CHARACTERIZATION OF CERVICOVAGINAL HPV VIROME AND BACTERIOME IN HIV-INFECTED WOMEN IN NORTHERN SOUTH AFRICA

BY

Ratshilindela Mpho (15012299)

A dissertation submitted in fulfilment of the requirements

For

The award of a Master of Science degree in Microbiology

To

The Department of Biochemistry and Microbiology

Faculty of Science, Engineering and Agriculture

University of Venda

Private Bag X5050

Thohoyandou

0950

South Africa

Supervisor: Professor Pascal Obong Bessong

Co-supervisor: Dr Denis Tebit

DECLARATION

I, Ratshilindela Mpho, hereby declare that this dissertation for the award of Master of Science degree in Microbiology at the University of Venda is my own work. It has not been submitted before for the degree examination at this or any other University. It is my own work at execution and the reference materials contained are therein have been duly acknowledged.

Signature:  ..... Date: 25 July 2022

ACKNOWLEDGEMENTS

I would like to express my gratitude and appreciation to the following people or organization:

- I am grateful to my supervisor Prof. P.O Bessong for his endless support, encouragement, and opportunity to learn from his vast knowledge in the field of molecular microbiology. I will forever run with the knowledge, skills, and the words of wisdom you graced me with.

- I extend my gratitude to my Co-supervisor Dr D.M Tebit for all the contributions you made towards my work, I am very grateful.

- To the entire team of HIV/AIDS and Global Health Programme, thank you for all the scientific inputs you contributed towards my work.

- To my family at large for being there for me when I needed them the most.

- The National Research Foundation for their financial support throughout the study and providing me with a studentship (UID 122824).

- Special thanks to God almighty.

DEDICATION

I would like to dedicate this Master of Science dissertation to my father Ntanganedzeni Ratshilindela, my late mother and grandmother, Ramudzuli Agnes Nephiphidi and Thidziambi Rosina Ratshilindela.

ABSTACT

BACKGROUND: The presence of a highly diverse bacterial species in the cervicovaginal niche is linked to a higher risk of contracting human immunodeficiency virus (HIV), persistent infection with human papillomavirus (HPV) and consequently cervical cancer. It is unknown how cervicovaginal bacterial species associate and interact with other viruses. This study hypothesized that HIV/HPV co-infected women have an increased diversity of cervicovaginal virome and bacteriome.

OBJECTIVES: The study's primary objective was to characterize cervicovaginal HPV virome and bacteriome in HIV-infected women from selected health care facilities in Northern South Africa. Specific objectives were to determine the presence of HPV virome in HIV-infected women and HIV-noninfected women, to determine the presence of bacteriome in HIV-infected women and HIV-noninfected women, and to determine the relational occurrence of virome and bacteriome according to HIV status.

METHODOLOGY: Cervical swabs from 50 HIV/HPV co-infected women; 50 HIV-infected, HPV-noninfected women; and from 50 HPV-infected, HIV-noninfected women were used to extract total deoxyribonucleic acid (DNA). To determine HPV virome, total DNA was enriched by rolling circle amplification (RCA) using an illustra TempliPhi amplification kit. To determine bacteriome, a two-round polymerase chain reaction (PCR) was employed targeting a bacterial 16S rRNA gene. Amplification products obtained from RCA and PCR were purified and sequenced by Next Generation Sequencing (NGS) using a MiniSeq platform (Illumina). The quality of the sequences was validated using the FastQC program. Sequences of good quality were assigned to viral family and genera using the Dragen metagenomics online tool with BaseSpace Sequence Hub. Bacterial sequences were assigned and categorised into vaginal community state types (CSTs) using the Dragen metagenomics online tool with BaseSpace Sequence Hub. The relational occurrence of virome and bacteriome according to HIV status was assessed through Chi-square statistical analysis available in GraphPad Prism version 9.3.1. Differences in occurrence were expressed as probability (*P*)-values.

RESULTS: A diverse group of viral families was observed among HIV/HPV co-infected women. *Papillomaviridae* (14/34; 41%) was the most prevalent (*P*<0.0001), followed by *Herpesviridae* and *Phycodnaviridae* (12/34; 35%) each, *Poxviridae* (10/34; 29%), *Mimiviridae* (7/34; 20%), *Maiseilleviridae* (3/34; 9%) and *Anelloviridae* (2/34; 6%) in order of decreasing prevalence. A highly diverse group of bacteriophages was observed, with *Myoviridae* (31/34, 91%) being the most prevalent (*P*<0.0001). Among HPV-infected HIV-noninfected women,

Papillomaviridae (8/26; 31%) was the most prevalent (*P*<0.0001), followed by *Anelloviridae* (4/26; 15%), *Phycodnaviridae* (4/26; 15%), *Poxviridae* and *Herpesviridae* (2/26; 8%) each in order of decreasing prevalence. *Myoviridae* (6/26; 23%) was the most prevalent bacteriophage family detected (*P=0.0005)*. Among HIV-infected HPV-noninfected women, a small group of viral families was observed, with *Myoviridae* (3/11,27%) *and Siphoviridae* (3/11, 27%) being the most prevalent viral families detected *(P*=0.0005 each). HPV 16 was the most common high risk (hr) HPV type in HIV-infected women and HIV-noninfected women of this study. This genotype co-existed with other hrHPV or probable hr types including HPV 54, HPV 53, and HPV 26. Overall, hrHPV types were more prevalent in HIV-infected women than in HIV-noninfected women, although this difference was not statistically significant (*P*=0.2832). When the occurrence of hrHPV types were considered individually, HPV 54 occurred more significantly in HIV-noninfected women than in HIV-infected women (*P*<0.0003).

Bacteriome revealed the presence of community state types (CSTs) one, two, three, four and five among study participants. Among HIV/HPV co-infected women, CST one (*L. crispatus*), CST three (*L. iners*) and CST four (high bacterial diversity) were observed, with CST four being the most prevalent. Among HPV-infected, HIV-noninfected women, CST one, three, four and five (*L. jensenni*) were observed, with CST four, also being the most prevalent. Among HIV-infected, HPV noninfected women, CST three and four were observed. CST three and four were associated with viral infections. *Gardnerella vaginalis (75%), Pretovella spp (54%), Atopobium spp (50%), Bifidobacterium spp (27%), Porphyromonas spp (53%), Pseudomonas spp (50%), Faecalibacterium prausnitzii (47%) and Bacteroides spp (35%)* were the most prevalent anaerobic bacterial species detected in CST four. A diverse group of bacterial families occurred more significantly among HIV-infected women as compared to HIV-noninfected women for NGS data of RCA products (*P*<0.0001) and NGS data of 16S amplification products (*P*<0.0001).

CONCLUSION: In conclusion, this study showed a higher diversity of cervicovaginal virome and bacteriome in women who were HIV/HPV co-infected than in those singly infected with HIV or HPV. The relationship between viral infections and a high diversity of bacterial species (CST four) observed herein may be a useful indicator of the individual's disease state, indicating the likelihood of developing cervical lesions and cervical cancer. As a result, additional research is necessary to uncover the association between viral infections and CST four with disease state. Vaccine development and antiviral research should also target compounds that boost the cervicovaginal environment and maintain vaginal homeostasis.

KEYWORDS: Virome, Bacteriome, HPV, HIV, Cervical cancer, Northern South Africa**.**

TABLE OF CONTENT

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µl	Microliters
AE	Elusion buffer
AIDS	Acquired immunodeficiency syndrome
CC	Cervical cancer
CCR5+	Chemokine receptor type 5
CD4+	Cluster of differentiation 4
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
CSTs	Community State Types
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide triphosphates
ds	Double-stranded
E4 and E5	Early genes
EBV	Epstein Barr virus
H_2O_2	Hydrogen Peroxide
HIV	Immunodeficiency Virus
HPV	Human Papillomavirus
hr	High risk
HSIL	High Intraepithelial Lesion
HSV	Herpes Simplex Virus
Kb	Kilobases
L	Lactobacillus
lr	Low risk
LSIL	Low Grade Squamous Intraepithelial Lesions
$Mgcl_2$	Magnesium chloride
Min	Minutes
Mm	Millimolar

n	Number
NAOH	Sodium Hydroxide
ng	Nanogram
NGS	Next Generation Sequence
Nm	Nanomolar
P-value	Probable value
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
pM	Picomolar
QC	Quality control
RBS	Re-suspension Buffer
RCA	Rolling circle amplification
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
S	Seconds
spp	Species
ss	Single-stranded
STIs	Sexual transmitted infections
TNF	Tumour necrosis factor
UV	Ultraviolet
V	Voltage
α	Alpha

LIST OF FIGURES

LIST OF TABLES

Table 3.15: Bacterial community state types and their bacterial species detected among HIV-infected, HPV-noninfected study group women.

CHAPTER ONE: BACKGROUND AND LITERATURE REVIEW

1.1 BACKGROUND

The human microbiome is referred to as a collection of microorganisms including viruses, bacteria, and fungus that colonize nearly every part of the human body in a symbiotic relationship (Siqueira et al., 2019). This microbiome has distinct profiles in each of the body's anatomical region (Wylie et al., 2014; Costello et al., 2009). The bacterial and viral profiles in the female anogenital tract are complex, resulting in significant interpersonal differences (Costello et al., 2009; Wylie et al., 2014; Perez et al., 2016).

In healthy women, *Papillomaviridae* is regarded as the most frequent viral family and its occurrence ranges from 38% to 100% (Santiago-Rodriguez et al., 2015; Siqueira et al., 2019). Anelloviruses are also frequently detected, with herpesviruses, polyomaviruses, and adenoviruses detected in small proportion of individuals (Swenson et al., 1995; Berntsson et al., 2013; Siqueira et al., 2019). A similar diversity of viruses is found in HIV-infected women, with papillomaviruses being more prevalent (Ameur et al., 2014; Madeddu et al., 2014).

The vaginal microenvironment is important for reproductive health. This is because it is believed that resident vaginal *Lactobacillus species (spp.)* guard against pathogens and sexually transmitted infections (STI) by maintaining a favourable pH and producing species-specific metabolites called bacteriocins (Boskey et al., 2001; McMillan et al., 2011). However, activities such as the use of vaginal hygiene products or sexual behaviours may disturb the vaginal microbiome, potentially resulting in health consequences including premature birth and an increased likelihood of contracting HIV, HPV and other STIs (Huang et al., 2014).

Cervical microbiome research using Next Generation Sequencing (NGS) has identified five well-established bacterial community state types (CSTs one-five), which are classified based on their frequency profiles (Ravel et al., 2011). *Lactobacillus spp*. dominate CSTs one, two, three and five (*L. crispatus, L. gasseri, L. iners, and L. je*nsenii, respectively), whereas CST four has a small proportion of *Lactobacillus spp.* and a greater diversity of anaerobic bacteria such as *Pretovella, Dialister, Atopobium,* and *Gardnerella* (Ravel et al., 2011; Forney et al., 2014).

1.2 LITERATURE REVIEW

1.2.1 BACTERIOME

1.2.1.1 Cervicovaginal microbiome classification

Based on 16S rRNA high-throughput sequencing data, the bacterial species present in the cervicovaginal microbiome can be classified into five groups. These groups are termed community state types (CSTs) (Ravel et al., 2011). CSTs were named from one to five based on bacterial frequency profiles. *Lactobacillus crispatus*, *L. gasseri, L. iners, and L. jensenni* are the dominant species in CSTs one, two, three, and five, respectively. CST four has a high bacterial diversity, with anaerobic bacteria including *Gardnerella*, *Megasphera, Atopobium*, and *Pretovella* present at high proportion of about 65%.

1.2.1.2 Healthy vaginal microbiome

The bacteria that make up the vaginal microbiome are inseparably linked to the acidity of the vaginal environment. *Lactobacillus spp*. such as *L. crispatus and L. gasseri* dominate a balanced cervicovaginal microbiota (Curty et al., 2017). By creating lactic acid, hydrogen peroxide (H_2O_2), and bacteriocins, these microorganisms are believed to maintain a healthy vaginal environment (Aroutcheva et al., 2001; Lambert et al., 2013). These metabolites act supportively to prevent the overgrowth of other, less favourable bacterial genera and viruses (Atassi and Servin, 2010; Kyrgiou et al., 2016).

Hydrogen peroxide employs its protective bactericidal effects through oxidative stress, which causes breaks in the DNA of the cells. These bactericidal effects are increased in the acidic environment that is created by a *Lactobacillus* metabolite, known as lactic acid (Atassi and Servin, 2010; Martin and Suarez, 2010). According to Hawes and Colleagues (1996), women who have a majority of H_2O_2 producing *Lactobacilli* showed a lower prevalence of symptomatic vaginosis when compared to women who were colonized by *Lactobacilli* that do not produce H_2O_2, or other bacterial species.

Lactic acid present at an active level promotes a healthy vaginal environment by reducing the pH of the vagina (O'Hanlonet al., 2013). Lactic acid production maintains vaginal pH at less than 4.5

(<4.5); this forms an unfavourable environment for pathogenic bacteria and prevents the overgrowth of anaerobic bacteria such as *Gardnerella*, *Prevotella, and Atopobium* through the disruption of the cell membrane (Arouchteva et al., 2001; Huang et al., 2014).

Additional *Lactobacillus* metabolites which promote a healthy vaginal environment are bacteriocins and biosurfactants. These antimicrobial peptides inhibit the growth and establishment of closely related *Lactobacillus spp.* as well as the growth of a wide range of anaerobic bacteria such as *Gardnerella, Prevotella, Megasphaera, Streptococcus, and Ureaplasma* (Arouchteva et al., 2001).

1.2.1.3 Risk factors of vaginal dysbiosis

Dysbiosis is characterized by an unbalanced cervicovaginal microbiome composition, which includes a highly diverse microbial flora with low *Lactobacillus* abundance, such as CST four. Certain women with dysbiosis exhibit vaginal discharge, irritation, and odour; and are identified with a condition referred to as vaginal dysbiosis. Although some women exhibit symptoms, the vast majority do not (Fettweis et al., 2014). However, both symptomatic and asymptomatic women are at an increased risk of a preterm birth, pelvic inflammatory disease, and infection with HIV, HPV, and other STIs (Sweet, 2000; van Schalkwyk and Yudin, 2015, Van de Wiigert and Jespers, 2017). The composition of the cervicovaginal microbiome is complex, fluctuating as a result of hormonal changes during the menstrual cycle, oral contraceptive use, breastfeeding, vaginal douching, diabetes mellitus, sexual activities and stress (Huang et al., 2014; Amabebe and Anumba, 2018).

Certain sexual behaviours and health practices have been linked to vaginal abnormalities. Recent sexual activities, multiple heterosexual sexual partners, vaginal intercourse following anal sex, unprotected sexual intercourse, lack of hormonal contraception, inconsistent condom use, sexual encounter with an uncircumcised partner, and use of intrauterine device are all examples of these behaviours (Cherpes et al., 2008; Bradshaw et al., 2012).

Hawes and Colleagues (1996) documented that having a new sexual partner was linked to a 2.5 times risk of having vaginal imbalance. Sexual intercourse with a male partner is regarded to pose a risk due to the semen's alkaline nature, which leads to a decrease of vaginal acidity shortly after sexual activity. Loss of acidity is likely to promote the proliferation of anaerobic bacteria and impair

the maintenance of H_2O_2 and *Lactobacilli* that produce lactic acid, resulting in an imbalanced vaginal environment (Cherpes et al., 2008). *Gardnerella vaginalis,* an anaerobic bacterium, produces vaginolysin, a pore-forming toxin that creates openings in the vaginal epithelium, impairing vaginal integrity and favouring infectious diseases (Macklaim et al., 2011; Rampersaud et al., 2011; Petrova et al., 2017).

Intravaginal applicants and practices such as feminine hygiene products, vaginal lubricant, vaginal douching, and gels may change the microbial composition and present varying impacts (Pyles et al., 2014; Li et al., 2014). Some douche products are comprised of surfactant detergents, which are substances that may inhibit *Lactobacilli* or disrupt cell membrane within the vaginal environment, resulting in the irritation of the mucosal surface, leading to an increased susceptibility to genital tract infections (Brotman et al., 2008; Pavlova et al., 2008).

1.2.2 VIROME

Human microbiome is comprised of other microorganisms other than bacteria. Human virome is a substantial and a complex human microbiome component that greatly influence a host physiology (Norman et al., 2015). Viruses are referred to as biological organisms that replicate only in the presence of a host cell (Abeles and Pride, 2014). They can be found everywhere as they infect almost every cell type (Abeles and Pride, 2014). The genome of viruses comprises RNA or DNA but not both, and the genome could be single or double stranded, contained within a protein capsid. The genomes encode proteins that control cell cycle and host gene expression, suppress, or disturb host immune response and encode microRNAs that control the processes of the cell (Wylie et al., 2014).

These dependent microorganisms interact with genetic materials of almost all cells on earth, including many bacteria found within human beings (Abeles and Pride, 2014). Regarding female anogenital tract, a study conducted by Wylie and Colleagues (2014) showed that vaginal specimens were dominated by papillomavirus, of which 37.5% of the specimens had one or more alphapapillomavirus with oncogenic type HPV 16 and HPV 18 included. In addition, this group proposed that viruses and bacteria may have a lively interaction within a microbiome. This is because alphapapillomavirus was more prevalent in individuals with a diverse bacterial group, with vaginal microbiome showing less than 85% of *Lactobacillus*-dominant bacteria and a high proportion of anaerobic bacteria (Wylie et al., 2014).

The virome population is extremely diverse like bacterial components of the microbiome (Wylie et al., 2014). Although there is little information on the vaginal virome, there are several persistent pathogenic viruses that are known to be present within female anogenital tract of some women such as Cytomegalovirus, Herpes simplex virus, and HPV.

1.2.2.1 Cytomegalovirus (CMV)

Cytomegalovirus is a causative agent of congenital infections, an opportunistic pathogen that is responsible for causing severe consequences in immunocompromised individuals (Ross et al., 2005; Adler, 2011). CMV is a double-stranded DNA virus that belongs to *Herpesviridae* family (Arvin et al., 2007; Azer and Limaiem, 2021). It is extremely complex and comprised of varying strains that have been distributed into four different groups based on their genomic differences in the amino-terminal region of the envelope glycogen (Ross et al., 2005). It can be detected in both immunocompromised and immunocompetent individuals; however, literature has shown that immunocompromised populations are more likely to harbour the viral infection (Dvorak, 2001).

This virus is present in approximately 40-100% of the population and can be found in the genital tract of women irrespective of whether they have vaginal imbalance or not. A study conducted by Ross and Colleagues (2005) documented that woman with vaginal imbalance have a higher prevalence of CMV and alteration. Furthermore, women with vaginal imbalance are more likely to have CMV than women without vaginal imbalance, irrespective of the infection being asymptomatic. The occurrence of vaginal imbalance among women may trigger replication of CMV within the genital tract (Ross et al., 2005).

An interaction between CMV and HPV also exists. The frequency of CMV in healthy women is 1-2%, however, it is present in 30% and 53% of patients with low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL), respectively (Mougin et al., 1995; Sekhon et al., 2004). Findings suggest that CMV is transmitted together with HPV or successively, enabling it to encourage dysplastic changes in the cervix that is induced by HPV (Sekhon et al., 2004). This is probably due to CMV replication involving the expression of immediate-early (IE1 and IE2) proteins, early, and late genes (Sekhon et al., 2004; Adler, 2011).

1.2.2.2 Herpes Simplex Virus (HSV)

Herpes simplex virus is a second most predominant sexually transmitted viral infection globally and the most common causative agent of genital ulcers (Fife et al., 2006; Masase et al., 2013). HSV-2 infection is estimated to affect 25% of women in the United States and more than 50% of women in Sub-Saharan Africa, respectively. However, HSV-1 infection and prevalence are increasing globally (Masase et al., 2013). HSV-2 infection relates to age, years of sexual intercourse, number of sexual partners in one's lifetime, and a history of STIs (Fife et al., 2006). Individuals with more than 10 sexual partners are at a higher risk of contracting genital herpes (Steben et al., 2008). Women are more likely to be at a higher risk of genital herpes infections (Masase et al., 2013; Alsamarai and Aljumaili, 2013). Furthermore, Women who are infected with HSV-2 have an increased prevalence of vaginal imbalance compared to those that are uninfected. It is assumed that vaginal microbiome is disturbed as a result of immune activation by HSV-2. Vaginal microbial imbalance also increases the chance of transmitting and acquiring HSV-1 and 2 (Allsworth et al., 2008). Women infected with HSV-2 are more likely to acquire HIV.

1.2.2.3 Human Papillomavirus (HPV)

Human papillomaviruses are a group of non-enveloped, circular, double stranded DNA (dsDNA) viruses with nearly 8 kilobases (kb) of genome in size (Morshed et al., 2014). HPV infects both mucosal and cutaneous epithelia (Fernandes and de Medeiros Fernandes, 2012). To date, over 228 HPV types have been identified (Jee et al., 2021) and based on their genetic and oncogenic potential, HPV is further stratified into two main groups: low risk (lr) and high risk (hr) types (Alemi et al., 2014).The most frequently encountered lr types are 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81. They are frequently linked with warts and are seldom isolated in cancerous lesions (Haedicke et al., 2013; Lu et al., 2015). HPV 6 and 11 account for about 90% of genital warts. The most common hrHPV types worldwide are 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 and they are associated with cervical cancer (CC) with hr types 16 and 18 being implicated in 70% of CC cases (Meloni et al., 2014; Lu et al., 2015).

Once the hr-HPV types are incorporated into the host genome, they exert their oncogenic effect by continuously expressing E4 and E5 HPV proteins (Haedicke et al., 2013). Persistent hrHPV types have been reported to be the causative agent of CC, the second most prevalent type of cancer among women in South Africa (Jemal et al., 2011). HPV has been defined as an AIDS

associated malignancy (Vangipuram et al., 2019). South Africa has the highest burden of HIV infection (20.6%) with women being the most infected population (Johnson et al., 2016; Simbayi et al., 2019). Women living with HIV are at a higher risk of contracting HPV, with a high rate of hrHPV persistence, which increases the chance of developing abnormalities in their cervix (Lima et al., 2014).

1.2.3 THE SIGNIFICANCE OF STUDYING CERVICOVAGINAL VIROME AND BACTERIOME

Arrival of novel sequencing methodologies such as the next generation deep sequencing has greatly improved research in the microbiome. These studies which dig deep into the genetic diversity of the human microbiome, collectively shed light on the complexity of cervicovaginal virome and bacteriome in both health and sickness. This is crucial for HIV-infected persons, as HIV/AIDS has been related to altered enteric and blood microbiomes (Li et al., 2013; Monaco et al., 2016). Additionally, there also seem to be an interaction between cervicovaginal virome and bacteriome of which we do not fully know.

1.3 STUDY RATIONALE

It is estimated that approximately 604,000 women worldwide develop CC annually, with more than 342,000 cases of death (Sung et al., 2020). In South Africa, 5,743 new cases of CC are reported yearly, with 3,027 associated deaths (Nyangu and Moteane, 2021). Persistent hrHPV infection is a principal risk factor for developing CC (Curty et al., 2017). Several risk factors influence the persistence of HPV infection such as HIV, contraceptive use, smoking, and cervicovaginal microflora imbalance (Mitra et al., 2016).

Lactobacillus spp. dominate the normal cervicovaginal flora and produces hydrogen peroxide (H_2O_2) and lactic acids that inhibit the growth of pathogenic microbes (Curty et al., 2017). In an imbalanced state, however, there is a significant decrease of *Lactobacillus spp*. and an increase in bacterial diversity, with anaerobic bacteria present in high proportion (Ravel et al., 2011; Liu et al., 2013; Borgdorff et al., 2016). This microbial imbalance increases the likelihood of premature birth, sexually transmitted infections, and pelvic inflammation in women (Brotman et al., 2011).

The cervical microbiome has been shown to have five district community states (CSTs) spanning from one to five, according to previous studies (Ravel et al., 2011). The *Lactobacillus spp. (L. crispatus, L. gasseri, L. iners*, or *L. jensenii*, respectively) dominate CSTs one, two, three and five; however, CST four is distinguished by a high diversity and prevalence of anaerobic bacteria, such as *Pretovella, Atopobium, Streptococcus, Gardnerella*, and *Ureaplasma* (Ravel et al., 2011). Brotman and Colleagues (2019) observed that women with a cervicovaginal microbiota that was exceptionally diverse or dominated by *L. iners* were more likely to be HPV-infected, while women with a microbiota dominated by *L. gasseri* cleared HPV infection more quickly.

Community state type four in the cervicovaginal niche has been linked with an increased likelihood of developing persistent HPV infection and, as a result, cervical lesions (Mitra et al., 2016; Kyrgiou et al., 2017). *Gardnerella vaginalis,* for instance, which is prevalent in CST four, can secrete sealidase which degrades vaginal mucus by cleaving its glycoprotein (Amabebe and Anumba, 2018). The vaginal mucus contains a protein known as mucin that provides a physical barrier to the mucosal vaginal surface and hinders bacterial-host interactions; nevertheless, its degradation compromises the mucosal barrier and favours genital tract infections (Mitra et al., 2016; Amabebe and Anumba, 2018).

Additionally, Gossman and Colleagues (2017) demonstrated that women who have CST four produce an increased number of inflammatory cytokines, that promotes the recruiting of stimulated CD4+ CCR5+ cells to the vaginal mucosa, increasing the likelihood of HIV infection. It has been found that women with CST four are more susceptible to HPV infection because of cytokine production, such as TNF-alpha (Audirac-Chalifour et al., 2016). The relationship between CSTs with other viruses, on the other hand, is poorly understood.

Thus, it is critical to screen and genotype HPV virome and bacteriome in HIV-infected women in order to understand what is circulating and the relationship between HPV virome and bacteriome. This could pave the way for the use of interacting microorganisms as diagnostic markers for cytological abnormalities indicative of vaginal disease state.

1.4 RESEARCH QUESTIONS, HYPOTHESIS AND OBJECTIVES

1.4.1 RESEARCH QUESTIONS

The research questions arising from literature review comprised:

1. What is the burden and diversity of HPV virome in HIV-infected women?
2. What is the burden and diversity of bacteriome in HIV-infected women?
3. What is the relational occurrence of virome and bacteriome according to HIV status?

1.4.2 HYPOTHESIS

The current study hypothesized that HIV/HPV co-infected women have an increased diversity of cervicovaginal HPV virome and bacteriome.

1.4.3 MAIN OBJECTIVE OF THE STUDY

The main objective of the study was to characterize cervicovaginal HPV virome and bacteriome in HIV-infected women from selected health care facilities in Northern South Africa.

1.4.4 SPECIFIC OBJECTIVES

The specific objectives of the study were:

1. To determine HPV virome in HIV-infected women and HIV-noninfected women.
2. To determine bacteriome in HIV-infected women and HIV-noninfected women.
3. To determine the relational occurrence of virome and bacteriome according to HIV status.

CHAPTER TWO: MATERIALS AND METHODS

2.1 ETHICAL CONSIDERATIONS

The ethical approval for the use of total DNA obtained from cervical specimens emanating from a prior study was obtained from the Human and Clinical Trial Research Ethics Committee of the University of Venda (Reference number SMNS/20/MBY/04/0104). No personal identifiers of the individuals who provided specimens was used in the current study.

2.2 STUDY SITE AND STUDY POPULATION

A total of 150 archived cervical DNA specimens were used for this current study. Out of this, three different study populations were designated namely: HIV/HPV co-infected women, HIV-infected HPV-noninfected women, and HPV-infected HIV-noninfected women. Each of this study population comprised DNA specimens from 50 women. The individuals who provided specimens were recruited from four health centres namely: Rethabile Community Health Centre, Polokwane; Seshego Clinic; Thohoyandou Integrated Health Centre; and Donald Frazer Hospital. These sites are situated in Limpopo Province, in the northern part of South Africa. Figure 2.1 shows the spatial location of these sites.

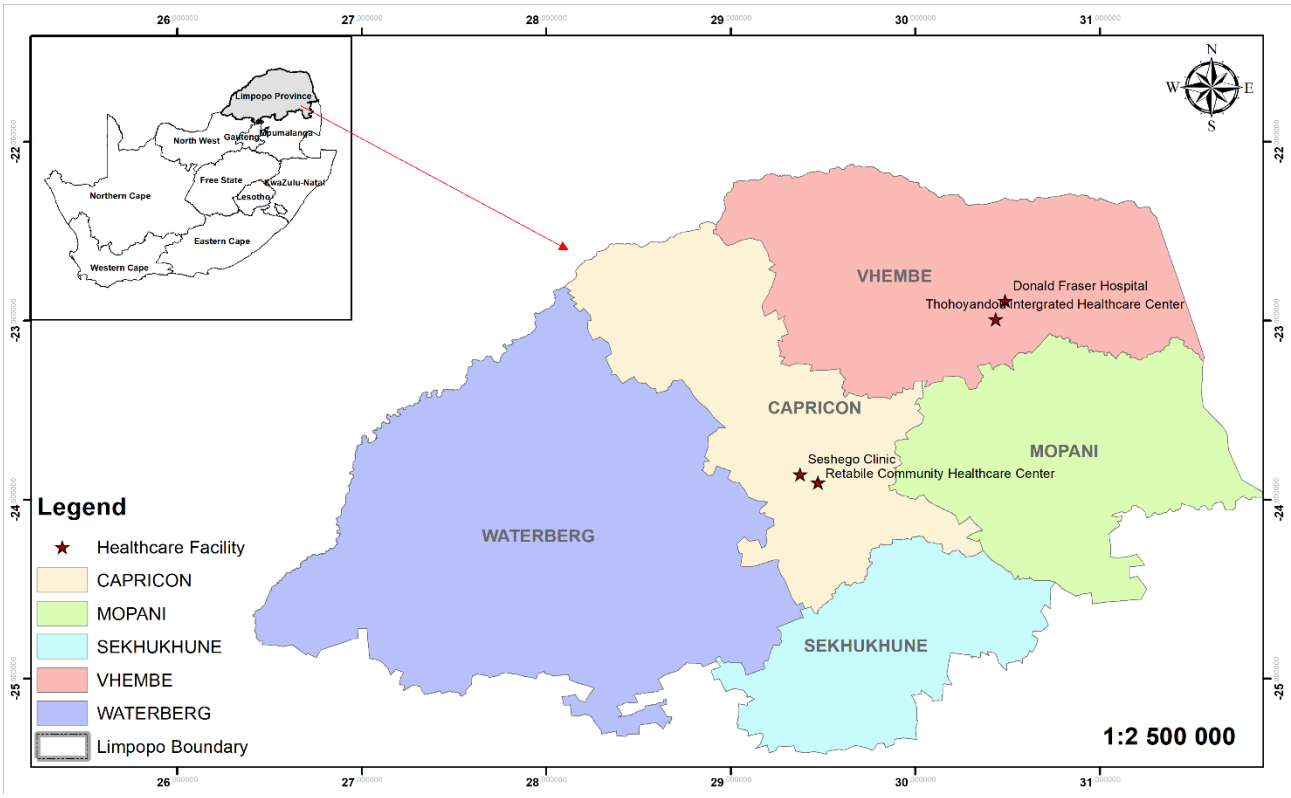

Figure 2.1: A map showing spatial location of the four study sites (Rethabile community health centre and Seshego clinic in Capricorn district; Thohoyandou Integrated Health Centre, and Donald Frazer Hospital in Vhembe district) indicated with red asterisks. On the left, the upper box indicates the location of Limpopo Province within South Africa, and the middle map shows a reduced map of Limpopo Province with study sites. The map legend is on the lower left corner while the map scale is on the lower left corner.

2.3 DETERMINATION OF HPV VIROME IN HIV-INFECTED WOMEN AND HIV-NONINFECTED WOMEN

APPROACH:

2.3.1 PROCESSING AND ISOLATION OF TOTAL DNA

Following the manufacturer's instructions, the isolation of total DNA from cervical pellets was performed using QIAamp DNA mini kit. The tubes were centrifuged and re-suspended into phosphate-buffer saline (PBS). This was followed by adding QIAGEN Proteinase K. To obtain a maximum yield of DNA, the tubes were incubated at 56ºC for 10 minutes. The tubes were then centrifuged to remove any remaining drips from the lid. Two hundred microlitres (200µl) of ethanol (100%) was added and mixed for 15s. Following mixing, the tubes were centrifuged to remove the drips from inside of the lid. After carefully applying the mixture to the QIAamp spin columns, it was centrifuged for 1 min. The QIAamp spin column was then transferred to a clean 2ml collection tube, and the filtrate was discarded. Five hundred microliters (500µl) of AW1 and AW2 were added subsequently to the DNA centrifugated at a speed of 8000 and 14000 rpm respectively. One hundred and thirty microliters (130µl) of elution buffer (AE) were directly added to a QIAamp spin column, this was the only step that was adjusted; the tubes were then incubated at room temperature and centrifuged to elute the trapped DNA.

2.3.2 ENRICHMENT OF DNA THROUGH ROLLING CYCLE AMPLIFICATION

To detect circular DNA molecules, DNA specimens from each of the study groups were exposed to rolling cycle amplification (RCA) utilizing the illustra TempliPhi Amplification kit (GE Health Life Science, Piscataway, NJ, USA) following the manufacturer's protocol. One microliter (1µl) of the starting material was added to 5µl of sample buffer. The mixture was heated at 95ºC for 3 minutes. After cooling to 4ºC, the mixture was combined with a 5 µl of a master mix obtained from a premix of 5µl reaction buffer and 0.2µl of enzyme mix, and thereafter incubated at 30ºC for 18 hours. Following incubation, DNA polymerase was deactivated by heating at 65ºC for 10 minutes. The amplification products were resolved electrophoretically on a 0.6% agarose gel, at 100V for 40 minutes and were visualized under UV transillumination.

2.4 DETERMINATION OF BACTERIOME IN HIV-INFECTED WOMEN AND HIV-NON-INFECTED WOMEN

2.3.2 BACTERIAL 16S rRNA POLYMERASE CHAIN REACTION

To detect bacterial species, a two-round conventional PCR was employed using the 338F(5'-ACTYCTACGGRAGGCWGC-3') and 1061R primers (5'-CRRCACGAGCTGACGAC-3') that target the V3-V6 segment (~700bp-4500kb) of the bacterial 16S rRNA gene. In a total of 25 µl, the PCR reaction contained 1x of buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.12 µM of each primer, and 0.03u/µl of Taq Platinum DNA polymerase. The cycle conditions used to amplify bacteria are listed in Table 2.1.

Table 2.1: Cycling conditions for bacterial 16S rRNA gene amplification in the V3-V6 segment.

CYCLING CONDITIONS	TEMPERATURE (°C)	TIME (min/s)
Initial denaturation	95	5 min
Amplification 35 Cycles		
Denaturation	95	30s
Annealing	59	30s
Elongation	72	1min
Final elongation	72	6 min

2.3.3 PURIFICATION AND QUANTIFICATION OF AMPLIFIED PRODUCTS

Purification of amplification products generated from rolling circle amplification and PCR was performed following the manufacturer's protocol using a Marchery Nagel NGS purification kit. Purified products were quantified following the manufacturer's procedure using a dsDNA high sensitivity assay kit and Qubit 3.0 Fluorometer.

2.3.4 NEXT GENERATION SEQUENCING

2.3.4.1 Library preparation and sequencing

The amplicons were diluted into a final concentration of 2ng of normalized genomic DNA and were used as initial input for tagmentation. To fragment the individual amplicons, Nextera XT DNA Sample Preparation kit comprising the transposon technology was used to randomly fragment DNA and add the sequencing adapter in the process. Following tagmentation, amplification of libraries was performed to add the molecular tags. To remove all the unadded tags, libraries were cleaned up using Marchery Nagel NGS purification kit following the manufacturer's protocol. This was followed by quantification using Qubit ds high sensitivity kit. To verify the average size of the fragmented amplicons, amplicons were subjected to a 1% E-gel electrophoresis. Depending on the library size, all the specimens were manually pooled at an equimolar ratio of 1 nM per specimen. The pooled libraries were denatured with 0.1 N NaOH and diluted to a final volume of 500 µl at 1.8 pM, following the Illumina MiniSeq instructions. As a control, 20% of the denatured PhiX genome was added to the dilution. The libraries were then loaded into MiniSeq High Output cartridge 300 cycles and sequenced using the Local Run Manager option on the MiniSeq instrument.

2.3.5 SEQUENCE ANALYSIS

2.3.5.1 Sequence quality evaluation

The Illumina MiniSeq platform demultiplexed the sequences automatically during data processing and end pairing. For each specimen, FastQ files containing the two paired end reads were created. The FastQC tool was used to ensure the sequences were of high quality.

2.3.5.2 Virome and bacteriome sequence data analysis

The sequences were assigned to viral families and genera using Dragen metagenomics online tool with BaseSpace Sequence hub. Bacteriome sequence data were assigned and classified according to the vaginal community state types using Dragen metagenomics with BaseSpace Sequence hub tool.

2.9 DETERMINATION OF THE RELATIONAL OCCURRENCE OF VIROME AND BACTERIOME ACCORDING TO HIV STATUS

APPROACH:

2.9.1 STATISTICAL ANALYSIS

Graph pad prism version 9.3.1, an online tool was utilized to determine the relational occurrence of virome and bacteriome according to HIV status. Statistical significance was defined as a P-value of <0.05.

CHAPTER THREE: RESULTS

3.1 THE STUDY POPULATION'S CHARACTERISTICS

This study enrolled a total of 150 women, with 50 participants each from HIV/HPV co-infected women; HIV-infected, HPV-noninfected women; and HPV-infected, HIV-noninfected women. Complete demographic data was available for 147/150 (98%) of the study participants. Of note, some participants were not comfortable in disclosing some of the required information. The age range of the participants was 18-84 years. More than half of the participants reported to be single (51%), while widows were the least represented group (3%). Additionally, nearly all the study participants (95%) were not vaccinated against HPV infection. Sixty percent (60%) reported to have had their sexual debut at between 12-18 years old. Characteristics of the study participants are shown in table 3.1.

Table 3.1: Demographic and clinical characteristics of the study participants.

Characteristics	**Distribution (%)**		
	HIV/HPV co-infected (n=47)	**HIV+/HPV- (n=50)**	**HIV-/HPV+ (n=50)**
Age (years)			
Range	24-63	18-84	19-68
Mean	41,9	38,2	44,2
Median	42	40	46
Marital status			
Single	25 (53%)	20 (40%)	31 (62%)
Married	19 (40%)	23 (46%)	16 (32%)
Divorced	2 (4%)	2 (4%)	3 (6%)
Widow	0	5 (10%)	0
No response	1 (2%)		1 (2%)
Highest level of education			
Grade 1-7	9 (19%)	7 (14%)	3 (6%)
Grade 8-12	29 (61%)	32 (64%)	42 (84%)
Varsity/college	9 (19%)	7 (14%)	5 (10%)
Not educated	0	4 (8%)	0
Occupation			
Employed	23 (49%)	27 (54%)	18 (36%)
Unemployed	24 (51%)	22 (44%)	31 (62%)
No response	0	0	1 (2%)
Smoking status			
Smokers	2 (4%)	3 (6%)	0
Non-smoker	44(94%)	47 (94%)	50 (100%)
No response	1 (2%)	0	0
Age of first sexual intercourse			
13-18	23 (49%)	29 (58%)	37 (74%)
19-24	14 (30%)	14 (28%)	13 (26%)
>25	3 (6%)	5 (10%)	0
No response	10 (21%)	2 (4%)	0
Number of sexual partners (s)			
0	5 (11%)	2 (4%)	0
1	30 (64%)	41 (82%)	50 (100%)
2-4	4 (9%)	3 (6%)	0
Celibate	2 (4%)	1 (2%)	0
No response	9 (19%)	3 (6%)	
HPV vaccination status			
Never vaccinated	42 (89%)	48 (96%)	49 (98%)
No response	5 (11%)	2 (4%)	0

3.2 DETERMINATION OF HPV VIROME IN HIV-INFECTED WOMEN AND HIV-NONINFECTED WOMEN

3.2.1 ENRICHMENT OF CIRCULAR DNA MOLECULES USING RCA

Rolling circle amplification (RCA) was performed for all 150 DNA specimens. Of these, 95 specimens (63%) were successfully enriched. Out of 95 specimens, 40 (42%) were from HIV/HPV co-infected women, 38 (40%) from HPV-infected, HIV-noninfected women; and 17 (18%) were from women who were HIV-infected, HPV-noninfected. Figure 3.1 shows a representative gel picture of the amplified products.

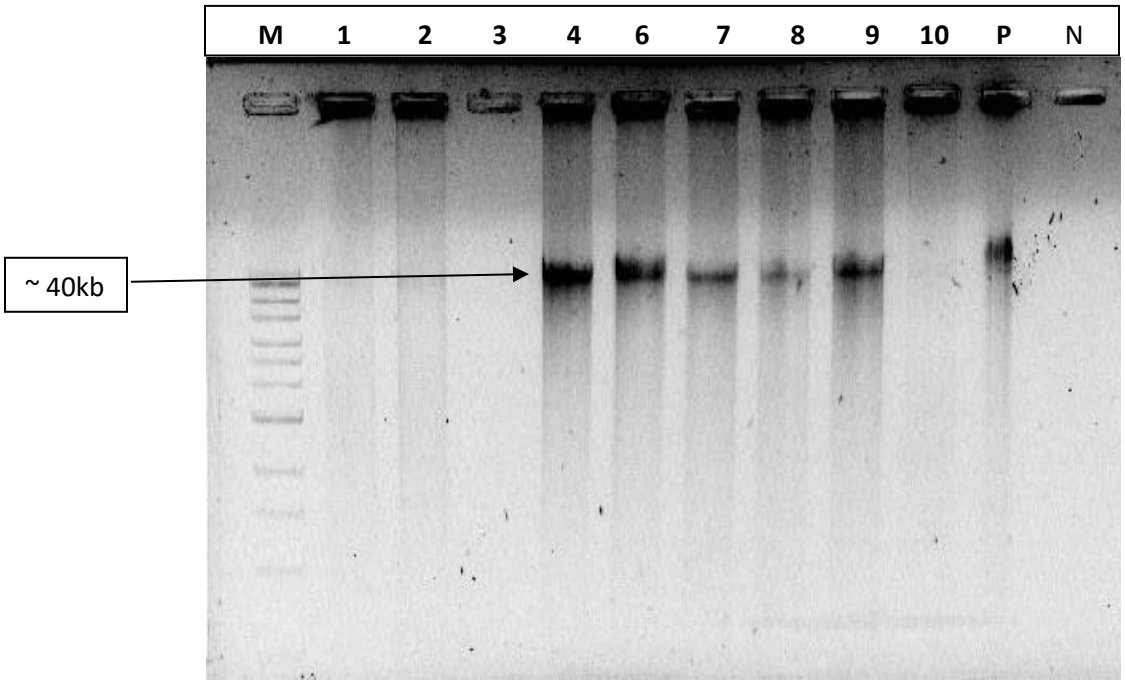

Figure 3.1: Representation of electrophoresed RCA amplicons loaded in a 0.6% ethidium bromide agarose-stained gel. **M:** molecular marker- 1 kb extend, **1-9** are test specimens, **P**: positive control (E-coli plasmid-pUC19), **N**: negative control (nuclease-free water).

3.2.2 SEQUENCING AND SEQUENCE READ QUALITY CONTROL

Of the 95 enriched DNA specimens, 74 (78%) were successfully sequenced. Quality control (QC) of the sequence reads is important to have reliable sequences for analysis. FASTQC assists in determining the quality score, the length of the reads, and how good the sequence reads are. Sequence reads are referred to as short fragments of genetic information, stored in a FASTQ file.

FASTQ reports QC metrics, with a traffic light warning system: normal (green), abnormal (orange), or bad (red). These can be used to quickly identify common problems with NGS data and QC individual sequencing runs before starting a biological analysis. In figure 3.2, the X-axis shows the individual bases for the reads, while the Y-axis shows the quality scores, which should be above 20%.

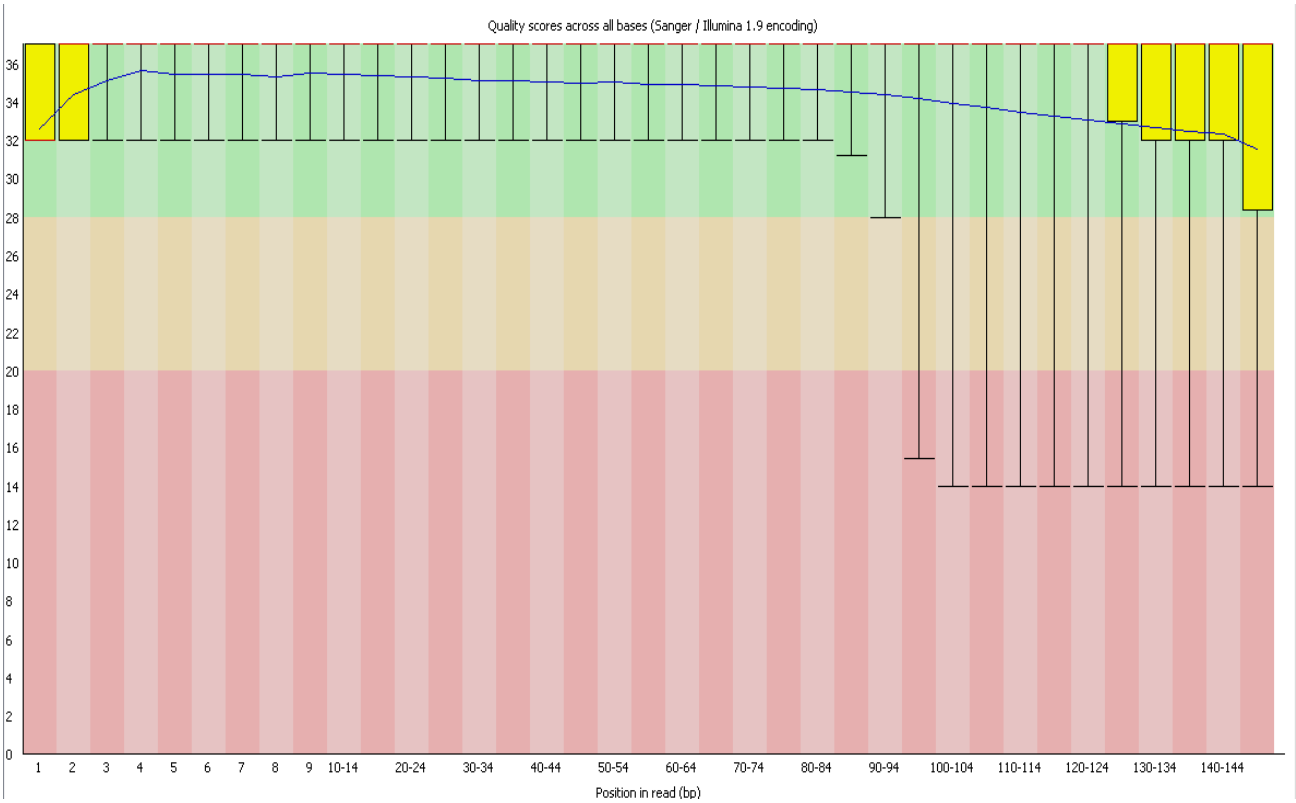

Figure 3.2: Sequence quality of the samples after sequencing using FastQC platform

The sequenced specimens (n=74) had reads that were above the threshold of > 100,000 reads per specimen, which is considered sufficient for metagenomics survey by Illumina. The average number of reads that were obtained per specimen was 308,584 (Range:100,920 - 536,522).

3.2.3 CHARACTERIZATION OF VAGINAL VIROME

The vaginal virome was characterized and reported as per the three study groups (HIV/HPV co-infected women; HIV-infected, HPV-noninfected women; and HPV-infected, HIV-noninfected women).

3.2.3.1 HIV/HPV co-infected study group

Reliable reads for analysis were available for 34 women who were HIV/HPV co-infected. Among this group, seven eukaryote-infecting viral families were detected: *Papillomaviridae, Herpesviridae, Anelloviridae, Poxviridae, Phycodnaviridae, Mimiviridae*, and *Maiselleviridae*. The prevalence of the eukaryote-infecting viral families among this study group are shown in table 3.2. *Papillomaviridae* was the most prevalent, and this was statistically significant (P<0.0001), followed by *Herpesviridae*, *Phycodnaviridae*, *Poxviridae*, and *Mimiviridae*. *Maiselleviridae* and *Anelloviridae* were the least prevalent viral families.

Table 3.2: The prevalence of the eukaryote-infecting viral families detected among HIV/HPV co-infected study group.

VIRAL FAMILIES	**PREVALENCE (%)**
Papillomaviridae	(14/34; 41%)
Herpesviridae	(12/34; 35%)
Phycodnaviridae	(12/34; 35%)
Poxviridae	(10/34; 29%)
Mimiviridae	(7/34; 20%)
Maiseilleviridae	(3/34; 9%)
Anelloviridae	(2/34; 6%)

In *Papillomaviridae* family, the most prevalent genotypes detected in order of decreasing frequency were HPV 16 (8/14; 57%), HPV 32 (7/14; 50%), HPV 53 and 85 (6/14; 43%), HPV 26 (5/14; 36%), and HPV 34 (4/14; 29%); all belonging to genus alphapapillomavirus.

In *Herpesviridae* family, only two human herpesviruses were detected: Herpes simplex virus type 2 (2/12; 17%) and Epstein Barr virus (1/12; 8%).

In *Anelloviridae* family, multiple Anellovirus types belonging to genus Alphatorquevirus were detected; this includes Torque teno virus types 2, 3, 4, 10,11,12, 17, 19, 22, 29 in one participant (ORHC-005) while the other participant (ORHC-055) had Torque teno virus types 6, 10, 11, 17.

Orphovirus IHUMI-LCo2 (3/12; 25%), Tokyovirus (3/3; 100%), Christoneura blennis virus (2/10; 20%) and Cafeteria roenbergensis virus (2/7; 28%) were the most frequently detected viral species in *Phycodnaviridae*, *Maiselleviridae*, *Poxviridae* and *Mimiviridae* family, respectively. The viral/species of the eukaryote-infecting viral families that were detected among this study group are shown in table 3.3.

Table 3.3: The viral species/types of the eukaryote-infecting viral families detected among HIV/HPV co-infected study group.

EUKARYOTE-INFECTING VIRAL FAMILIES	SPECIES
Papillomaviridae	HPV 16 (8/14; 57%), HPV 32 (7/14; 50%), HPV 53 and 85 (6/14; 43% each), HPV 26 (5/14; 36%), HPV 34 (4/14; 29%), HPV 6, HPV 10; and HPV 90 (3/14; 21% each); HPV 7 and HPV 92 (2/14; 14% each); HPV 5, HPV 54, HPV 71, HPV 131, HPV 129, HPV 154, HPV 156, and HPV 166 (1/14; 14% each)
Herpesviridae	Herpes simplex virus type 2 (2/12; 17%) Epstein Barr virus (1/12; 8%)
Anelloviridae	Torque teno virus types 2, 3, 4, 6, 12, 19, 22, 29 (1/2; 50% each) and Torque teno virus types 10, 11, 17 (2/2; 100% each)
Phycodnaviridae	Orphovirus IHUMI-LCo2 (3/12; 25%), Phaeocystis globosa virus, Ostrecoccus virus, Micromonas virus, Chrysochromulina ericina virus, and Heterosigma akashiwo virus 01 (1/12; 8% each)
Maiseilleviridae	Tokyovirus (3/3; 100%)
Poxviridae	Christoneura blennis virus (2/10; 20%), Swine poxvirus, Fowl pox virus, Variola virus, and Ny-014 pox virus (1/10; 10% each)
Mimiviridae	Cafeteria roenbergensis virus (2/7; 28%), Acanthoeba polyphoga, Mimivirus, Moumouvirus, and Megavirus chiliensis (1/7; 14% each)

Four Prokaryote-infecting viral families referred to as bacteriophages were identified: *Myoviridae* (31/34, 91%,) *Siphoviridae* (27/34, 79%) and *Podoviridae* (17/34, 50%), with *Myoviridae* being the most prevalent, and this was statistically significant (P<0.0001). More than one viral species of the *Myoviridae* and *Siphoviridae* family were detected per participant of this study group. The viral species of the prokaryote-infecting viral families that were frequently detected among HIV/HPV co-infected study group are shown in table 3.4.

Table 3.4: The viral species of the prokaryote-infecting viral families detected among HIV/HPV co-infected study group.

BACTERIOPHAGE VIRAL FAMILIES	VIRAL SPECIES
Myoviridae	Flavobacterium virus (11/31; 35%), Tequatrovirus (9/31; 29%), Clostridium phage (3/31; 10%), Bacillus virus B (2/31; 6%).
Siphoviridae	Rhodobacter virus RcCronus (10/27; 37%), Lactobacillus phage (5/27; 18%), Gordonia phage (4/27; 15%), Streptococcus phage (4/27; 15%), Escherichia phage (3/27; 11%); Salmonella phage (3/27; 11%).
Podoviridae	Uncultured crAss phage (4/17; 23%)

3.2.3.2 HPV-infected, HIV-noninfected women

Reliable reads for analysis were available for 26 women who were HPV-infected, HIV-noninfected. Among this group, four eukaryote-infecting viral families were detected: Papillomaviridae, *Anelloviridae*, *Phycodnaviridae*, *Poxviridae*, and *Herpesviridae*. The prevalence of the eukaryote-infecting viral families among HPV-infected, HIV-noninfected study group are shown in table 3.5. *Papillomaviridae* was the most prevalent, and this was statistically significant (P<0.0001), followed by *Anelloviridae*, and *Phycodnaviridae*. *Poxviridae and Herpesviridae* were the least prevalent viral families detected.

Table 3.5: The prevalence of the eukaryote-infecting viral families detected among HPV-infected, HIV-noninfected study group.

EUKARYOTE-INFECTING VIRAL FAMILIES	PREVALENCE (%)
Papillomaviridae	31% (8/26)
Anelloviridae	15% (4/26)
Phycodnaviridae	15% (4/26)
Poxviridae	8% (2/26)
Herpesviridae	8% (2/26)

In *Papillomaviridae* family, the most prevalent HPV types were HPV 16 (5/8, 62%), HPV 26 (3/8, 37%) followed by HPV 32, 34, 53 and 54 (2/8, 25% each), all belong to genus alphapapillomavirus.

In *Anelloviridae* family, Torque teno virus (types 11, 12, 13 and 19) belonging to genus Alphatorquevirus and torque teno midi virus 8 belonging to genus gamma torgue virus were detected.

In *Herpesviridae* family, Herpes simplex virus type 2 (2/2; 8%) and Human cytomegalovirus (1/2; 4%) were detected, with Human cytomegalovirus detected in one participant (ORHC-195) with a relative abundance of 55%.

In *Phycodnaviridae* and *Poxviridae* families, viral species such as Chrysochromulina ericina virus (1/4; 25%) and Vacunia virus (1/2; 50%) were detected respectively. The viral species/types of the eukaryote-infecting viral families that were detected among HPV-infected, HIV-noninfected study group are shown in table 3.6.

Table 3.6: The viral species/types of the eukaryote-infecting viral families detected among HPV-infected, HIV-noninfected study group.

EUKARYOTE-INFECTING VIRAL FAMILIES	VIRAL SPECIES/TYPES
Papillomaviridae	HPV 16 (5/8; 62%), HPV 26 (3/8; 37%) followed by HPV 32, 34, 53, 54 and 90 (2/8; 25% each), HPV 7, 30, 54, 66, 87, and 144 (1/8; 13% each).
Anelloviridae	Torque teno virus types 11, 12,13, 19 and Torque teno midi virus 8 (1/4; 25% each)
Herpesviridae	Herpes simplex virus type 2 (2/2; 8%) Human cytomegalovirus (1/2; 4%)
Phycodnaviridae	Chrysochromulina ericina virus (1/4; 25) Only Syngin Nebraska (1/4; 25%) Ostrecoccus lucimarinus virus (2/4; 50%)
Poxviridae	Vacunia virus (1/2; 50%) Murmansk poxvirus (1/2; 50%)

Prokaryote-infecting viral families detected were *Myoviridae* (6/26; 23%), *Siphoviridae* (5/26; 19%), and *Podoviridae* (4/26; 15%); with *Myoviridae* being the most prevalent (*P*=0.0005). The viral species of the prokaryote-infecting viral families that were frequently detected among HPV-infected, HIV-infected study group are shown in table 3.7.

Table 3.7: The viral species of the prokaryote-infecting viral families detected among HPV-infected, HIV-noninfected study group.

Prokaryote-infecting viral families	Viral Species (%)
Myoviridae	Shigella phage (1/6; 17%) Flavobacterium (1/6; 17%) Arthrobacter virus Sonny (1/6; 17%) Klebsiella virus Matisse (1/6; 17%) Lactobacillus phage S40 (1/6; 17%) Shigella phage (1/6; 17%)
Siphoviridae	Rhodobacter virus RcCronus (3/5; 60%) Flavobacterium virus FCV1 (1/5; 20%) Caulobacter phage CcrColossus (1/5; 20%) Streptomyces virus Bing (1/5; 20%)
Podoviridae	E-coli pallock (1/4; 25%) Dinoroseobacter virus (1/4; 25%) unclassified crAss-like virus (1/4; 25%) Rosenblumvirus (1/4; 25%)

3.2.3.3 HIV-infected, HPV-noninfected women

Reliable reads for analysis were available for 11 women who were HIV-infected, HPV-noninfected. Among this group, a small group of viral families was detected: *Herpesviridae*, *Phycodnaviridae*, *Mimiviridae*, *Siphoviridae* and *Myoviridae*. The prevalence of the prokaryote and the eukaryote-infecting viral families among HIV-infected, HPV-noninfected study group are shown in table 3.8.

Table 3.8: The prevalence of the viral families detected among HIV-infected, HPV-noninfected study group.

EUKARYOTE-INFECTING VIRAL FAMILIES	**PREVALENCE (%)**
Herpesviridae	9% (1/11)
Phycodnaviridae	9% (1/11)
Mimiviridae	9% (1/11)
PROKARYOTE-INFECTING VIRAL FAMILIES	
Siphoviridae	27% (3/11)
Myoviridae	27% (3/11)

In *Herpesviridae*, *Phycodnaviridae* and *Mimiviridae* families; Herpes simplex virus type 2 (1/1; 100%), Orpheovirus IHUMI-LCC2 (1/1; 100%) and Megavirus chiliensis (1/1; 100%) were detected respectively.

In *Siphoviridae*, viral species such as Caulobacter virus roque (1/3; 33%), Lactobacillus phage LV-1 (1/3; 33%) and Rhodobacter phage RcRhea (1/3; 33%) were detected. In *Myoviridae* family, Flavobacterium virus FCV1 (1/3; 33%), Escherichia virus VR20 (1/3; 33%), Morganella phage Vb-MmoM-MP1 (1/3; 33%) were some of the viral species that were detected in the family. The viral species/types of the eukaryote and the prokaryote-infecting viral families that were detected among HIV-infected, HPV-noninfected study group are shown in table 3.9.

Table 3.9: The viral species/types of the eukaryote and prokaryote-infecting viral families detected among HIV-infected, HPV-noninfected study group.

EUKARYOTE-INFECTING VIRAL FAMILIES	**VIRAL SPECIES/TYPES**
Herpesviridae	Herpes simplex virus type 2 (1/1; 100%)
Phycodnaviridae	Orpheovirus IHUMI-LCC2 (1/1; 100%)
Mimiviridae	Megavirus chiliensis (1/1; 100%)
PROKARYOTE-INFECTING VIRAL FAMILIES	
Siphoviridae	Caulobacter virus roque (1/3; 33%) Lactobacillus phage LV-1 (1/3; 33%) Rhodobacter phage RcRhea (1/3; 33%) Streptococcus phage 5093 (1/3; 33%) Vibrio phage (1/3; 33%)
Myoviridae	Flavobacterium virus FCV1 (1/3; 33%) Escherichia virus VR20 (1/3; 33%) Morganella phage Vb-MmoM-MP1 (1/3; 33%) Aeromas phage (1/3; 33%) Citrobacter virus merlin (1/3; 33%) Tequatrovirus (1/3; 33%)

3.2.3.4 The prevalence of high risk/probable hrHPV types according to HIV status

More than one high risk or probable hrHPV types were detected in 34% of the study participants. HPV 16 was the most predominant type in both HIV-infected women and HIV-noninfected women (Figure 3.3). Overall, the hrHPV types were more prevalent in HIV-noninfected women when compared to HIV-infected women, however, this was not statistically significant (P=0.2832; χ^2=3.80). When the hrHPV or probable hrHPV types were analyzed individually, only the occurrence of HPV 54 was statistically significant in HIV-noninfected women as compared to HIV-infected women (P<0,0003; χ^2=12.981; Figure 3.3).

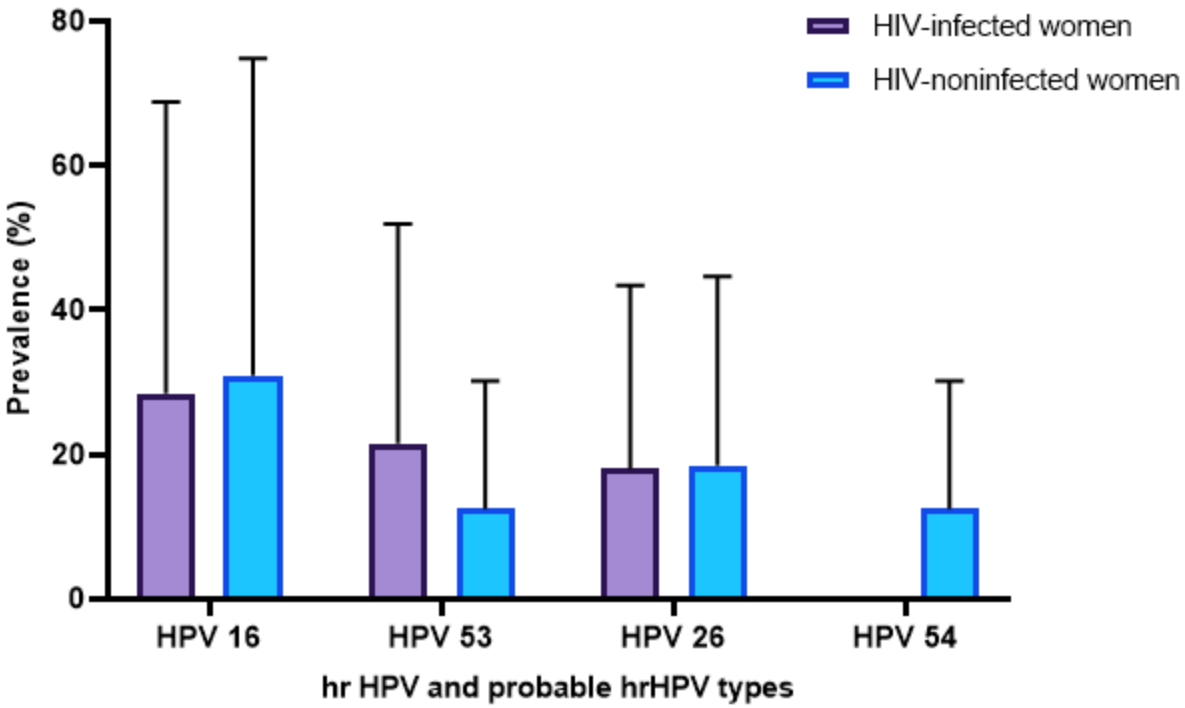

Figure 3.3: A bar graph showing a mean prevalence of hrHPV and probable hrHPV types according to HIV status. HPV 16 and 54 are hrHPV types and HPV 26 and 53 are probable hrHPV types. Only HPV 54 occurred significantly in HIV-infected women as compared to HIV-noninfected women (*P*<0,0003). Purple bars indicate HIV-infected women, and blue bars indicate HIV-noninfected women.

3.3 DETERMINATION OF BACTERIOME FROM HIV-INFECTED AND HIV-NONINFECTED WOMEN

3.3.1 AMPLIFICATION AND SEQUENCING OF BACTERIAL 16S rRNA GENE

A total of 79/150 (53%) specimens screened for 16S rRNA fragment were successfully amplified. Out of the 79 specimens, 24 (30%) were from HIV/HPV co-infected women, 21 (27%) from HPV-infected, HIV-noninfected women; and 34 (43%) from HIV-infected HPV-noninfected women.

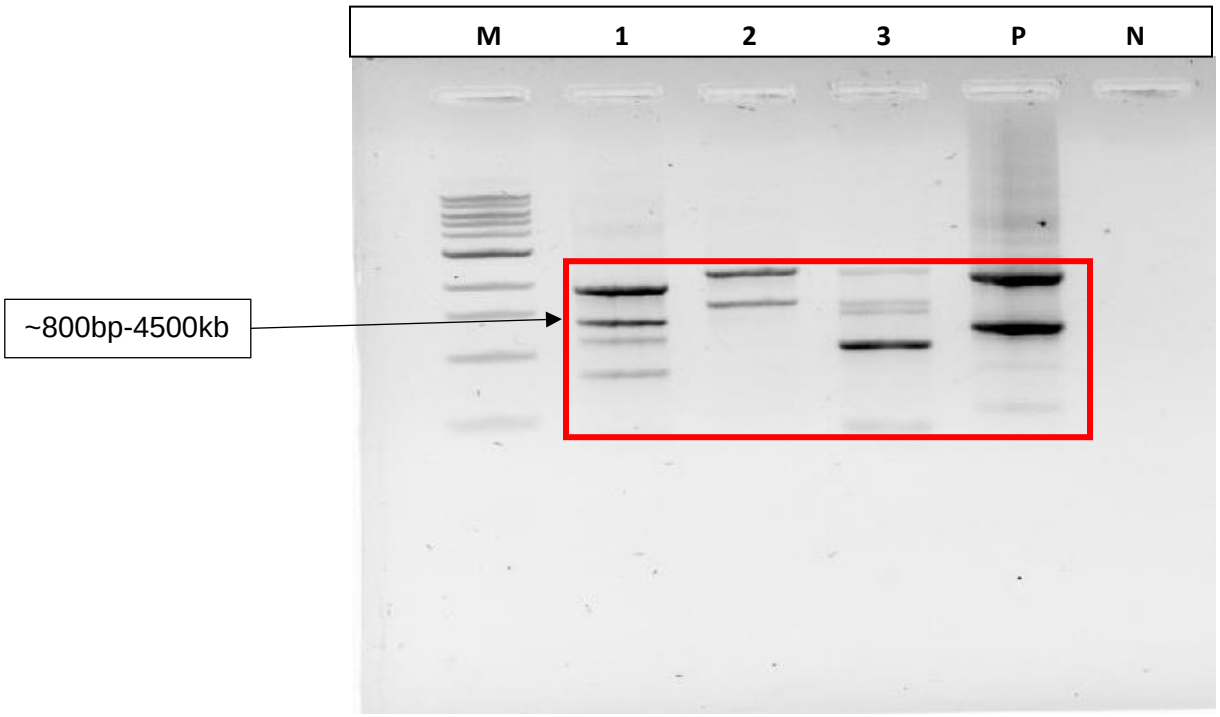

Figure 3.4: Amplification of bacterial 16S rRNA gene using a two-round PCR protocol (338F and 1061R universal primers) from cervical specimens. A red box shows the fragment size between approximately 800bp-4500kb of amplicons obtained and subjected to downstream analysis. **M:** molecular weight marker (1 kb), **1-3** refers to the test specimens, **P:** positive control (E-coli) **N:** negative control (nuclease-free water).

3.3.2 SEQUENCING AND PRE-PROCESSING OF THE SEQUENCE READS

Out of the 79 DNA specimens amplified, 60 (78%) amplicons were successfully sequenced. The sequence reads were assessed for quality using FastQC tool as was done for the virome. Ninety-eight percent (98%, 59/60) of the sequenced libraries had a threshold of >100,000 reads per

specimen. The average number of reads obtained was 258,268 (range: 127,124-888,232). Of note, 8/60 (13%) sequences were of bad quality and thus, were excluded from the final analysis.

3.3.3 BACTERIAL COMMUNITY STATE TYPE CHARACTERIZATION

To characterize bacterial species, the sequence reads from NGS data of 16S amplification products and RCA products were classified into five bacterial community state types (CSTs) based on previous description of the vaginal microbiome. Briefly, CSTs one, two, three, and five comprises *L. crispatus, L. gasseri, L. iners, and L. jensenni* as the prevailing species, respectively. CST four presents high bacterial diversity, with anaerobic bacterial species including *Gardnerella, Megasphera, Atopobium, and Pretovella* present in high proportion. The classification was carried out as per the three study groups (HIV/HPV co-infected women; HPV-infected, HIV-noninfected women; and HIV-infected, HPV-noninfected women).

3.3.3.1 Bacterial community state type characterization from NGS data of 16S amplification products.

3.3.3.1.1 HIV/HPV co-infected study group

Reliable reads for analysis were available for 22 women who were HIV/HPV co-infected. Among this group, CST one, three, and four were observed. In CST one, the relative abundance of *L. crispatus* was 14% (3/22), while the relative abundance of *L. iners* in CST three was 59% (13/22). In CST four, *Gardnerella vaginalis* was the most predominant anaerobic bacterial species (16/22; 73%) and this was statistically significant *(P*<0.0001), followed by *Pretovella* spp (12/22; 54%), *Pseudomonas spp (*11/22*; 50%)*, *Atopobium parvum* (11/22; 50%) as shown in table 3.10.

Table 3.10: Bacterial community state types and their bacterial species detected among HIV/HPV co-infected study group.

BACTERIAL COMMUNITY STATE TYPES (CSTs)	BACTERIAL SPECIES
CST one	*L. crispatus* (3/22; 14%)
CST three	*L. iners* (13/22; 59%)
CST four	*Staphylococcus spp* (22/22; 100%) *Gardnerella vaginalis* (16/22; 73%) *Pretovella spp* (16/22; 73%) *Pseudomonas spp* (11/22; 50%) *Atopobium parvum* (11/22; 50%) *Bifidobacterium spp* (6/22; 27%) *Porphyromonas spp* (5/22; 23%) *Polynucleobacter spp* (4/22; 18%)

Staphylococcus spp was detected in all participants although with varying relative abundance. *Lactobacillus vaginalis* was highly prevalent in one of the participants (ORHC-086), constituting 88% of relative abundance. Additionally, *Lactobacillus vaginalis* also co-existed with *L. plantarum*, in the absence of anaerobic bacteria such as *Gardnerella vaginalis*, *Pretovella,* and *Prophyromonas spp* as shown in figure 3.5. *Pseudomonas* was also highly prevalent in one participant (ORHC-095), with a relative abundance of 90%.

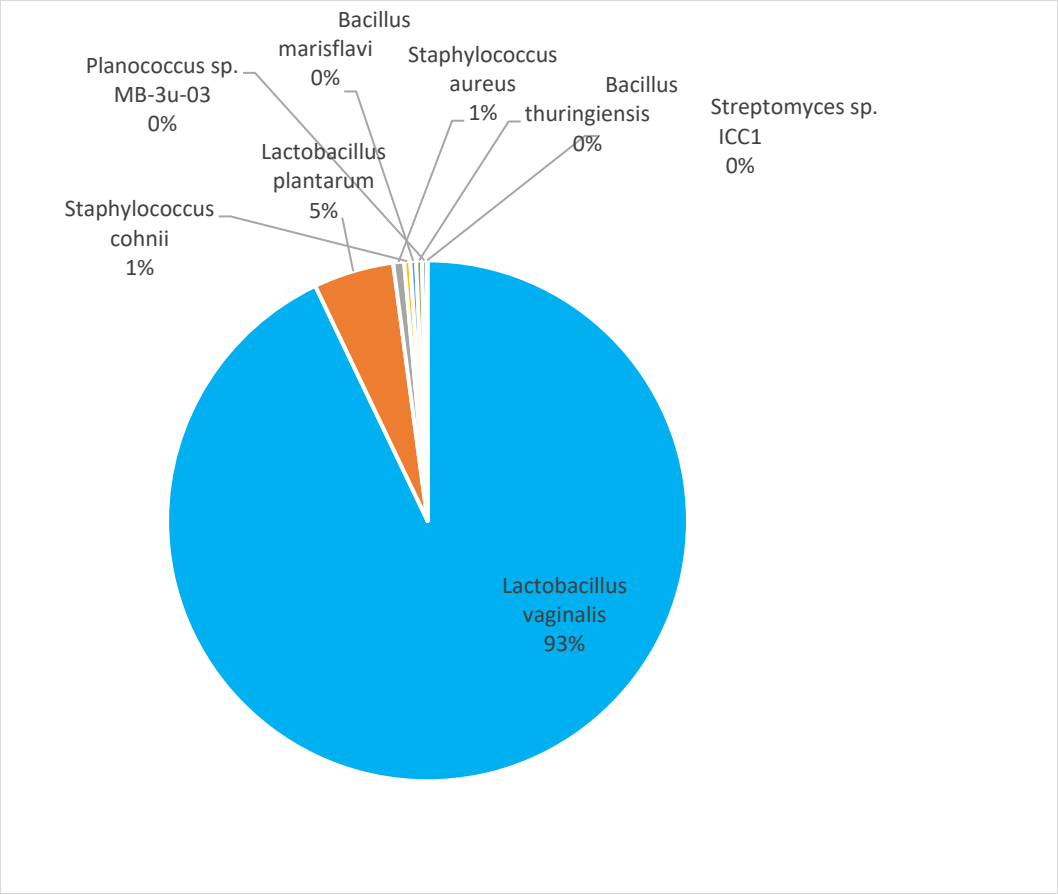

Figure 3.5: A pie chart showing the relative abundance of bacterial species that were detected in one of the study participants (ORH-036-2017). *Lactobacillus vaginalis* was the most abundant species in this study participant. Additionally, *Lactobacillus vaginalis* also co-existed with *L. plantarum*, in the absence of anaerobic bacteria such as *Gardnerella vaginalis*, *Pretovella,* and *Prophyromonas spp*. However, this was not the case for all study participants of this study group. *Gardnerella vaginalis* was the most predominant anaerobic bacterial species among participants of this study group.

3.3.3.1.2 HPV-INFECTED, HIV-NONINFECTED STUDY GROUP

Reliable reads for analysis were available for 15 women who were HPV-infected, HIV-noninfected. Among this group, CST three and CST four were observed. Bacterial community state types (CSTs) and their bacterial species that were frequently detected among women of this study group are shown in table 3.11. In CST three, the relative abundance of *L. iners* was 31% (5/15). In CST four, *Gardnerella vaginalis* was the most predominant anaerobic bacterial species

(10/15; 66%) and this was statistically significant (*P*<0.001), followed by *Polynucleobacter spp* (7/15; 47%), *Pseudomonas spp* (6/15;40%) and *Pretovella spp* (5/15; 31%).

Table 3.11: Bacterial community state types and their bacterial species frequently detected among HPV-infected, HIV-noninfected study group.

BACTERIAL COMMUNITY STATE TYPES (CSTs)	**BACTERIAL SPECIES**
CST three	*L. iners* (5/15; 33%)
CST four	*Klebsiella spp (13/15; 87%)* *Staphylococcus spp (11/15; 73%)* *Xanthomonas citri (11/15; 73%)* *Gardnerella vaginalis* (10/15; 66%) *Polynucleobacter spp* (7/15; 47%) *Pseudomonas spp* (6/15; 40%) *Pretovella spp (5/15; 31%)* *Porphyromonas spp (3/15; 20%)* *Bifidobacterium spp (2/15; 13%)*

One participant (OSHC-198) had a high relative abundance of *L. iners* (CST three) co-existing with other *Lactobacillus spp*. such as *L. acidophilus* and *L. delbrueckii* and *L. acetotolerans* as shown in figure 3.6.

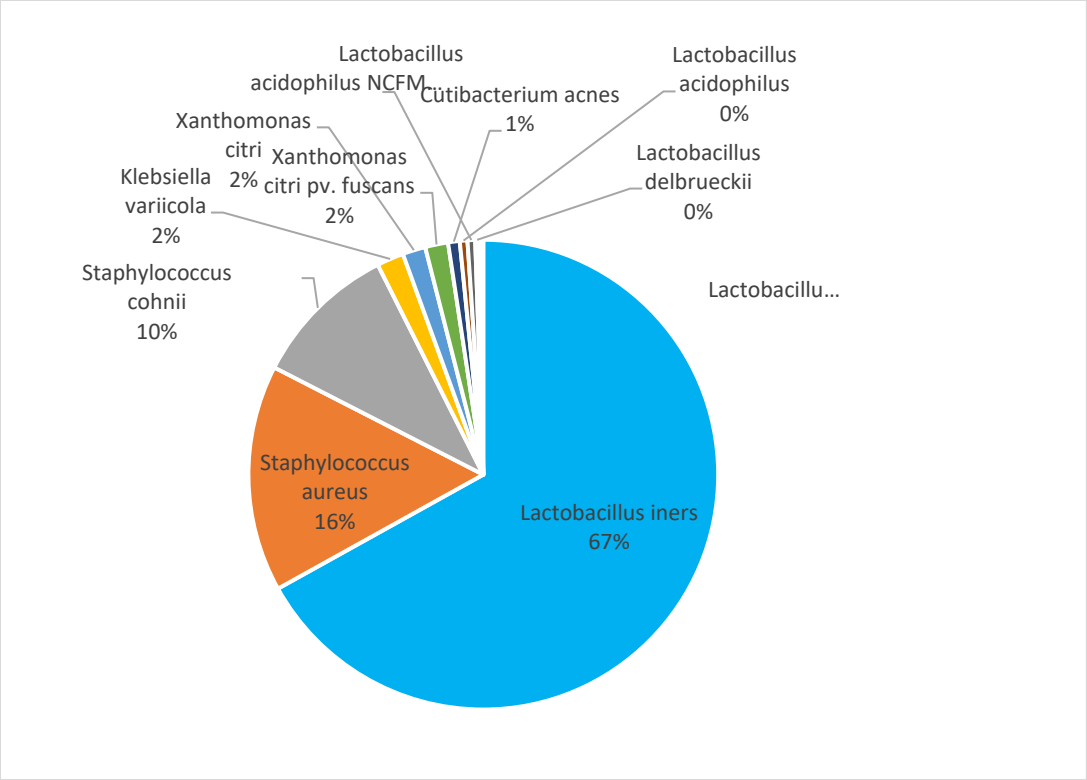

Figure 3.6: A pie chart showing the relative abundance of bacterial species that were present in one of the study participants (OSHC-198-2017). *Lactobacillus iners* (CST three) was the most predominant bacterial species detected in this participant, co-existing with other *Lactobacillus spp.* such as *L. acidophilus* and *L. delbrueckii* and *L. acetotolerans*. However, this was not the case for all the study participants. *Gardnerella vaginalis* was the most predominant bacterial species detected among participants of this study group.

3.3.3.1.3 HIV-infected, HPV non-infected study group

Reliable reads for analysis were available for 15 women who were HIV-infected, HPV-noninfected. Among this group, CST three and four were observed. Bacterial community state types and their bacterial species that were detected in this study group are shown in table 3.12. In CST three, the relative abundance of *L. iners* was 40% (6/15). In CST four, *Gardnerella vaginalis* (66%; 10/15) was the most predominant anaerobic bacterial species detected (as shown in figure 3.7 in one of the study participants-ODF-014-2017) and this was statistically significant (*P*< 0.0001), followed by *Porphyromonas asaccharolytica (8/15; 53%), Pretovella* (6/15; 40%), *Polynucleobacter* (4/15; 27%).

Table 3.12: Bacterial community state types and their bacterial species frequently detected among HIV-infected, HPV non-infected study group.

BACTERIAL COMMUNITY STATE TYPES (CSTs)	BACTERIAL SPECIES
CST three	*L. iners* (6/15;40%)
CST four	*Gardnerella vaginalis (*10/15; 66%) *Porphyromonas asaccharolytica (8/15; 53%)* *Pretovella spp (*6/15; 40%) *Polynucleobacter spp (*4/15; 27%), *Sneathia spp (4/15; 27%)* *Bifidobacterium spp (3/15; 20%).*

Thirty percent of the participants (30%; 3/10) presented *Gardnerella vaginalis* in moderate amount. In CST three, *L. iners* was predominately detected in two participants (13%; 2/15) but had a moderate relative abundance in three participants (20%; 3/15).

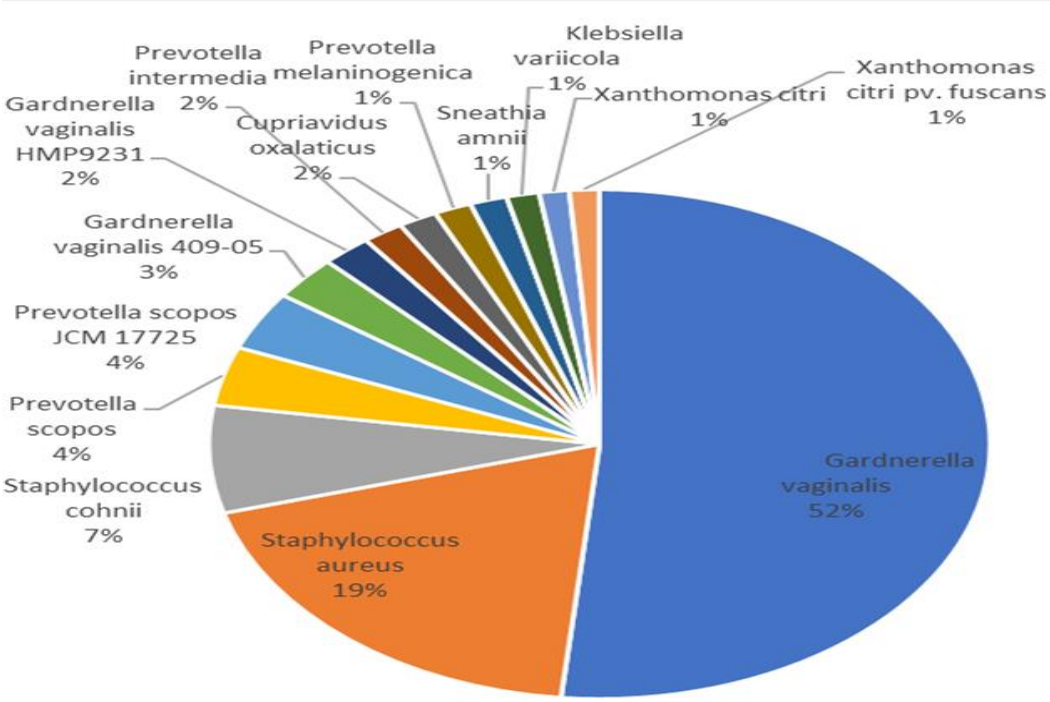

Figure 3.7: A pie chart showing the relative abundance of bacterial species that were detected in one of the study participants (ODF-014-2017). *Gardnerella vaginalis* was the most abundant bacterial species detected in this participant as was observed among other participants of this study group.

3.3.4.1 Bacterial community state type characterization from NGS data of RCA products

3.3.4.1.1 HIV/HPV co-infected study group

Reliable sequence reads for analysis were available for 34 women who were HIV/HPV co-infected. Among this group, CST one, three and four were observed. Bacterial CST and their species that were detected among this study group are shown in table 3.13. In CST one, the relative abundance of *L. crispatus* was 9% (3/34), while in CST three, the relative abundance of *L. iners* was 35% (12/34) as shown in table 3.13. In CST four, *Gardnerella vaginalis* had a relative abundance of 44% (15/34).

Table 3.13: Bacterial community state types and their bacterial species detected among HIV/HPV co-infected study group.

BACTERIAL COMMUNITY STATE TYPES	BACTERIAL SPECIES
CST one	*L. crispatus* (3/34; 9%)
CST three	*L. iners* (12/34; 44%)
CST four	*Polynucleobacter (17/34; 50%)* *Faecalibacterium Prausnitzii (16/34; 47%)* *Gardnerella vaginalis* (15/34; 44%) *Bacteroides spp (15/34; 44%)* *Pretovella spp (12/34; 35%)* *Bifidobacterial spp (9/34; 26%)* *Clostridium spp (5/34; 15%)* *E. coli (5/34; 15%)* *Streptococcus spp (4/34; 12%)*

Six participants (such as ODF-004-2017 shown in figure 3.8) provided an exception since microbiome was characterised by a massive colonization of *Bifidobacterial spp*, classified as B group (6/34; 18%). CST four, as expected showed a higher bacterial heterogeneity, including colonization by a higher rate of gram-positive microorganisms dominated by *Faecalibacterium Prausnitzii* (16/34; 47%) and by gram-negative microorganisms such as *Bacteroides spp.* (12/34; 35%) and *Pretovella spp* (12/34; 35%), which are compatible with a status of dysbiosis and biofilm production.

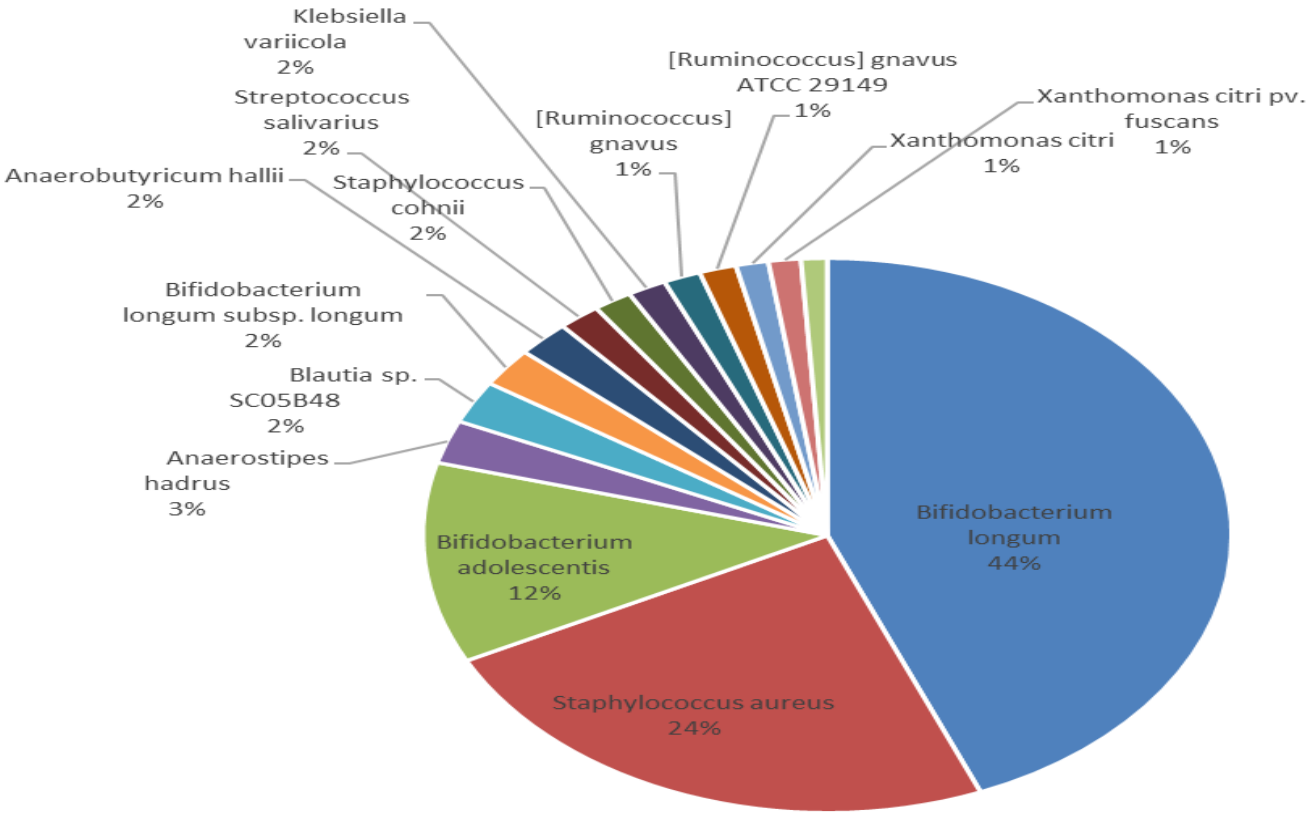

Figure 3.8: A pie chart showing the relative abundance of different bacterial species that were present in one of the study participants (ODF-004-2017). *Bifidobacterium spp* were the most predominant bacterial species detected in this participant. However, in other members of this study group, *Faecalibacterium Prausnitzii, Bacteroides spp* and *Pretovella spp* were the most prevalent bacterial species detected.

3.3.4.1.2 HPV-infected, HIV-noninfected study group

Reliable sequence reads for analysis were available for 26 women who were HPV-infected, HIV -noninfected. Among this group, CST one, three, four and five were observed. Bacterial community state types and their bacterial species that were frequently detected among women of this study group are shown in table 15. In CST one, the relative abundance of *L. crispatus* was 19% (5/26), while in CST three, the relative abundance of *L. iners* was 42% (11/26) as shown in table 3.14. In CST four, the relative abundance of *Gardnerella vaginalis* was 75% (18/26). In CST five, the relative abundance of *L. jensenni* was 8% (2/26).

Table 3.14: Bacterial community state types and their bacterial species detected among HPV-infected, HIV-noninfected study group.

BACTERIAL COMMUNITY STATE TYPES (CSTs)	BACTERIAL SPECIES
CST one	*L. crispatus* (5/26; 19%)
CST three	*L. iners* (11/26; 42%)
CST four	*Staphylococcus spp (26/26; 100%)* *Klebsiella varicola (19/26; 73%)* *Xanthomonas Citri (19/26; 73%)* *Gardnerella vaginalis (18/26; 75%)* *Pseudomonas spp (16/26;61%)* *Polynucleobacter necessarius (17/26; 65%)* *Burkholderia spp (14/26; 54%)* *Pretovella spp (10/26; 38%)* *Serratia spp* (10/26; 38%) *Sneathia spp* (6/26; 23%)
CST five	*L. Jensenni* (8%; 2/26)

In the presence of *L. crispatus* (CST one), a diverse group of *Lactobacillus spp.* was also detected in high proportion as shown in figure 3.9. The presence of *Gardnerella vaginalis* was mostly associated with the presence of *L. iners*.

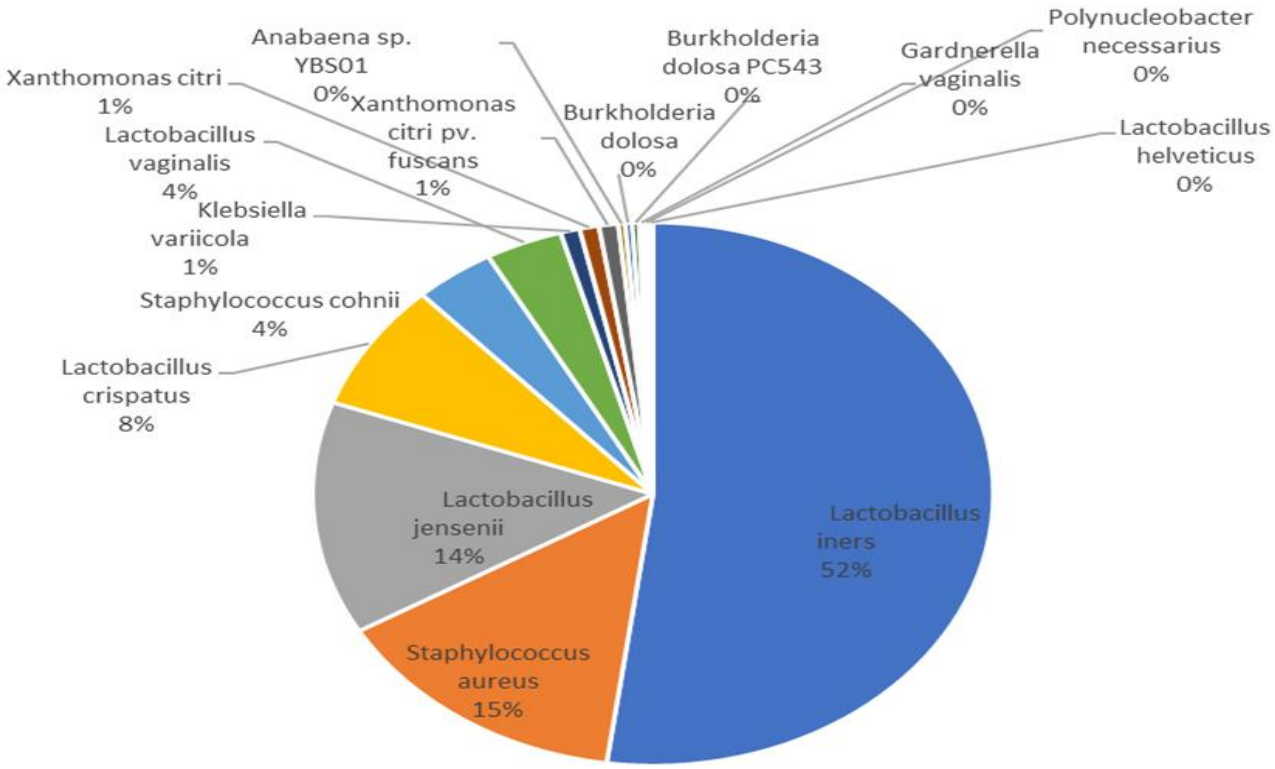

Figure 3.9: A pie chart showing the relative abundance of bacterial species that were present in one of the study participants (OSHC-075-2017). *Lactobacillus iners* was the most abundant species in this participant, However, in other members of this group, *Staphylococcus aureus* and *Gardnerella vaginalis* were the most prevalent species detected.

3.3.4.3 HIV-INFECTED, HPV-NONINFECTED STUDY GROUP

Reliable sequence reads for analysis were available for 11 women who were HIV-infected, HPV-noninfected. Among this group, CST three, four and five were observed. Bacterial community state types and their bacterial species that were frequently detected among women of this study group are shown in table 3.15. In CST three, the relative abundance of *L. iners* was 45% (5/11) while in CST four, *Gardnerella vaginalis* was the most predominant anaerobic bacterial species (8/11; 73%), and this was statistically significant *(P*< 0.001), followed by *Pretovella* (4/11; 36%), and *Corynebacterium spp* (4/11; 36%). In CST five, *L. Jensenni* was only detected in one participant (1/11; 11%) (ORHC-019), which was also colonized by a diverse group of other *Lactobacillus spp.*

Table 3.15: Bacterial community state types and their bacterial species detected among HIV-infected, HPV-noninfected study group women.

BACTERIAL COMMUNITY STATE TYPES (CSTs)	BACTERIAL SPECIES
CST three	*L. iners (5/11; 45%))*
CST four	*Gardnerella vaginalis (8/11; 73%* *Corynebacterium spp (4/11; 36%)* *Mobiluncus Curtisii (4/11; 36%)* *Pretovella spp (4/11; 36%)* *Schaalia cardiffensis (3/11; 27%)* *Pseudomonas spp (3/11; 27%)* *Atopobium parvum (3/11; 27%)* *Schaalia spp (3/11; 18%)*
CST five	*L. jensenni (3/11; 11%)*

3.4 DETERMINATION OF THE RELATIONAL OCCURRENCE OF VIROME AND BACTERIOME ACCORDING TO HIV STATUS.

The relational occurrence of cervicovaginal virome and bacteriome according to HIV status was assessed through Chi-square statistical analysis available in GraphPad Prism version 9.3.1. Differences in occurrence were expressed as probability (*P*)-values.

3.4.1 OCCURRENCE OF VIROME ACCORDING TO HIV STATUS

Overall, a higher prevalence of viral families was detected in HIV-infected women compared to HIV-noninfected women, and this was statistically significant (P<0,0001; χ^2=28.18). However, when the viral occurrence was considered individually, only *Mimiviridae* family occurred more significantly in the HIV-infected women as compared to the HIV-noninfected women (P= 0.0047; χ^2= 7.973).

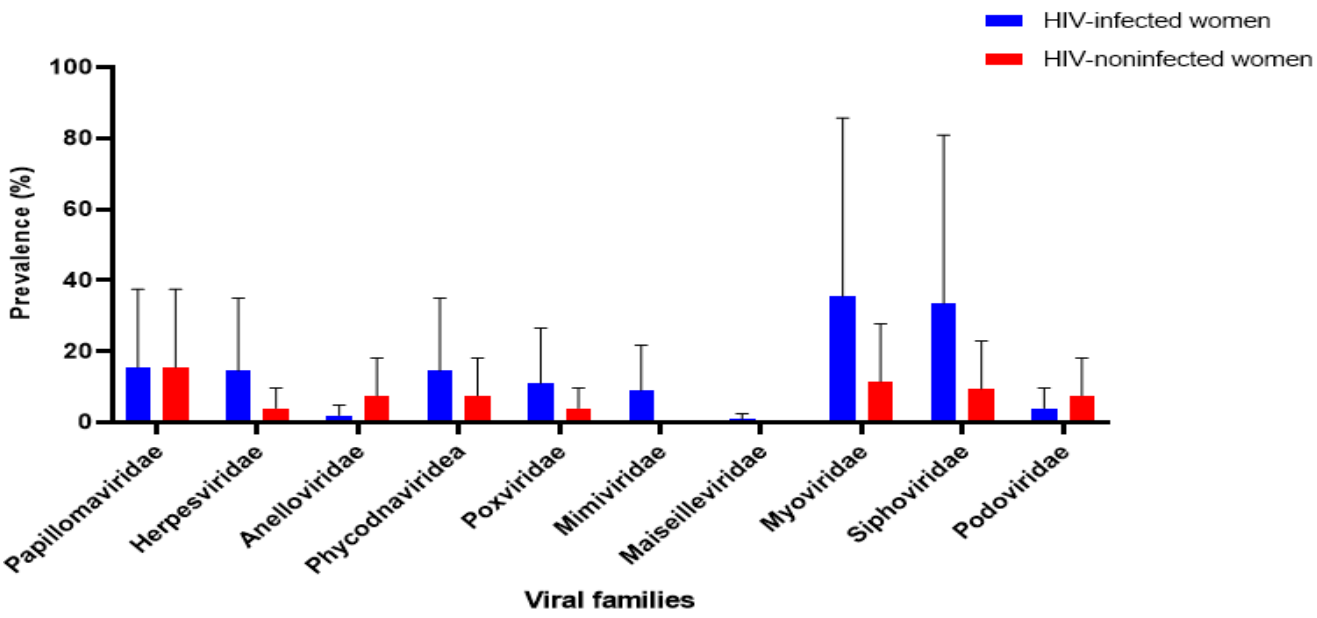

Figure 3.10: A bar graph of mean prevalence of viral families that were detected according to HIV status. The viral families occurred more significantly in the HIV-infected women as compared to HIV-noninfected women *(P*<0.0001). Blue bars indicate HIV-infected women, and red bars indicate HIV-noninfected women.

3.4.2 OCCURRENCE OF BACTERIOME ACCORDING TO HIV STATUS.

In this study, two amplification methods were used to detect bacterial species from total nucleic acid namely, rolling circle amplification (RCA) and conventional PCR with generic primers to amplify the 16S rRNA gene. Amplification products obtained from the two methods were sequenced by Next Generation Sequencing (NGS). The sequences obtained were analysed using Dragen metagenomics tool and bacterial families with varying relative abundance were obtained. To determine the relational occurrence of bacteria according to HIV status, bacterial families with at least a relative abundance of >1% were used for the analysis. NGS data of RCA products revealed a wide variety of bacterial families with a relative abundance of >1% as compared to that of 16S amplification products. Overall, bacterial families occurred more significantly in HIV-infected women than in HIV-noninfected women for NGS data of RCA products *(P*<0.0001; χ^2=177.5) and for NGS data of 16S amplification products (*P*< 0.0001; χ^2=130.8). However, when the bacterial families were considered individually, only the occurrence of *Rickenellanceae* (*P*=0.0472;χ^2=3.938), *Atopobiaceaea (P*=0.0083;χ^2=6.964), *Erysinelotrichaceae* (*P*=0.0472;χ^2=0.0472), *Leuconostoaceae* (*P*<0.001;χ^2=2.049) and *Veillonellaceae* (*P*=0,0261;χ^2=4.950) obtained from NGS data of RCA products and

Porphyromonadaceae(P=0,0004;χ^2=12.48),*Lachnospiraceae(P=0.0036;*χ^2=8.471),*Clostridiacea e (P=0.0472;*χ^2=3.938),*Selenomonaceae (P=0.0350;*χ^2=4.444) obtained from NGS data of 16S amplification products occurred significantly in HIV-infected women than in HIV-noninfected women. *Streptococcaceae (P*=0.0146; χ^2=5,958) obtained from NGS data of 16S amplification products occurred significantly in HIV-noninfected women than in HIV-infected women.

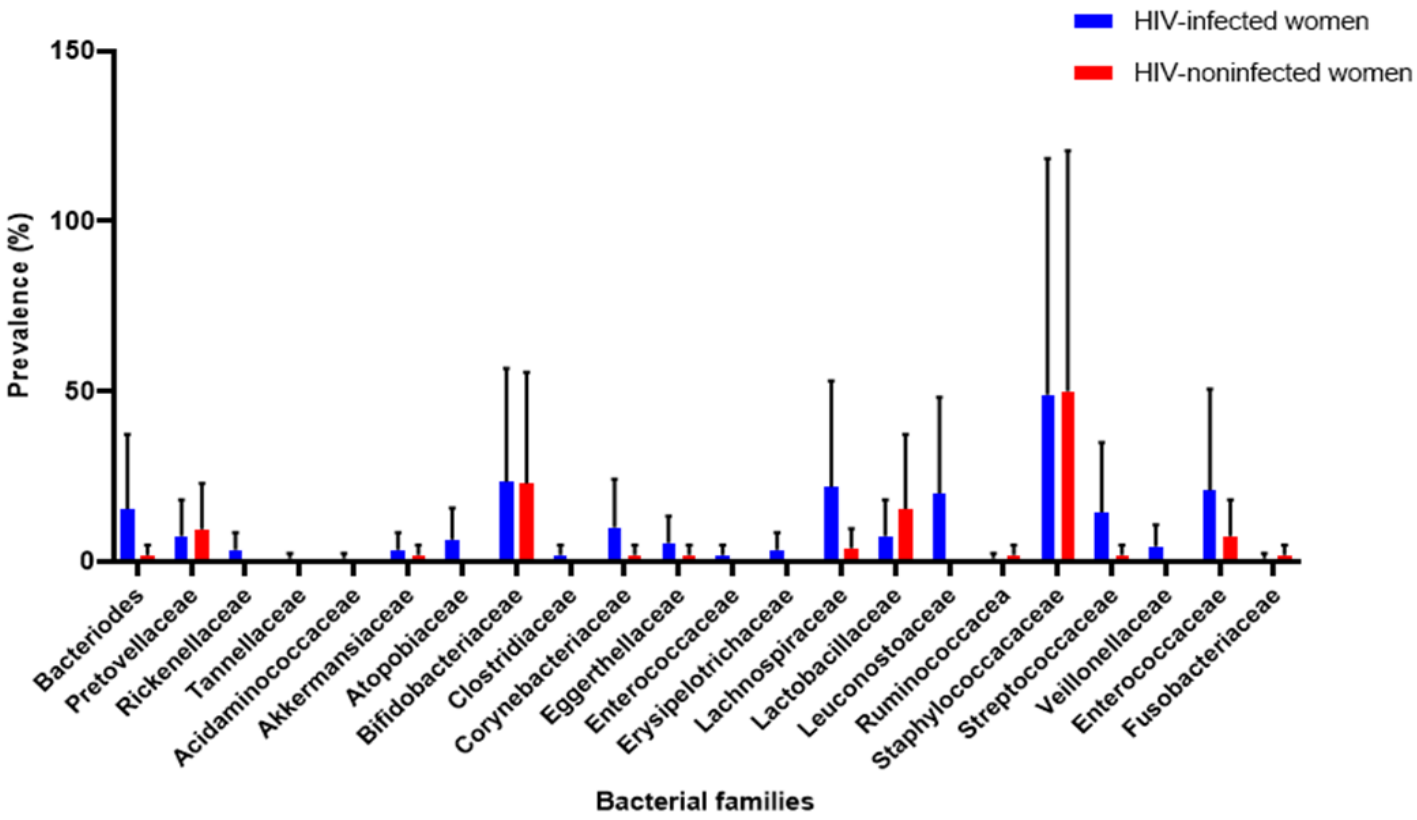

Figure 3.11: A bar graph of mean prevalence of bacterial families obtained from NGS data of rolling circle amplification (RCA) products according to HIV status. In RCA, total nucleic acid was enriched and sequenced by Next Generation Sequencing (NGS). Bacterial families occurred more significantly in HIV-infected women than in HIV-noninfected women *(P*<0.0001). Blue bars indicate HIV-infected women, and red bars indicate HIV-noninfected women.

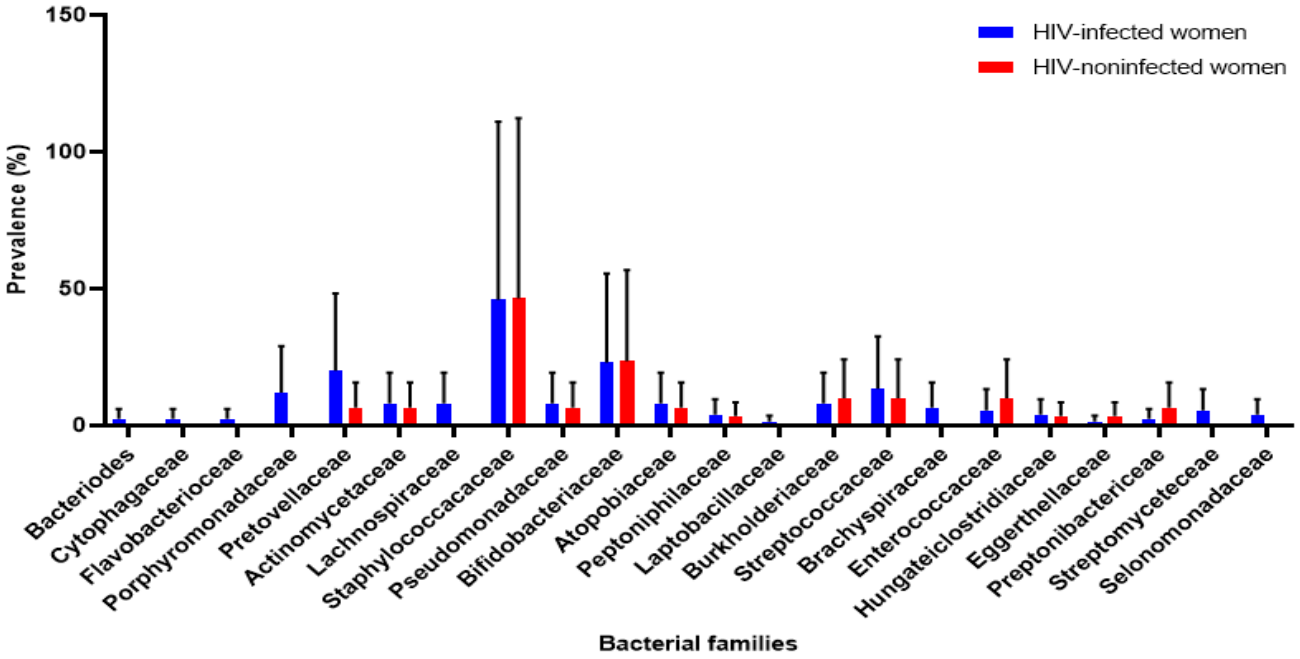

Figure 3.12: A bar graph of mean prevalence of bacterial families obtained from NGS data of 16S amplification products. Generic primers were used to amplify the 16S rRNA gene from total nucleic acids and sequenced by NGS. Bacterial families occurred more significantly in HIV-infected women than in HIV-noninfected women. Blue bars indicate HIV-infected women, and red bars indicate HIV-noninfected women.

Bacteria families that were only detected from RCA NGS data includes *Rickenellanceae* (*P*=0.0472;χ^2=3.938), *Acidaminococcaceae (P=0.2482; χ^2=1.333), Akkermansiaceae (P=0.7154; χ^2=0.7154), Bacillaceae (P=0.1213; χ^2=2.400), Enterococcaceae (P=0.1213; χ^2=2.400, Erysinelotrichaceae (P=0.0472; χ^2=3.938), Fusobacterioceaea (P=0.4945; χ^2=0.4667), Leptotrichiceae (P=0.1551; χ^2=0,3556), Pasterellaceae (P=0.2482; χ^2=0.1333), Cellulomonadaceae (P=0.7154; χ^2=0.7154), Mycoplasmataceae (P=0.2482; χ^2=1.333)* whereas bacteria families that were only detected from 16S NGS data includes *Cytophagaceae (P=0.0877; χ^2=2.917), Flavobacteriaceae (P=0.0877; χ^2=2.977), Hymenobactereceae (P=0.1709; χ^2=1.875), Brachyspiraceae (P=0.1709; χ^2=1.875), Propionibacteriaceae (P=0.1305; χ^2=2.287), Chitinophagaceae (P=0.1709; χ^2=1.875),* and *Dermabacteraceae (P=0.1709; χ^2=1.875)*.

CHAPTER FOUR: DISCUSSION, STUDY LIMITATIONS AND CONCLUSION

The microbial community is highly dynamic and its function in the wellbeing of an individual is only starting to be uncovered. Numerous recent research has unravelled the interaction between human virome and bacteriome, however, the relationship between the two microbiome components has received little attention or evaluation. In this study, DNA viruses and bacterial species obtained from the female genital tract of HIV-infected women and HIV-noninfected women were characterised by next generation sequencing (NGS), enabling the evaluation of their mutual relationship. Majority of the viral reads (34%) were assigned to *Papillomaviridae* family irrespective of whether the participants were HIV-infected or not. Papillomaviruses have been found to be the most prevalent (86%) eukaryote-infecting viruses colonising the female genital tract (Siqueira et al., 2019; Happel et al., 2020).

Multiple high risk (hr) HPV types were also prevalent in the two study groups. Human papillomavirus type 16, which has been connected to more than 50% cases of cervical cancer, was the most prevalent HPV type detected in the current study. This genotype co-existed with other hrHPV or probable hr types such as HPV 54, HPV 53, and HPV 26 in this study. Concurrent infection with multiple HPV types have been linked to a higher likelihood of cervical lesions (Datta et al., 2012; Adler et al.,2016; Kim et al., 2021). A study conducted by Siqueira and Colleagues (2019) found that women with at least three hr or probable hrHPV types had a higher risk of cervical abnormalities. Thus, these results could imply that participants in whom these hrHPV types were detected may be at risk of developing cervical abnormalities. Therefore, HPV health education, and continuous screening for HPV DNA may be of great importance for early diagnosis of cancer risks. Human papillomavirus type 18 is one of the most frequent hr HPV types in South Africa, following HPV 16 (Mbulawa et al., 2021). Seventy percent (70%) of cervical cancer cases are caused by HPV 16 and 18 (Clifford et al., 2006; Schwarz et al., 2008) with HPV 18 accounting for 10-15% of cervical cancer cases (Borch and de Sanjose, 2003; Clifford et al., 2003). However, among participants of this study, HPV 18 was not detected. Therefore, administration of HPV vaccines containing HPV 18 such as Gardacil-9 may provide protection against future infection with HPV 18 and thereby reduce the likelihood of cervical cancer development.

Anelloviruses are commonly detected in the female cervical region independent of the cervix's abnormalities (Calcaterra et al., 2001, Wells et al., 2020). Torque teno viruses (*Anelloviridae)* are prevalent in HIV-infected women, with significant viral loads seen in the blood of immunocompromised patients (Christensen et al., 2000; Touinssi et al., 2001; Focosi et al.,2010). With AIDS progression in the individual, the viral load of these viruses increases, and this suggests a negative relationship with the host immune response (Thom and Petrik, 2007; Li et al., 2013; Siqueira et al., 2019). In the current study, multiple torque teno virus types were observed in participants that were infected with HPV irrespective of HIV status. This suggests its association with HPV regardless of HIV status.

Herpesviridae (HSV-2, CMV and EBV) have been linked to a variety of adverse health outcomes such as cervical cancer, genital ulcers (HSV-2), postnatal herpes (HSV-2) and liver disease (hepatitis B) (Si et al., 2019; Spicknall et al., 2019; Happel et al., 2020). Herpes simplex virus type 2 (HSV-2), Human cytomegalovirus (HCMV), and Epstein-bar virus (EBV) were detected in this study, with HSV-2 detected in all three study groups. The literature demonstrates a solid biological

link among HIV and HSV-2 infections, most notably because HSV-2 infections speed up HIV acquisition. In addition, as HIV and HSV-2 increase the transmission of each other, HSV-2 disease has been observed to be more severe among individuals with HIV-related immunosuppression (Van de Perre et al., 2008; Schiffer et al., 2019). This shows that women who are not infected with HIV but are infected with HSV-2 and other viruses may be at an increased risk of contracting HIV. This is because ulcerative episodes and recurrent HSV-2 infection disrupt the epithelial barrier, allowing HIV to enter. In addition, by recruiting immune cells to regulate early HSV-2 infections and reactivations, HIV target cells are concentrated in the vaginal area (Zhu et al., 2009; Looker et al., 2020). As a result, HSV-2 monitoring could be a valuable additional tool in efforts for the control of HIV infection.

Human cytomegalovirus (CMV) was detected among HPV-infected, HIV-noninfected women. Literature suggests that CMV is transmitted together with HPV or successively, enabling it to encourage dysplastic changes in the cervix that is induced by HPV (Sekhon et al., 2004; Paradowska et al., 2019). This is probably due to CMV replication involving the expression of immediate-early, early, and late genes (Adler, 2011). As a result, these women are likely to be at an increased risk of cervical intraepithelial changes which may lead to cervical cancer if not monitored or treated.

Epstein-Barr virus (EBV) is a mucosal pathogen that can remain latent within a host for a lifetime, which may play a role in modulating the immune system of the host (Djaoud et al., 2017). In this current study, Epstein-Barr virus (EBV) was observed among HIV/HPV co-infected women. Several studies have established a relationship concerning EBV infections and aberrant cervical cytology, an increase in EBV positivity with lesion severity, and increased incidence of cervical cancer in EBV infected women compared to EBV noninfected women (Feng et al., 2021). Cameroon and Colleagues (2020) found that HIV-infected women who had both cervical EBV and hrHPV had a fourfold greater probability of aberrant cytology compared to women who only had hrHPV. This shows that among women who are HIV/HPV co-infected, cervical EBV infection may be associated with an increased risk of cervical dysplasia.

Bacteriophages (prokaryote-infecting viruses) are also the largest and the most predominant cervicovaginal viral group; and have been found to regulate bacterial composition and abundance (Cadwell, 2015; Parmar et al., 2017). In this study, *Myoviridae, Siphoviridae*, and *Podoviridae* were detected from all three study groups, with *Myoviridae* and *Siphoviridae* highly prevalent

among HIV/HPV co-infected women. These results are in line with a University of Cape Town HIV/HPV study that also documented a high number of sequences assigned to *Myoviridae, Siphoviridae*, and *Podoviridae* (Madere et al., 2021). Jakobsen and Colleagues in 2019 also revealed the presence of dsDNA bacteriophages of the family *Myo*-, *Podo*- and *Siphoviridae,* with only 4% of eukaryote-infecting viruses identified, supporting the high prevalence of prokaryote-infecting viruses in the female genital tract. Bacteriophage may be temperate towards some bacteria while lytic towards others, rapidly depleting important bacterial species. This contributes to the inexplicable reduction of *Lactobacillus spp* that occurs favouring the overgrowth of anaerobic bacteria characteristic of bacterial vaginosis (BV) (Kiliç et al., 2001; Madere et al., 2021). Future research is required to fully comprehend the role of bacteriophages in health and diseases in the South Africa population.

The bacteriome has been identified as a significant factor that may influence the increase or decrease of viral infection (Vyshenska et al., 2017). The lower reproductive tract of a female carries a complex community of microorganisms that are critical for sustaining and providing protection against viral infections. As previously stated, bacterial cervical populations are grouped into five CST. *Lactobacillus spp.* predominate in community state type one, two, three and five *(L. crispatus, L. gasseri, L. iners and L. jensenni,* respectively*)*, whereas CST four has a highly diverse bacterial community and a high proportion of multiple anaerobic bacteria (Ravel et al., 2011; Kyrgiou et al., 2017). In the current study, CST one, three, four and five were detected. Herein also, viral families were linked to CST three and CST four. Furthermore, multiple hrHPV types were found to be associated with CST three and CST four. These findings are in line with studies that have linked CST three and four to an increased incidence of HIV, HPV, and HSV-2 infections (Cherpes et al., 2003; Borgdorff et al., 2014). The Siqueira and Colleagues (2019) study among HIV/HPV co-infected women reported a higher frequency of samples with *Herpesviridae* family in CST four, with a 47% increased probability of getting the virus. These concurrent infections may contribute to vaginal dysbiosis, resulting in chronic cervicovaginal inflammation and an increased risk of cervical cancer development.

A high prevalence of CST four was observed among HPV-infected, HIV-noninfected women in the present study. This is in line with a study conducted by Dareng and Colleagues (2016) that also reported a higher prevalence of CST four among women who were infected with HPV but HIV noninfected. Additionally, Dareng and Colleagues (2016) reported insignificant variation in the bacteriome irrespective of HPV status. This was also observed in the current study. This data

agrees with previous reports indicating HIV-infected women maintain a stable microbial community with a high diversity of bacteria that is unaffected by the presence of cervical intraepithelial neoplasia status or HPV infection (Dareng et al., 2016; Gossman et al., 2017).

Anaerobic bacteria that were highly prevalent in CST four were *Gardnerella vaginalis, Pretovella, Atopobium, Bifidobacterium spp, Porphyromonas spp, Pseudomonas, Faecalibacterium prausnitzii and Bacteroides* in this study groups. Carrying *Atopobium vaginae, Gardnerella vaginalis,* and *L. iners* in the absence of *L. crispatus* (CST one) elevated the incidence of cervical intraepithelial lesions (CIN) about sixfold in Korean women with and without CIN. Additionally, they found a symbiotic microbiological pattern and an oncogenic HPV infection associated with an increased incidence of CIN (34.1 odd ratio) (Brodman et al., 2011). According to these studies, high bacterial diversity is associated with HPV status and varying degree of cervical dysplasia, implying that it may be useful for cervical dysplasia prediction.

The 16S rRNA gene sequence has been the most frequently employed housekeeping hereditary marker in studies of bacterial phylogeny and classification. This is because of its presence in almost all bacteria, where it is frequently found as a multigene family with a function that has not changed overtime (Patel, 2001; Janda and Abbott, 2007). In this study, NGS data of 16S amplification products and rolling circle amplification (RCA) products were used to characterize cervicovaginal bacteria and were compared against each other. The two molecular targets generated a surprisingly similar microbial profile, with only a minor difference in the proportion of species found. For example, *Streptococcus spp, Bacteroides* spp, and *Bifidobacterium spp* were observed in higher proportions in NGS data of the RCA generated products than in NGS data of 16S PCR amplification products. Some studies have demonstrated that 16S rRNA sequencing is less likely to detect some microorganisms, for example *Streptococcus, Proteobacteria,* and *Actinobacteria*. This may explain why some elements of the vaginal microbiome have received little attention. *Mollicutes*, for example, are commonly missed in 16S rRNA-based studies due to bias in the use of universal primer sets (Hummelen et al., 2010) and have been missed in the vaginal microbiome using cpn60 UT (a sequence universal target), because the target sequences are absent in almost all Mollicutes species (Hill et al., 2005; Schellenberg et al., 2009; Schellenberg et al., 2011). In this case, RCA can be of great importance in the detection of such microorganisms since no specific primers are required during the amplification process.

LIMITATIONS OF THE STUDY

The strengths of this study included the use of sensitive approaches and technologies such as rolling circle amplification (RCA) and metagenomic Next Generation Sequencing (NGS). These are highly appropriate in the description of the microbiota in a particular compartment. In the current study, a highly diverse group of viral and bacterial species from cervical specimens were observed. However, despite these strengths, the findings described here must be seen in the context of some limitations. Firstly, proinflammatory cytokines, such as IL-8, IL-1α and TNFα which have been shown to influence microbial diversity (Mirmosef et al., 2012; Sabo et al., 2020) were not investigated. Therefore, it is challenging to provide some of the reasons for the diversity observed. Secondly, the presence of fungi such as the abundance of *Candidaceae, Sporidiobalaceae* and *Malasseziaceae* known to be associated with hrHPV infections and microbial diversity (Godoy-Vitorino et al., 2018) was not investigated. Therefore, future studies focusing on the detection of fungi from cervical specimens may provide an insight in the occurrence of fungi in relation to highly diverse bacterial species and viral infections. It will also be important in future to look at the characteristics of the full genomes that were identified in this compartment.

CONCLUSION AND RECOMNMENDATIONS

In conclusion, this study showed a higher diversity of cervicovaginal virome and bacteriome in women who were HIV/HPV co-infected. The relationship between viral infections and a high diversity of bacterial species (CST four) observed herein may be a useful indicator of an individual's disease state, indicating the likelihood of developing cervical lesions and cervical cancer. As a result, additional research is necessary to uncover the association between viral infections and CST four with disease state. Vaccine development and antiviral research should also target compounds that boost the cervicovaginal environment and maintain vaginal homeostasis.

REFERENCES

Arvin, A., Campadelli-Fiume, G., Mocarski, E., Moore, P.S., Roizman, B., Whitley, R. and Yamanishi, K. eds., 2007. Human herpesviruses: biology, therapy, and immunoprophylaxis.

Abeles, S.R. and Pride, D.T., 2014. Molecular bases and role of viruses in the human microbiome. *Journal of Molecular Biology*, *426*(23), pp.3892-3906.

Adler, D.H., Wallace, M., Bennie, T., Abar, B., Meiring, T.L., Williamson, A.L. and Bekker, L.G., 2016. Cumulative impact of HIV and multiple concurrent human papillomavirus infections on the risk of cervical dysplasia. *Advances in Virology*, *2016*.

Adler, S.P., 2011. Screening for cytomegalovirus during pregnancy. *Infectious Diseases in Obstetrics and Gynaecology*, *2011*.

Alemi, M., Mohabatkar, H. and Behbahani, M., 2014. In silico comparison of low-and high-risk human papillomavirus proteins. *Applied Biochemistry and Biotechnology*, *172*(1), pp.188-195.

Allsworth, J.E., Lewis, V.A. and Peipert, J.F., 2008. Viral sexually transmitted infections and bacterial vaginosis: 2001–2004 National Health and Nutrition Examination Survey data. *Sexually Transmitted Diseases*, *35*(9), pp.791-796.

Alsamarai, M. and Aljumaili, Z., 2013. Seroepidemiology of toxoplasma, rubella, cytomegalovirus and herpes simplex virus-2 in women with bad obstetric history. PART II. Cytomegalovirus and herpes simplex virus infections. *Dermatology*, *4*, pp.536-544.

Ameur, A., Meiring, T.L., Bunikis, I., Häggqvist, S., Lindau, C., Lindberg, J.H., Gustavsson, I., Mbulawa, Z.Z., Williamson, A.L. and Gyllensten, U., 2014. Comprehensive profiling of the vaginal microbiome in HIV positive women using massive parallel semiconductor sequencing. *Scientific Reports*, *4*, p.4398.

Aroutcheva, A., Gariti, D., Simon, M., Shott, S., Faro, J., Simoes, J.A., Gurguis, A. and Faro, S., 2001. Defense factors of vaginal lactobacilli. *American Journal Of Obstetrics And Gynaecology*, *185*(2), pp.375-379.

Atassi, F. and Servin, A.L., 2010. Individual and co-operative roles of lactic acid and hydrogen peroxide in the killing activity of enteric strain Lactobacillus johnsonii NCC933 and vaginal strain

Lactobacillus gasseri KS120. 1 against enteric, uropathogenic and vaginosis-associated pathogens. *Federation Of European Microbiology Society Microbiology Letters*, *304*(1), pp.29-38.

Berntsson, M., Dubicanac, L., Tunbäck, P., Ellström, A., Löwhagen, G.B. and Bergström, T., 2013. Frequent detection of cytomegalovirus and Epstein–Barr virus in cervical secretions from healthy young women. *Acta Obstetricia Et Gynecologica Scandinavica*, *92*(6), pp.706-710.

Borgdorff, H., Armstrong, S.D., Tytgat, H.L., Xia, D., Ndayisaba, G.F., Wastling, J.M. and van de Wijgert, J.H., 2016. Unique insights in the cervicovaginal Lactobacillus iners and L. crispatus proteomes and their associations with microbiota dysbiosis. *PLoS One*, *11*(3), p.e0150767.

Borgdorff, H., Tsivtsivadze, E., Verhelst, R., Marzorati, M., Jurriaans, S., Ndayisaba, G.F., Schuren, F.H. and Van De Wijgert, J.H., 2014. Lactobacillus-dominated cervicovaginal microbiota associated with reduced HIV/STI prevalence and genital HIV viral load in African women. *The International Society For Microbial Ecology journal*, *8*(9), pp.1781-1793.

Boskey, E.R., Cone, R.A., Whaley, K.J. and Moench, T.R., 2001. Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. *Human Reproduction*, *16*(9), pp.1809-1813.

Bradshaw, C.S., Vodstrcil, L.A., Hocking, J.S., Law, M., Pirotta, M., Garland, S.M., De Guingand, D., Morton, A.N. and Fairley, C.K., 2012. Recurrence of bacterial vaginosis is significantly associated with posttreatment sexual activities and hormonal contraceptive use. *Clinical Infectious Diseases*, *56*(6), pp.777-786.

Brotman, R.M., 2011. Vaginal microbiome and sexually transmitted infections: an epidemiologic perspective. *The Journal of clinical investigation*, *121*(12), pp.4610-4617.

Brotman, R.M., Klebanoff, M.A., Nansel, T.R., Andrews, W.W., Schwebke, J.R., Zhang, J., Yu, K.F., Zenilman, J.M. and Scharfstein, D.O., 2008. A longitudinal study of vaginal douching and bacterial vaginosis—a marginal structural modelling analysis. *American Journal Of Epidemiology*, *168*(2), pp.188-196.

Cadwell, K., 2015. The virome in host health and disease. *Immunity*, *42*(5), pp.805-813.

Cherpes, T.L., Hillier, S.L., Meyn, L.A., Busch, J.L. and Krohn, M.A., 2008. A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen

peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. *Sexually Transmitted Diseases*, *35*(1), pp.78-83.

Cherpes, T.L., Meyn, L.A., Krohn, M.A., Lurie, J.G. and Hillier, S.L., 2003. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. *Clinical Infectious Diseases*, *37*(3), pp.319-325.

Costello, E.K., Lauber, C.L., Hamady, M., Fierer, N., Gordon, J.I. and Knight, R., 2009. Bacterial community variation in human body habitats across space and time. *Science*, *326*(5960), pp.1694-1697.

Curty, G., Costa, R.L., Siqueira, J.D., Meyrelles, A.I., Machado, E.S., Soares, E.A. and Soares, M.A., 2017. Analysis of the cervical microbiome and potential biomarkers from postpartum HIV-positive women displaying cervical intraepithelial lesions. *Scientific Reports*, *7*(1), p.17364.

Dareng, E.O., Ma, B., Famooto, A.O., Akarolo-Anthony, S.N., Offiong, R.A., Olaniyan, O., Dakum, P.S., Wheeler, C.M., Fadrosh, D., Yang, H. and Gajer, P., 2016. Prevalent high-risk HPV infection and vaginal microbiota in Nigerian women. *Epidemiology And Infection*, *144*(1), pp.123-137.

Dvorak, P., 2001. Cytomegalovirus Infection in Immunocompetent and Immunocompromised Individuals-A Review Cytomegalovirus Infection in Immunocompetent and Immunocompromised Individuals-A Review. *Current Drug Targets-Immune, Endocrine & Metabolic Disorders*, *1*(2), pp.179-187.

Fernandes, J.V. and de Medeiros Fernandes, T.A.A., 2012. Human papillomavirus: biology and pathogenesis. In *Human Papillomavirus and Related Diseases-From Bench to Bedside-A Clinical Perspective*. InTech.

Fife, K.H., Fortenberry, J.D., Ofner, S., Katz, B.P., Morrow, R.A. and Orr, D.P., 2006. Incidence and prevalence of herpes simplex virus infections in adolescent women. *Sexually Transmitted Diseases*, *33*(7), pp.441-444.

Forney, L.J., 2014. Microbial Ecology in States of Health and Disease.

Gosmann, C., Anahtar, M.N., Handley, S.A., Farcasanu, M., Abu-Ali, G., Bowman, B.A., Padavattan, N., Desai, C., Droit, L., Moodley, A. and Dong, M., 2017. Lactobacillus-deficient

cervicovaginal bacterial communities are associated with increased HIV acquisition in young South African women. *Immunity*, *46*(1), pp.29-37.

Haedicke, J. and Iftner, T., 2013. Human papillomaviruses and cancer. *Radiotherapy And Oncology*, *108*(3), pp.397-402.

Hawes, S.E., Hillier, S.L., Benedetti, J., Stevens, C.E., Koutsky, L.A., Wølner-Hanssen, P. and Holmes, K.K., 1996. Hydrogen peroxide—producing lactobacilli and acquisition of vaginal infections. *Journal Of Infectious Diseases*, *174*(5), pp.1058-1063.

Hill, J.E., Goh, S.H., Money, D.M., Doyle, M., Li, A., Crosby, W.L., Links, M., Leung, A., Chan, D. and Hemmingsen, S.M., 2005. Characterization of vaginal microflora of healthy, nonpregnant women by chaperonin-60 sequence-based methods. *American Journal Of Obstetrics And Gynaecology*, *193*(3), pp.682-692.

Huang, B., Fettweis, J.M., Brooks, J.P., Jefferson, K.K. and Buck, G.A., 2014. The changing landscape of the vaginal microbiome. *Clinics In Laboratory Medicine*, *34*(4), pp.747-761.

Hummelen, R., Fernandes, A.D., Macklaim, J.M., Dickson, R.J., Changalucha, J., Gloor, G.B. and Reid, G., 2010. Deep sequencing of the vaginal microbiota of women with HIV. *PloS one*, *5*(8), p.e12078.

Jakobsen, R.R., Haahr, T., Humaidan, P., Jensen, J.S., Kot, W., Castro-Mejia, J., Deng, L., Leser, T.D. and Nielsen, D.S., 2019. Characterization of the vaginal DNA virome in health and dysbiosis: an opening study in patients with non-female factor infertility. *bioRxiv*, p.755710.

Janda, J.M. and Abbott, S.L., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal Of Clinical Microbiology*, *45*(9), pp.2761-2764.

Jemal, A., Bray, F., Centre, M.M., Ferlay, J., Ward, E. and Forman, D., 2011. Global cancer statistics. *CA: A Cancer Journal For Clinicians*, *61*(2), pp.69-90.

Johnson, L.F., Chiu, C., Myer, L., Davies, M.A., Dorrington, R.E., Bekker, L.G., Boulle, A. and Meyer-Rath, G., 2016. Prospects for HIV control in South Africa: a model-based analysis. *Global Health Action*, *9*(1), p.30314.

Kiliç, A.O., Pavlova, S.I., Alpay, S., Kiliç, S.S. and Tao, L., 2001. Comparative study of vaginal Lactobacillus phages isolated from women in the United States and Turkey: prevalence, morphology, host range, and DNA homology. *Clinical Diagnostic Laboratory Immunology*, *8*(1), pp.31-39.

Kyrgiou, M., Mitra, A. and Moscicki, A.B., 2017. Does the vaginal microbiota play a role in the development of cervical cancer? *Translational Research*, *179*, pp.168-182.

Lambert, J.A., John, S., Sobel, J.D. and Akins, R.A., 2013. Longitudinal analysis of vaginal microbiome dynamics in women with recurrent bacterial vaginosis: recognition of the conversion process. *PloS one*, *8*(12), p.e82599.

Li, L., Deng, X., Linsuwanon, P., Bangsberg, D., Bwana, M.B., Hunt, P., Martin, J.N., Deeks, S.G. and Delwart, E., 2013. AIDS alters the commensal plasma virome. *Journal Of Virology*, *87*(19), pp.10912-10915.

Li, X.D., Wang, C.C., Zhang, X.J., Gao, G.P., Tong, F., Li, X., Hou, S., Sun, L. and Sun, Y.H., 2014. Risk factors for bacterial vaginosis: results from a cross-sectional study having a sample of 53,652 women. *European Journal Of Clinical Microbiology And Infectious Diseases*, *33*(9), pp.1525-1532.

Lima, M.D., Braz-Silva, P.H., Pereira, S.M., Riera, C., Coelho, A.C. and Gallottini, M., 2014. Oral and cervical HPV infection in HIV-positive and HIV-negative women attending a sexual health clinic in São Paulo, Brazil. *International Journal Of Gynaecology And Obstetrics*, *126*(1), pp.33-36.

Liu, M.B., Xu, S.R., He, Y., Deng, G.H., Sheng, H.F., Huang, X.M., Ouyang, C.Y. and Zhou, H.W., 2013. Diverse vaginal microbiomes in reproductive-age women with vulvovaginal candidiasis. *PloS one*, *8*(11), p.e79812.

Looker, K.J., Welton, N.J., Sabin, K.M., Dalal, S., Vickerman, P., Turner, K.M., Boily, M.C. and Gottlieb, S.L., 2020. Global and regional estimates of the contribution of herpes simplex virus type 2 infection to HIV incidence: a population attributable fraction analysis using published epidemiological data. *The Lancet Infectious Diseases*, *20*(2), pp.240-249.

Lu, S., Cong, X., Li, M., Chang, F., Ma, L. and Cao, Y.T., 2015. Distribution of high-risk human papillomavirus genotypes in HPV-infected women in Beijing, China. *Journal Of Medical Virology*, *87*(3), pp.504-507.

Macklaim, J.M., Gloor, G.B., Anukam, K.C., Cribby, S. and Reid, G., 2011. At the crossroads of vaginal health and disease, the genome sequence of Lactobacillus iners AB-1. *Proceedings Of The National Academy Of Sciences*, *108*(Supplement 1), pp.4688-4695.

Madeddu, G., Mameli, G., Capobianco, G., Babudieri, S., Maida, I., Bagella, P., Rocca, G., Cherchi, P.L., Sechi, L.A., Zanetti, S. and Nunnari, G., 2014. HPV infection in HIV-positive females: the need for cervical cancer screening including HPV-DNA detection despite successful HAART. *European Review For Medical And Pharmacological Sciences*, *18*(8), pp.1277-1285.

Madere, F.S., Sohn, M., Winbush, A., Barr, B., Grier, A., Java, J., Meiring, T., Williamson, A.L., Bekker, L.G., Adler, D.H. and Monaco, C.L., 2021. Transkingdom Analysis of the Female Reproductive Tract Reveals Bacteriophages Form Communities. *BioRxiv*.

Martín, R. and Suárez, J.E., 2010. Biosynthesis and degradation of H2O2 by vaginal lactobacilli. *Applied and Environmental Microbiology.*, *76*(2), pp.400-405.

Masese, L., Baeten, J.M., Richardson, B.A., Bukusi, E., John-Stewart, G., Jaoko, W., Shafi, J., Kiarie, J. and McClelland, R.S., 2013. Incident herpes simplex virus type 2 infection increases the risk of subsequent episodes of bacterial vaginosis. *The Journal of Infectious Diseases*, *209*(7), pp.1023-1027.

McMillan, A., Dell, M., Zellar, M.P., Cribby, S., Martz, S., Hong, E., Fu, J., Abbas, A., Dang, T., Miller, W. and Reid, G., 2011. Disruption of urogenital biofilms by lactobacilli. *Colloids and Surfaces B: Biointerfaces*, *86*(1), pp.58-64.

Meloni, A., Pilia, R., Campagna, M., Usai, A., Masia, G., Caredda, V. and Coppola, R.C., 2014. Prevalence and molecular epidemiology of human papillomavirus infection in Italian women with cervical cytological abnormalities. *Journal of Public Health Research*, *3*(1).

Mirmonsef, P., Krass, L., Landay, A. and T Spear, G., 2012. The role of bacterial vaginosis and trichomonas in HIV transmission across the female genital tract. *Current HIV research*, *10*(3), pp.202-210.

Mitra, A., MacIntyre, D.A., Marchesi, J.R., Lee, Y.S., Bennett, P.R. and Kyrgiou, M., 2016. The vaginal microbiota, human papillomavirus infection and cervical intraepithelial neoplasia: what do we know and where are we going next? *Microbiome*, *4*(1), p.58.

Monaco, C.L., Gootenberg, D.B., Zhao, G., Handley, S.A., Ghebremichael, M.S., Lim, E.S., Lankowski, A., Baldridge, M.T., Wilen, C.B., Flagg, M. and Norman, J.M., 2016. Altered virome and bacterial microbiome in human immunodeficiency virus-associated acquired immunodeficiency syndrome. *Cell Host & Microbe*, *19*(3), pp.311-322.

Morshed, K., Polz-Gruszka, D., Szymański, M. and Polz-Dacewicz, M., 2014. Human Papillomavirus (HPV)–structure, epidemiology and pathogenesis. *Otolaryngologia Polska*, *68*(5), pp.213-219.

Mougin, C., Bernard, C., Schaal, J.P., Laurent, R. and Carbillet, J.P., 1995. Detection of human cytomegalovirus in female and male anogenital lesions with papillomavirus-associated dysplastic changes. *Cancer Detection and Prevention*, *19*(4), pp.316-324

Norman, J.M., Handley, S.A., Baldridge, M.T., Droit, L., Liu, C.Y., Keller, B.C., Kambal, A., Monaco, C.L., Zhao, G., Fleshner, P. and Stappenbeck, T.S., 2015. Disease-specific alterations in the enteric virome in inflammatory bowel disease. *Cell*, *160*(3), pp.447-460.

Nyangu, I. and Moteane, T., 2021. Young women's knowledge and attitudes towards cervical cancer screening at a selected higher education institution in Lesotho: a cross-sectional survey.

O'Hanlon, D.E., Moench, T.R. and Cone, R.A., 2011. In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. *BioMed Central Infectious Diseases*, *11*(1), p.200.

Patel, J.B., 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Molecular Diagnosis*, *6*(4), pp.313-321.

Pavlova, S.I. and Tao, L., 2000. In vitro inhibition of commercial douche products against vaginal microflora. *Infectious Diseases in Obstetrics and Gynaecology*, *8*(2), pp.99-104.

Perez, G.I.P., Gao, Z., Jourdain, R., Ramirez, J., Gany, F., Clavaud, C., Demaude, J., Breton, L. and Blaser, M.J., 2016. Body site is a more determinant factor than human population diversity in the healthy skin microbiome. *PLoS One*, *11*(4), p.e0151990.

Petrova, M.I., Reid, G., Vaneechoutte, M. and Lebeer, S., 2017. Lactobacillus iners: friend or foe? *Trends in Microbiology*, *25*(3), pp.182-191.

Pyles, R.B., Vincent, K.L., Baum, M.M., Elsom, B., Miller, A.L., Maxwell, C., Eaves-Pyles, T.D., Li, G., Popov, V.L., Nusbaum, R.J. and Ferguson, M.R., 2014. Cultivated vaginal microbiomes alter HIV-1 infection and antiretroviral efficacy in colonized epithelial multilayer cultures. *PloS one*, *9*(3), p.e93419.

Rampersaud, R., Planet, P.J., Randis, T.M., Kulkarni, R., Aguilar, J.L., Lehrer, R.I., and Ratner, A.J., 2011. Inerolysin, a cholesterol-dependent cytolysin produced by Lactobacillus iners. *Journal of Bacteriology*, *193*(5), pp.1034-1041.

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M. and Koenig, S.S., 2011. Vaginal microbiome of reproductive-age women. *Proceeding of the National Academy of Sciences of the United States of America.*

Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., McCulle, S.L., Karlebach, S., Gorle, R., Russell, J., Tacket, C.O. and Brotman, R.M., 2011. Vaginal microbiome of reproductive-age women. *Proceedings Of the National Academy of Sciences*, *108*(Supplement 1), pp.4680-4687.

Ross S a, Novak Z, Ashrith G, et al. Association between genital tract cytomegalovirus infection and bacterial vaginosis. *Journal of Infectious Diseases*. 2005; 192:1727-1730.

Santiago-Rodriguez, T.M., Ly, M., Bonilla, N. and Pride, D.T., 2015. The human urine virome in association with urinary tract infections. *Frontiers In Microbiology*, *6*, p.14.

Schellenberg, J., Links, M.G., Hill, J.E., Dumonceaux, T.J., Peters, G.A., Tyler, S., Ball, T.B., Severini, A., and Plummer, F.A., 2009. Pyrosequencing of the chaperonin-60 universal target as a tool for determining microbial community composition. *Applied and environmental microbiology*, *75*(9), pp.2889-2898.

Schellenberg, J.J., Links, M.G., Hill, J.E., Dumonceaux, T.J., Kimani, J., Jaoko, W., Wachihi, C., Mungai, J.N., Peters, G.A., Tyler, S. and Graham, M., 2011. Molecular definition of vaginal microbiota in East African commercial sex workers. *Applied and Environmental Microbiology*, *77*(12), pp.4066-4074.

Schiffer, J.T. and Gottlieb, S.L., 2019. Biologic interactions between HSV-2 and HIV-1 and possible implications for HSV vaccine development. *Vaccine*, *37*(50), pp.7363-7371.

Sekhon, H.S., Press, R.D., Schmidt, W.A., Hawley, M. and Rader, A., 2004. Identification of cytomegalovirus in a liquid-based gynecologic sample using morphology, immunohistochemistry, and DNA real-time PCR detection. *Diagnostic Cytopathology*, *30*(6), pp.411-417.

Si, J., Yu, C., Guo, Y., Bian, Z., Meng, R., Yang, L., Chen, Y., Jin, J., Liu, J., Guo, Z. and Chen, J., 2019. Chronic hepatitis B virus infection and total and cause-specific mortality: a prospective cohort study of 0.5 million people. *British Medical Journal open*, *9*(4), p.e027696.

Simbayi, L., Zuma, K., Zungu, N., Moyo, S., Marinda, E., Jooste, S., Mabaso, M., Ramlagan, S., North, A., Van Zyl, J. and Mohlabane, N., 2019. South African national HIV prevalence, incidence, behaviour, and communication survey, 2017: towards achieving the UNAIDS 90-90-90 targets.

Siqueira, J.D., Curty, G., Xutao, D., Hofer, C.B., Machado, E.S., Seuánez, H.N., Soares, M.A., Delwart, E. and Soares, E.A., 2019. Composite analysis of the virome and bacteriome of HIV/HPV co-infected women reveals proxies for immunodeficiency. *Viruses*, *11*(5), p.422.

Spicknall, I.H., Looker, K.J., Gottlieb, S.L., Chesson, H.W., Schiffer, J.T., Elmes, J. and Boily, M.C., 2019. Review of mathematical models of HSV-2 vaccination: Implications for vaccine development. *Vaccine*, *37*(50), pp.7396-7407.

Steben, M. and Money, D., 2008. Genital herpes: gynaecological aspects. *Journal of Obstetrics and Gynaecology Canada : JOGC= Journal d'obstetrique et gynecologie du Canada : JOGC*, *30*(9), p.768.

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A. and Bray, F., 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *71*(3), pp.209-249.

Sweet, R.L., 2000. Gynecologic conditions and bacterial vaginosis: implications for the non-pregnant patient. *Infectious Diseases in Obstetrics and Gynaecology*, *8*(3-4), pp.184-190.

Swenson, P.D., Lowens, M.S., Celum, C.L. and Hierholzer, J.C., 1995. Adenovirus types 2, 8, and 37 associated with genital infections in patients attending a sexually transmitted disease clinic. *Journal of Clinical Microbiology*, *33*(10), pp.2728-2731.

Van de Perre, P., Segondy, M., Foulongne, V., Ouedraogo, A., Konate, I., Huraux, J.M., Mayaud, P. and Nagot, N., 2008. Herpes simplex virus and HIV-1: deciphering viral synergy. *The Lancet Infectious Diseases*, *8*(8), pp.490-497.

Van Schalkwyk, J., Yudin, M.H., Allen, V., Bouchard, C., Boucher, M., Boucoiran, I., Caddy, S., Castillo, E., Kennedy, V.L., Money, D.M. and Murphy, K., 2015. Vulvovaginitis: screening for and management of trichomoniasis, vulvovaginal candidiasis, and bacterial vaginosis. *Journal of Obstetrics and Gynaecology Canada*, *37*(3), pp.266-274.

Vangipuram, R. and Tyring, S.K., 2019. AIDS-associated malignancies. *HIV/AIDS-Associated Viral Oncogenesis*, pp.1-21.

Vyshenska, D., Lam, K.C., Shulzhenko, N. and Morgun, A., 2017, August. Interplay between viruses and bacterial microbiota in cancer development. In *Seminars in immunology* (Vol. 32, pp. 14-24). Academic Press.

Wylie, K.M., Mihindukulasuriya, K.A., Zhou, Y., Sodergren, E., Storch, G.A. and Weinstock, G.M., 2014. Metagenomic analysis of double-stranded DNA viruses in healthy adults. *BioMed Central Biology*, *12*(1), p.71.

Zhu, J., Hladik, F., Woodward, A., Klock, A., Peng, T., Johnston, C., Remington, M., Magaret, A., Koelle, D.M., Wald, A. and Corey, L., 2009. Persistence of HIV-1 receptor–positive cells after HSV-2 reactivation is a potential mechanism for increased HIV-1 acquisition. *Nature medicine*, *15*(8), pp.886-892.

Fettweis, J.M., Serrano, M.G., Huang, B., Brooks, J.P., Glascock, A.L., Sheth, N.U., Vaginal Microbiome Consortium, Strauss III, J.F., Jefferson, K.K. and Buck, G.A., 2014. An emerging mycoplasma associated with trichomoniasis, vaginal infection and disease. *PloS one*, *9*(10), p.e110943.

Jee, B., Yadav, R., Pankaj, S. and Shahi, S.K., 2021. Immunology of HPV-mediated cervical cancer: current understanding. *International Reviews of Immunology*, *40*(5), pp.359-378.

Amabebe, E. and Anumba, D.O., 2018. The vaginal microenvironment: the physiologic role of lactobacilli. *Frontiers in Medicine*, *5*, p.181.

Audirac-Chalifour, A., Torres-Poveda, K., Bahena-Román, M., Téllez-Sosa, J., Martínez-Barnetche, J., Cortina-Ceballos, B., López-Estrada, G., Delgado-Romero, K., Burguete-García,

A.I., Cantú, D. and García-Carrancá, A., 2016. Cervical microbiome and cytokine profile at various stages of cervical cancer: a pilot study. *PloS one*, *11*(4), p.e0153274.

Happel, A.U., Kullin, B., Gamieldien, H., Wentzel, N., Zauchenberger, C.Z., Jaspan, H.B., Dabee, S., Barnabas, S.L., Jaumdally, S.Z., Dietrich, J. and Gray, G., 2020. Exploring potential of vaginal Lactobacillus isolates from South African women for enhancing treatment for bacterial vaginosis. *PLoS Pathogens*, *16*(6), p.e1008559.

Datta, P., Bhatla, N., Pandey, R.M., Dar, L., Patro, A.R., Vasisht, S., Kriplani, A. and Singh, N., 2012. Type-specific incidence and persistence of HPV infection among young women: a prospective study in North India. *Asian Pacific Journal of Cancer Prevention*, *13*(3), pp.1019-1024.

Adler, D.H., Wallace, M., Bennie, T., Abar, B., Meiring, T.L., Williamson, A.L. and Bekker, L.G., 2016. Cumulative impact of HIV and multiple concurrent human papillomavirus infections on the risk of cervical dysplasia. *Advances in Virology*, *2016*.

Kim, M., Park, N.J.Y., Jeong, J.Y. and Park, J.Y., 2021. Multiple Human Papilloma Virus (HPV) Infections Are Associated with HSIL and Persistent HPV Infection Status in Korean Patients. *Viruses*, *13*(7), p.1342.

Calcaterra, S., Zaniratti, M.S., Serraino, D., Peroni, M., Abbate, I., Cappiello, G., Piselli, P., Pavia, C., Rezza, G., Ippolito, G. and Capobianchi, M.R., 2001. Cervicovaginal shedding of TT virus in HIV-infected women. *Journal of Human Virology*, *4*(6), pp.343-345.

Wells, J.S., Chandler, R., Dunn, A. and Brewster, G., 2020. The vaginal microbiome in US Black Women: a systematic review. *Journal of Women's Health*, *29*(3), pp.362-375.

Christensen, J.K., Eugen-Olsen, J., Sørensen, M., Ullum, H., Gjedde, S.B., Pedersen, B.K., Nielsen, J.O. and Krogsgaard, K., 2000. Prevalence and prognostic significance of infection with TT virus in patients infected with human immunodeficiency virus. *The Journal of Infectious Diseases*, *181*(5), pp.1796-1799.

Paradowska, E., Jabłońska, A., Studzińska, M., Wilczyński, M. and Wilczyński, J.R., 2019. Detection and genotyping of CMV and HPV in tumors and fallopian tubes from epithelial ovarian cancer patients. *Scientific Reports*, *9*(1), pp.1-10.

Touinssi, M., Gallian, P., Biagini, P., Attoui, H., Vialettes, B., Berland, Y., Tamalet, C., Dhiver, C., Ravaux, I., De Micco, P. and De Lamballerie, X., 2001. TT virus infection: prevalence of elevated

viraemia and arguments for the immune control of viral load. *Journal of Clinical Virology*, *21*(2), pp.135-141.

Focosi, D., Maggi, F., Albani, M., Macera, L., Ricci, V., Gragnani, S., Di Beo, S., Ghimenti, M., Antonelli, G., Bendinelli, M. and Pistello, M., 2010. Torquetenovirus viremia kinetics after autologous stem cell transplantation are predictable and may serve as a surrogate marker of functional immune reconstitution. *Journal of Clinical Virology*, *47*(2), pp.189-192.

Li, L., Deng, X., Linsuwanon, P., Bangsberg, D., Bwana, M.B., Hunt, P., Martin, J.N., Deeks, S.G. and Delwart, E., 2013. AIDS alters the commensal plasma virome. *Journal of Virology*, *87*(19), pp.10912-10915.

Thom, K. and Petrik, J., 2007. Progression towards AIDS leads to increased Torque teno virus and Torque teno minivirus titers in tissues of HIV infected individuals. *Journal of Medical Virology*, *79*(1), pp.1-7.

Djaoud, Z., Guethlein, L.A., Horowitz, A., Azzi, T., Nemat-Gorgani, N., Olive, D., Nadal, D., Norman, P.J., Münz, C. and Parham, P., 2017. Two alternate strategies for innate immunity to Epstein-Barr virus: One using NK cells and the other NK cells and γδ T cells. *Journal of Experimental Medicine*, *214*(6), pp.1827-1841.

Feng, M., Duan, R., Gao, Y., Zhang, H., Qiao, Y., Li, Q. and Zhao, F., 2021. Role of Epstein-Barr Virus and Human Papillomavirus Coinfection in Cervical Intraepithelial Neoplasia in Chinese Women Living With HIV. *Frontiers in Cellular and Infection Microbiology*, p.847.

Cameron, J.E., Dennis, D.A.C., Herrel, N.R., Chapple, A.G. and Hagensee, M.E., 2020. Risk of abnormal cervical cytology in HIV-infected women testing positive for both human papillomavirus and Epstein-Barr virus in genital tract specimens. *Cancer Causes & Control*, *31*(4), pp.365-375.

Parmar, K.M., Gaikwad, S.L., Dhakephalkar, P.K., Kothari, R. and Singh, R.P., 2017. Intriguing interaction of bacteriophage-host association: an understanding in the era of omics. *Frontiers in Microbiology*, *8*, p.559.

Sabo, M.C., Lehman, D.A., Wang, B., Richardson, B.A., Srinivasan, S., Osborn, L., Matemo, D., Kinuthia, J., Fiedler, T.L., Munch, M.M. and Drake, A.L., 2020. Associations between vaginal bacteria implicated in HIV acquisition risk and proinflammatory cytokines and chemokines. *Sexually Transmitted Infections*, *96*(1), pp.3-9.

Godoy-Vitorino, F., Romaguera, J., Zhao, C., Vargas-Robles, D., Ortiz-Morales, G., Vázquez-Sánchez, F., Sanchez-Vázquez, M., De La Garza-Casillas, M., Martinez-Ferrer, M., White, J.R. and Bittinger, K., 2018. Cervicovaginal fungi and bacteria associated with cervical intraepithelial neoplasia and high-risk human papillomavirus infections in a hispanic population. *Frontiers in Microbiology*, p.2533.