600
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>gb|ON366690.1| Enterococcus faecium strain HBUAS62985 16S ribosomal RNA gene, partial sequence
Length=1483

Score = 2383.5 bits (2642), Expect = 0E00
Identities = 1324/1330 (99%), Gaps = 0/1330 (0%)
Strand = Plus/Minus

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Query  1     GTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1420  GTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACC  1361

Query  61    GCGGCGTGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1360  GCGGCGTGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTG  1301

Query  121   CAATCCGAACTGAGAGAAGCTTTAAGAGATTAGCTTAGCCTCGCGACTTCGCAACTCGTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1300  CAATCCGAACTGAGAGAAGCTTTAAGAGATTAGCTTAGCCTCGCGACTTCGCAACTCGTT  1241

Query  181   GTACTTCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1240  GTACTTCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTC  1181

Query  241   ATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTTGCTAGAGTGCCCAACTGAATGATG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1180  ATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTTGCTAGAGTGCCCAACTGAATGATG  1121

Query  301   GCAACTAACAATAAGGGKTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAG  360
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1120  GCAACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAG  1061

Query  361   CTGACGACAACCATGCACCACCTGTCACTTTGCCCCCGAAGGGGAAGCTCTATCTCTAGA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1060  CTGACGACAACCATGCACCACCTGTCACTTTGCCCCCGAAGGGGAAGCTCTATCTCTAGA  1001

Query  421   GTGGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1000  GTGGTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACAT  941
```

>gb|ON005263.1| Limosilactobacillus fermentum strain HBUAS62490 16S ribosomal RNA gene, partial sequence

Length=1498

Score = 2470.1 bits (2738), Expect = 0E00
Identities = 1370/1372 (99%), Gaps = 0/1372 (0%)
Strand = Plus/Minus

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Query  1     CGACTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1444  CGACTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAAC  1385

Query  61    GTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGCAGGCGAGT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1384  GTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGCAGGCGAGT  1325

Query  121   TGCAGCCTGCAGTCCGAACTGAGAACGGTTTTAAGAGATTTGCTTGCCCTCGCGAGTTCG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1324  TGCAGCCTGCAGTCCGAACTGAGAACGGTTTTAAGAGATTTGCTTGCCCTCGCGAGTTCG  1265

Query  181   CGACTCGTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1264  CGACTCGTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGA  1205

Query  241   TCTGACGTCGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAAC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1204  TCTGACGTCGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAAC  1145

Query  301   TTAATGCTGGCAACTAGTAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1144  TTAATGCTGGCAACTAGTAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCA  1085

Query  361   CGACACGAGCTGACGACGACCATGCACCACCTGTCATTGCGTTCCCGAAGGAAACGCCCT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084  CGACACGAGCTGACGACGACCATGCACCACCTGTCATTGCGTTCCCGAAGGAAACGCCCT  1025

Query  421   ATCTCTAGGGTTGGCGCAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1024  ATCTCTAGGGTTGGCGCAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATT  965
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>gb|ON005263.1| Limosilactobacillus fermentum strain HBUAS62490 16S ribosomal RNA gene, partial sequence

Length=1498

Score = 2426.8 bits (2690), Expect = 0E00
Identities = 1346/1348 (99%), Gaps = 0/1348 (0%)
Strand = Plus/Minus

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Query  1     GGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1436  GGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC  1377

Query  61    CGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGCAGGCGAGTTGCAGCCT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1376  CGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGCAGGCGAGTTGCAGCCT  1317

Query  121   GCAGTCCGAACTGAGAACGGTTTTAAGAGATTTGCTTGCCCTCGCGAGTTCGMGACTCGT  180
             |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  1316  GCAGTCCGAACTGAGAACGGTTTTAAGAGATTTGCTTGCCCTCGCGAGTTCGCGACTCGT  1257

Query  181   TGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATCTGACGT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1256  TGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATCTGACGT  1197

Query  241   CGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTTAATGCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1196  CGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTTAATGCT  1137

Query  301   GGCAACTAGTAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1136  GGCAACTAGTAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGA  1077

Query  361   GCTGACGACGACCATGCACCACCTGTCATTGCGTTCCCGAAGGAAACGCCCTATCTCTAG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1076  GCTGACGACGACCATGCACCACCTGTCATTGCGTTCCCGAAGGAAACGCCCTATCTCTAG  1017

Query  421   GGTTGGCGCAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1016  GGTTGGCGCAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACA  957
```

```
ALIGNMENTS
>gb|ON209902.1| Lactiplantibacillus pentosus strain HBUAS58109 16S ribosomal RNA gene, partial
sequence
          Length=1504

 Score = 2626.1 bits (2911),  Expect = 0E00
 Identities = 1456/1457 (99%), Gaps = 0/1457 (0%)
 Strand = Plus/Minus

Query  1     CCTAAAAGGTTACCCCACCGACTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1461  CCTAAAAGGTTACCCCACCGACTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTG  1402

Query  61    TGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1401  TGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCC  1342

Query  121   GACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGATTAG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1341  GACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGATTAG  1282

Query  181   CTTACTCTCGCGAGTTCGCAACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1281  CTTACTCTCGCGAGTTCGCAACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGG  1222

Query  241   TCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1221  TCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGT  1162

Query  301   CTCACCAGAGTGCCCAACTTAATGCTGGCAACTGATAATAAGGGTTGCGCTCGTTGCGGG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1161  CTCACCAGAGTGCCCAACTTAATGCTGGCAACTGATAATAAGGGTTGCGCTCGTTGCGGG  1102

Query  361   ACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTATCCATGT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1101  ACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTATCCATGT  1042

Query  421   CCCCGAAGGGAACGTCTAATCTCTTAGATTTGCATAGTATGTCAAGACCTGGTAAGGTTC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1041  CCCCGAAGGGAACGTCTAATCTCTTAGATTTGCATAGTATGTCAAGACCTGGTAAGGTTC  982

Query  481   TTCGCGTAGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  981   TTCGCGTAGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCC  922
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ALIGNMENTS
>gb|CP094655.1| Limosilactobacillus fermentum strain SCB0035 chromosome, complete genome
Length=2016236

Score = 2522.4 bits (2796), Expect = 0E00
Identities = 1403/1407 (99%), Gaps = 1/1407 (0%)
Strand = Plus/Minus

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Query  1       CTGGCTCCTAAAAGGTTACCCCACCGACTTTGGGTGTTACAAACTCTCATGGTGTGACGG  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265309  CTGGCTCCTAAAAGGTTACCCCACCGACTTTGGGTGTTACAAACTCTCATGGTGTGACGG  265250

Query  61      GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGC  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265249  GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTACTAGC  265190

Query  121     GATTCCGACTTCGTGCAGGCGAGTTGCAGCCTGCAGTCCGAACTGAGAACGGTTTTAAGA  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265189  GATTCCGACTTCGTGCAGGCGAGTTGCAGCCTGCAGTCCGAACTGAGAACGGTTTTAAGA  265130

Query  181     GATTTGCTTGCCCTCGCGAGTTCGCGACTCGTTGTACCGTCCATTGTAGCACGTGTGTAG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265129  GATTTGCTTGCCCTCGCGAGTTCGCGACTCGTTGTACCGTCCATTGTAGCACGTGTGTAG  265070

Query  241     CCCAGGTCATAAGGGGCATGATGATCTGACGTCGTCCCCACCTTCCTCCGGTTTGTCACC  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265069  CCCAGGTCATAAGGGGCATGATGATCTGACGTCGTCCCCACCTTCCTCCGGTTTGTCACC  265010

Query  301     GGCAGTCTCACTAGAGTGCCCAACTTAATGCTGGCAACTAGTAACAAGGGTTGCGCTCGT  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265009  GGCAGTCTCACTAGAGTGCCCAACTTAATGCTGGCAACTAGTAACAAGGGTTGCGCTCGT  264950

Query  361     TGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACGACCATGCACCACCTGTCA  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264949  TGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACGACCATGCACCACCTGTCA  264890

Query  421     TTGCGTTCCCGAAGGAAACGCCCTATCTCTAGGGTTGGCGCAAGATGTCAAGACCTGGTA  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264889  TTGCGTTCCCGAAGGAAACGCCCTATCTCTAGGGTTGGCGCAAGATGTCAAGACCTGGTA  264830
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ALIGNMENTS
>gb|CP094655.1| Limosilactobacillus fermentum strain SCB0035 chromosome, complete genome
Length=2016236

Score = 2489.9 bits (2760), Expect = 0E00
Identities = 1381/1383 (99%), Gaps = 0/1383 (0%)
Strand = Plus/Minus

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Query  1       GGCGGCTGGCTCCTAAAAGGTTACCCCACCGACTTTGGGTGTTACAAACTCTCATGGTGT  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265314  GGCGGCTGGCTCCTAAAAGGTTACCCCACCGACTTTGGGTGTTACAAACTCTCATGGTGT  265255

Query  61      GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTA  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265254  GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGATTA  265195

Query  121     CTAGCGATTCCGACTTCGTGCAGGCGAGTTGCAGCCTGCAGTCCGAACTGAGAACGGTTT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265194  CTAGCGATTCCGACTTCGTGCAGGCGAGTTGCAGCCTGCAGTCCGAACTGAGAACGGTTT  265135

Query  181     TAAGAGATTTGCTTGCCCTCGCGAGTTCGCGACTCGTTGTACCGTCCATTGTAGCACGTG  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265134  TAAGAGATTTGCTTGCCCTCGCGAGTTCGCGACTCGTTGTACCGTCCATTGTAGCACGTG  265075

Query  241     TGTAGCCCAGGTCATAAGGGGCATGATGATCTGACGTCGTCCCCACCTTCCTCCGGTTTG  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265074  TGTAGCCCAGGTCATAAGGGGCATGATGATCTGACGTCGTCCCCACCTTCCTCCGGTTTG  265015

Query  301     TCACCGGCAGTCTCACTAGAGTGCCCAACTTAATGCTGGCAACTAGTAACAAGGGTTGCG  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  265014  TCACCGGCAGTCTCACTAGAGTGCCCAACTTAATGCTGGCAACTAGTAACAAGGGTTGCG  264955

Query  361     CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACGACCATGCACCACC  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264954  CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACGACCATGCACCACC  264895
```

```
>gb|MH771720.1| Lactobacillus fermentum strain LJOSL 16S ribosomal RNA gene, partial sequence
          Length=1466

 Score = 2479.1 bits (2748),  Expect = 0E00
 Identities = 1377/1378 (99%), Gaps = 0/1378 (0%)
 Strand = Plus/Minus

Query  1     CTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTA  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1448  CTTTGGGTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTA  1389

Query  61    TTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGCAGGCGAGTTGC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1388  TTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGACTTCGTGCAGGCGAGTTGC  1329

Query  121   AGCCTGCAGTCCGAACTGAGAACGGTTTTAAGAGATTTGCTTGCCCTCGCGAGTTCGCGA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1328  AGCCTGCAGTCCGAACTGAGAACGGTTTTAAGAGATTTGCTTGCCCTCGCGAGTTCGCGA  1269

Query  181   CTCGTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATCT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1268  CTCGTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATCT  1209

Query  241   GACGTCGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTTA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1208  GACGTCGTCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACTAGAGTGCCCAACTTA  1149

Query  301   ATGCTGGCAACTAGTAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1148  ATGCTGGCAACTAGTAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGA  1089

Query  361   CACGAGCTGACGACGACCATGCACCACCTGTCATTGCGTTCCCGAAGGAAACGCCCTATC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1088  CACGAGCTGACGACGACCATGCACCACCTGTCATTGCGTTCCCGAAGGAAACGCCCTATC  1029

Query  421   TCTAGGGTTGGCGCAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1028  TCTAGGGTTGGCGCAAGATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAA  969

Query  481   CCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  968   CCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGT  909
```

Figure C1: The identification of the isolated gut bacteria. The sequences of the isolated gut bacteria were blasted on NCBI Blast website (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Appendix D

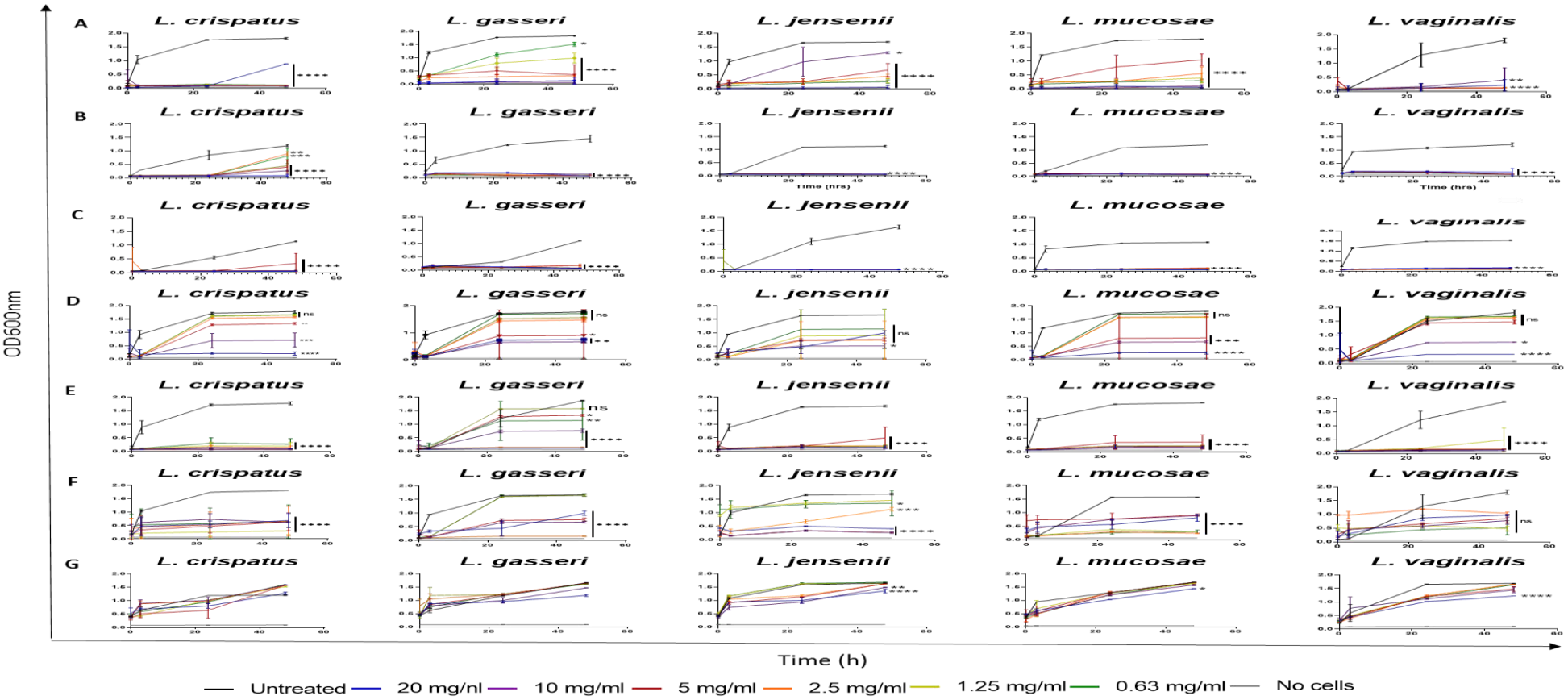

Figure D1: The minimal inhibitory concentration (MIC) of antibiotics against vaginal *Lactobacillus* species isolated from adolescent girls and young women. The MIC assay was carried out by means of micro dilution. *Lactobacillus crispatus, L. gasseri L. jensenii, L. mucosae* and *L. vaginalis* isolated from South African adolescent girls and young women aged 14-24 years were inoculated with 20, 10, 5, 2.5, 1.25, 0.63 and 0.315 mg/mL of amoxicillin (A), ampicillin (B), clindamycin (C), metronidazole (D), penicillin (E), rifampicin (F) and streptomycin (G). The bacterial growth at OD600 nm was measured after 0-, 3-, 24- and 48 hours of anaerobic incubation at 37°C using a microplate reader. Statistical significance was tested using Kruskal-Wallis two-way ANOVA (parametric) and the Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered as significant. The level of significance was denoted as ns=not significant, ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). This experiment was done in triplicate.

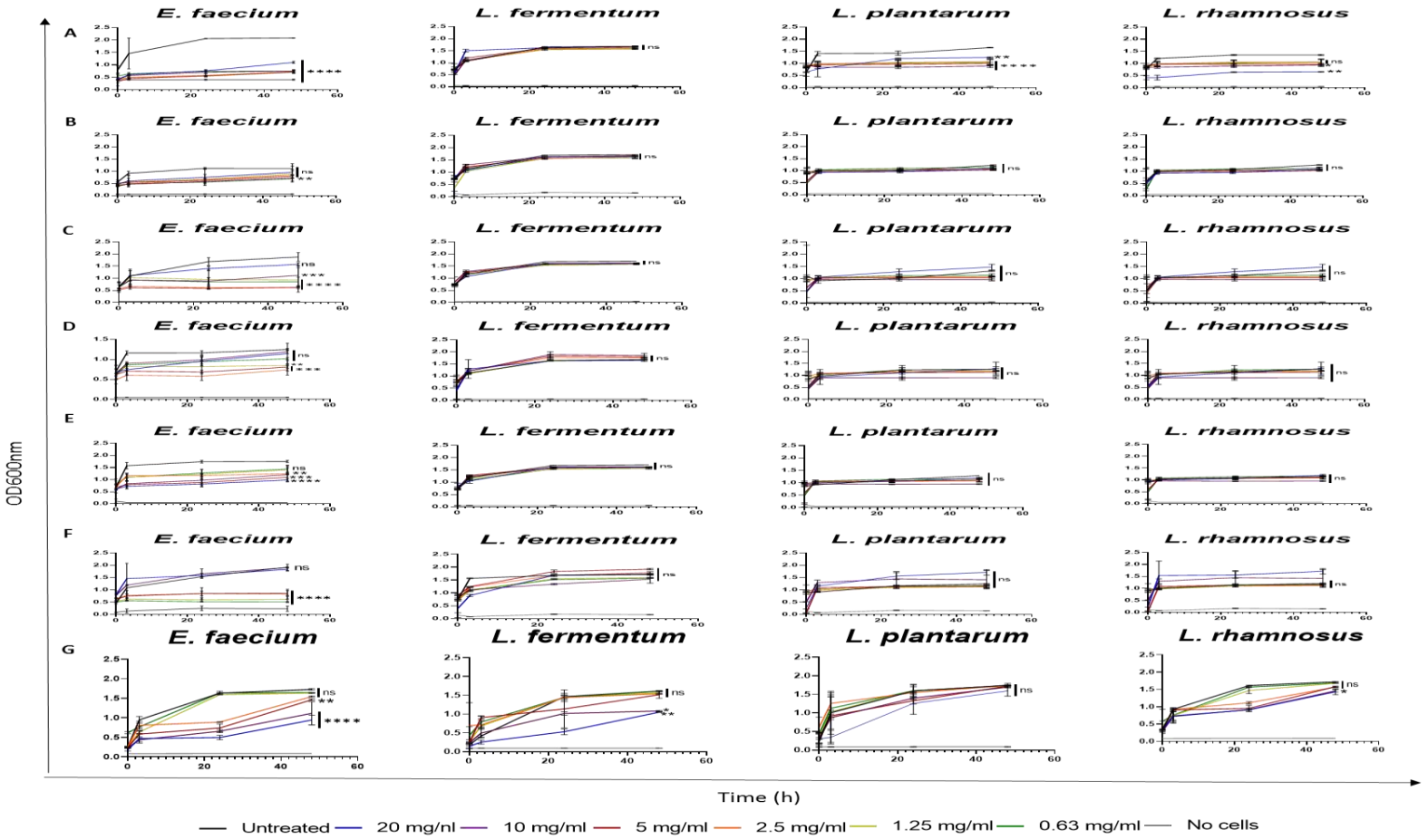

Figure D2: The minimal inhibitory concentration (MIC) of antibiotics against gut species isolated from adolescent girls and young women. The MIC assay was carried out by means of micro dilution. *Enterococcus faecium, L. fermentum, L. plantarum* and *L. rhamnosus* isolated from South African adolescent girls and young women aged 14-24 years were inoculated with 20, 10, 5, 2.5, 1.25, 0.63 and 0.315 mg/mL of amoxicillin (A), ampicillin (B), clindamycin (C), metronidazole (D), penicillin (E), rifampicin (F) and streptomycin (G). The bacterial growth was measured at OD600 nm after 0-, 3-, 24- and 48 hours of anaerobic incubation at 37°C using a microplate reader. Statistical significance was tested using Kruskal-Wallis two-way ANOVA (parametric) and the Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered as significant. The level of significance was denoted as ns=not significant, ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). This experiment was done in triplicate.

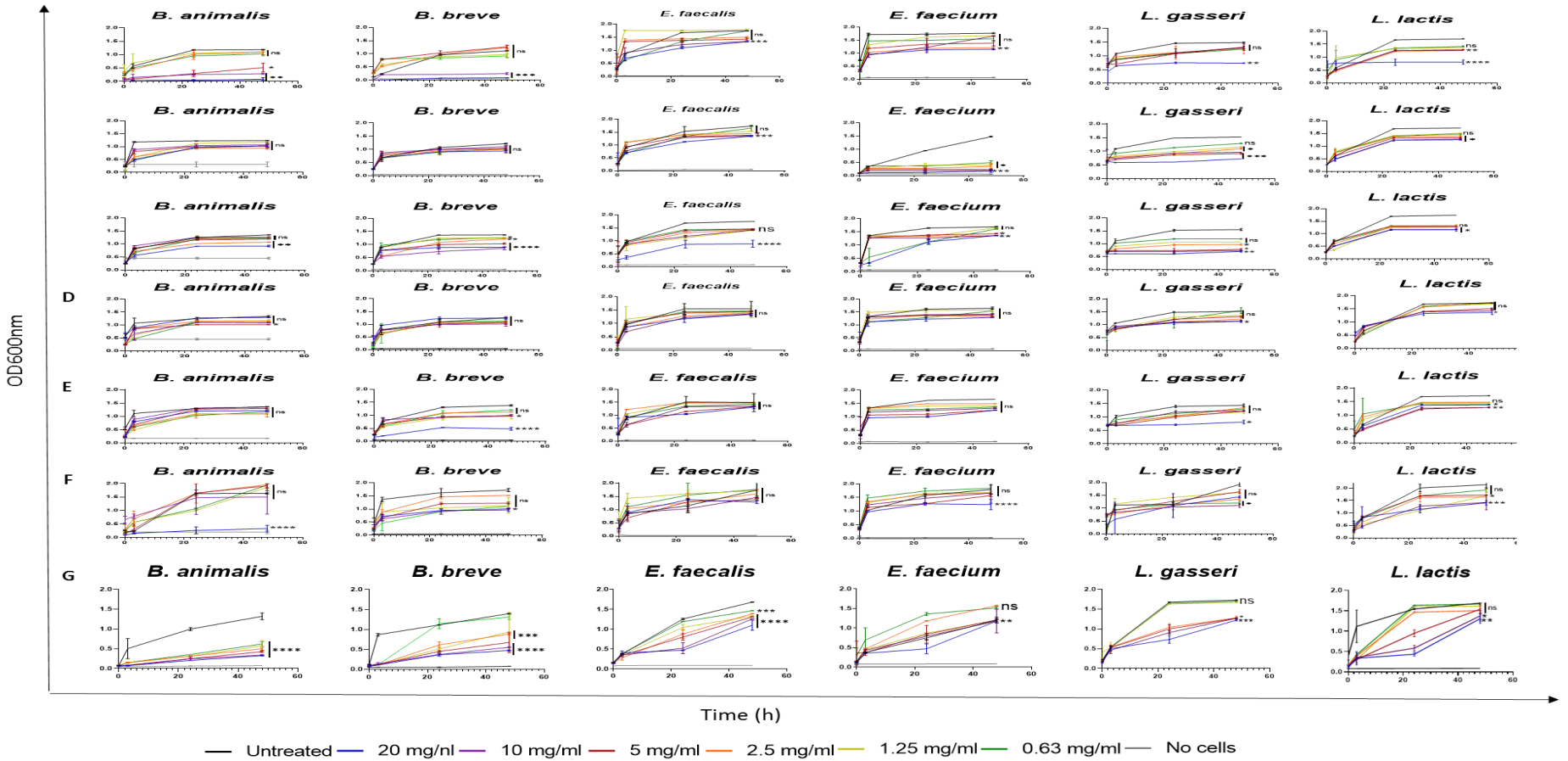

Figure D3: The minimal inhibitory concentration (MIC) of antibiotics against gut species isolated from adolescent girls and young women. The MIC assay was carried out by means of micro dilution. The gut ATCC strains of *B. animalis, B. breve, E. faecalis, E. faecium, L. gasseri* and *L. lactis* isolated from South African adolescent girls and young women aged 14-24 years were inoculated with 20, 10, 5, 2.5, 1.25, 0.63 and 0.315 mg/mL of amoxicillin (A), ampicillin (B), clindamycin (C), metronidazole (D), penicillin (E), rifampicin (F) and streptomycin (G). The bacterial growth at OD600 nm was measured after 0-, 3-, 24- and 48 hours of anaerobic incubation at 37°C using a microplate reader. Statistical significance was tested using Kruskal-Wallis two-way ANOVA (parametric) and the Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered as significant. The level of significance was denoted as ns=not significant, ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). This experiment was done in triplicate.

Appendix E

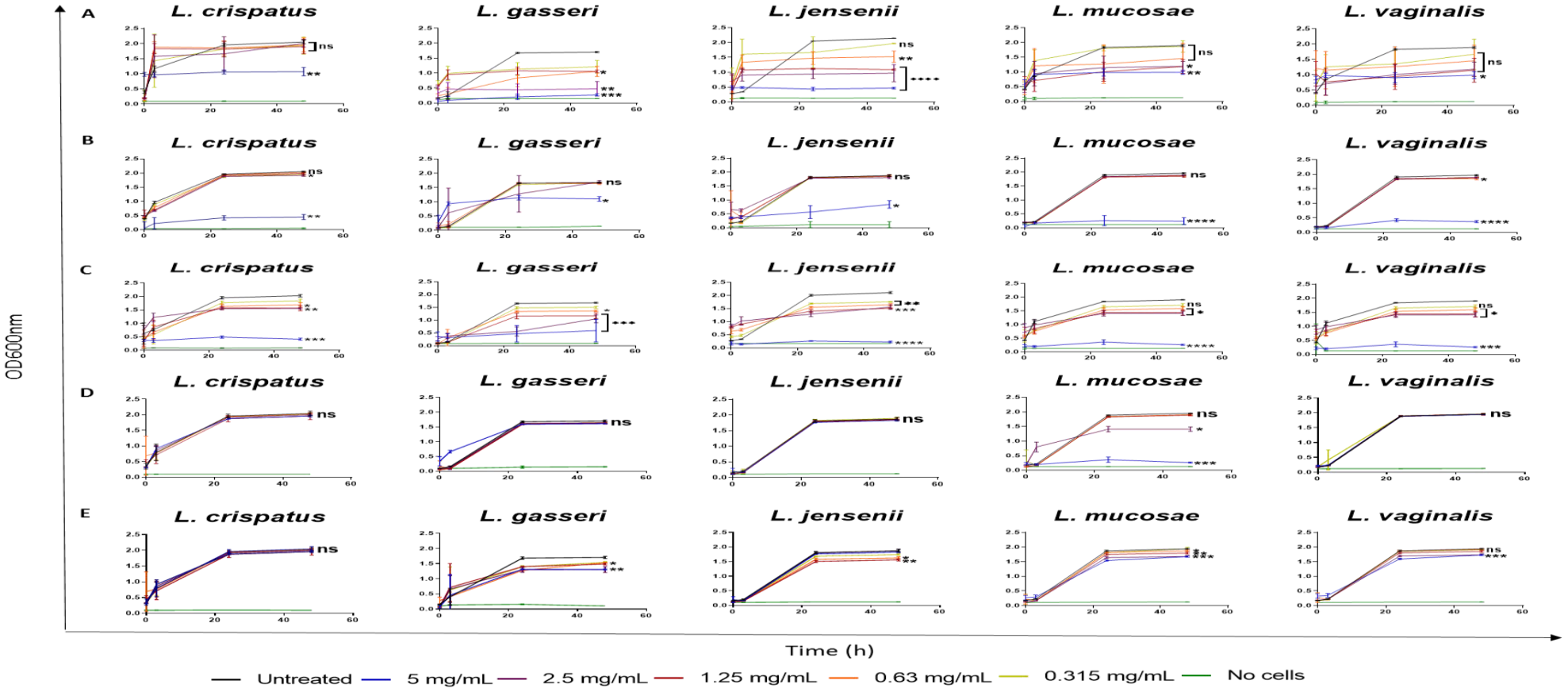

Figure E1: The minimal inhibitory concentration (MIC) of ARVs against vaginal *Lactobacillus* species isolated from adolescent girls and young women. The MIC assay was carried out by means of micro dilution. *Lactobacillus crispatus, L. gasseri L. jensenii, L. mucosae* and *L. vaginalis* isolated from South African adolescent girls and young women aged 14-24 years were inoculated with 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL/ 0.63 mg/mL and 0.315 mg/mL of dolutegravir (DTG, A), tenofovir disoproxil fumarate (TDF, B), Tenovifor/lamivudine/dolutegravir (TLD, C) Tenofovir alafenamide (TAF, D), cabotegravir (CAB, E). The bacterial growth at OD600 nm was measured after 0-, 3-, 24- and 48 hours of anaerobic incubation at 37°C using a microplate reader. Statistical significance was tested using Kruskal-Wallis two-way ANOVA (parametric) and the Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered as significant. The level of significance was denoted as ns=not significant, ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). This experiment was done in triplicate.

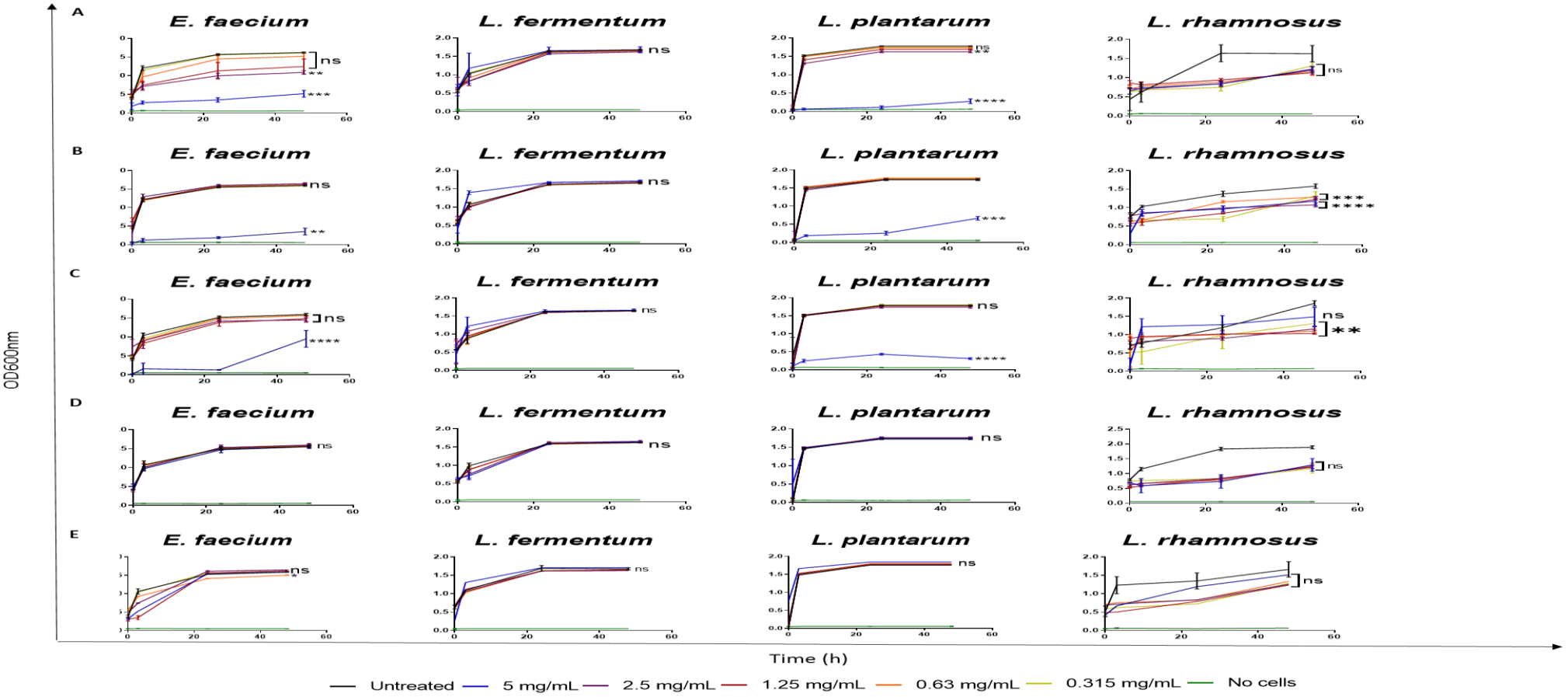

Figure E2: The minimal Inhibitory concentration (MIC) of ARVs against gut species isolated from South African adolescent girls and young women. The MIC assay was carried out by means of micro dilution. *Enterococcus faecium, L. fermentum, L. plantarum* and *L. rhamnosus* isolated from adolescent girls and young women between 14 – 24 years were inoculated with 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL/ 0.63 mg/mL and 0.315 mg/mL of dolutegravir (DTG, A), tenofovir disoproxil fumarate (TDF, B), Tenovifor/lamivudine/dolutegravir (TLD, C) Tenofovir alafenamide (TAF, D), cabotegravir (CAB, E). The bacterial growth at OD600 nm was measured after 0-, 3-, 24- and 48 hours of anaerobic incubation at 37°C using a microplate reader. Statistical significance was tested using Kruskal-Wallis two-way ANOVA (parametric) and the Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered as significant. The level of significance was denoted as ns=not significant, ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). This experiment was done in triplicate.

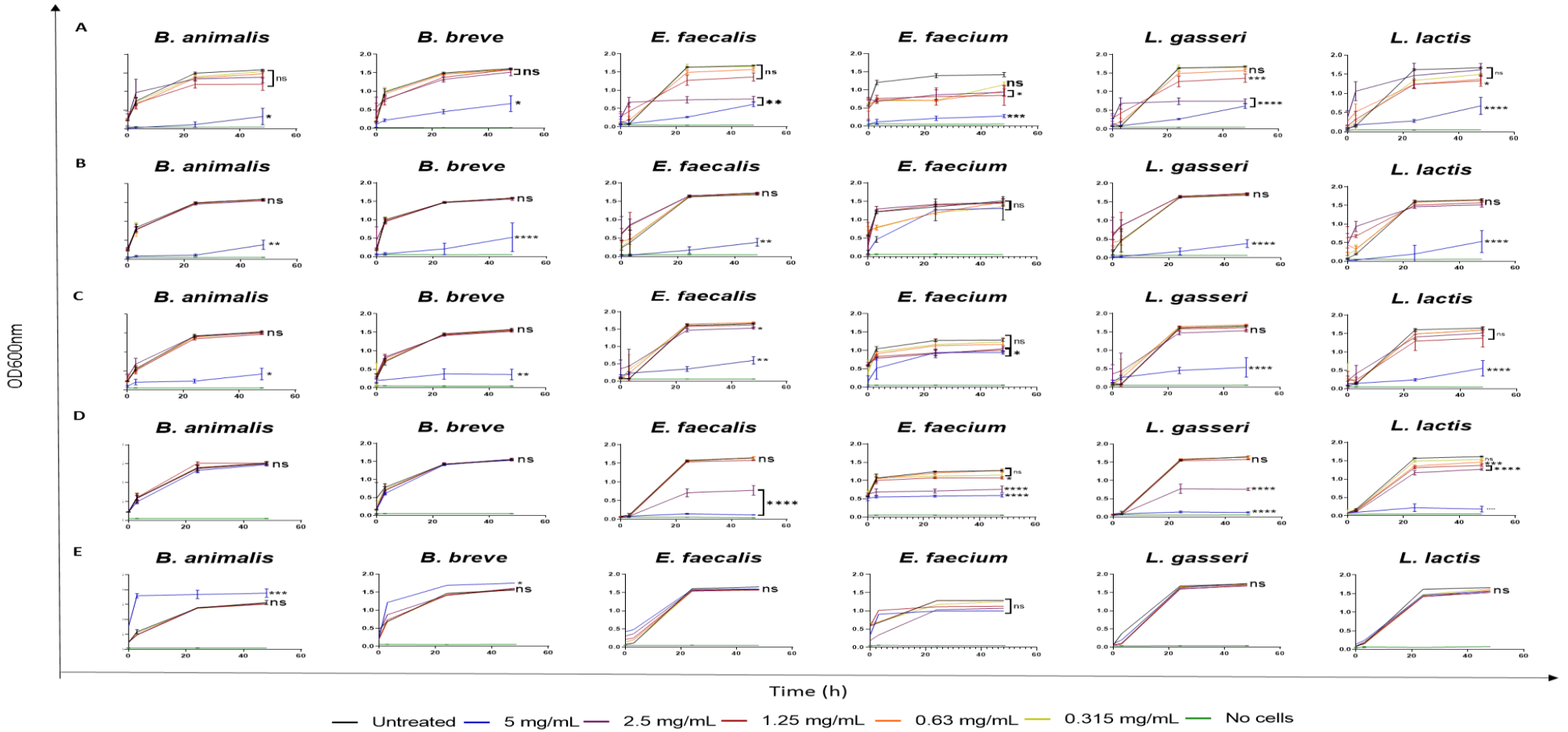

Figure E3: The minimal Inhibitory concentration (MIC) of ARVs against gut ATCC strains. The MIC assay was carried out by means of micro dilution. The gut ATCC strains of *B. animalis, B. breve, E. faecalis, E. faecium, L. gasseri* and *L. lactis* were inoculated with 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL/ 0.63 mg/mL and 0.315 mg/mL of dolutegravir (DTG, A), Tenofovir disoproxil fumarate (TDF, B), Tenovifor/lamivudine/dolutegravir (TLD, C) Tenofovir alafenamide (TAF, D), cabotegravir (CAB, E). The bacterial growth at OD600 nm was measured after 0-, 3-, 24- and 48 hours of anaerobic incubation at 37°C using a microplate reader. Statistical significance was tested using Kruskal-Wallis two-way ANOVA (parametric) and the Dunnett's multiple comparison test. An adjusted P-value of ≤0.05 was considered as significant. The level of significance was denoted as ns=not significant, ≤0.05 (*), 0.01 (**), <0.001 (***), 0.0001 (****). This experiment was done in triplicate.