VAGINAL BACTERIAL MICROBIOME PROFILES ASSOCIATED WITH HIGH-RISK HPV IN WOMEN INFECTED WITH HIV IN MERU, KENYA

THOMAS ATENYA MUTORO

A Thesis Submitted in Partial Fulfillment of the Requirements for the Conferment Master of Science in Molecular Biology of Meru University of Science and Technology

2025

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2025

DECLARATION

This thesis is my original work and has not been presented for a degree in any other institution.

SC410/202592/22

Signed _____ Date _____

Thomas Atenya Mutoro

DECLARATION BY SUPERVISORS

This thesis has been submitted with our approval as University supervisors.

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DEDICATION

This thesis is dedicated to my mother Mary, whose unwavering strength, resilience and determination for her kids' success have been the impactful force behind everything I have achieved. She made it her obligation to make sure that each one of us received a good education and an opportunity to dream big despite all the challenges, sacrifices and impossibilities she faced. Her constant encouragement and ardent belief in striving for excellence have shaped my academic journey as well as the person I have become. Therefore, this achievement is as much hers as it is mine. I also dedicate this work to my loved ones, Loise and Gavin, who stood by me through the experimental and writing process. They offered emotional support and a motivation to keep going when things felt challenging. To my wonderful siblings, Emmah, George and Nicholas, I cannot say enough thank you for the consistent help, encouragement and many ways you supported me. Your belief in my abilities always pushed me to keep going and do one more thing. Finally, I extend my heartfelt appreciation to Dr. Atunga Nyachieo, whose guidance, mentorship and encouragement came at the moment when life after campus felt overwhelming and so uncertain. His support helped me realign my focus and regain confidence in my academic and professional aspirations.

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to all those who have contributed to the successful completion of this thesis. First and foremost, I extend my heartfelt appreciation to the Center for Molecular Biosciences and Genomics (CMB) under the guidance of Dr. Frank Onyambu. Your expertise, resources, and unwavering support have been invaluable throughout my research journey. Much gratitude goes to my supervisors, Dr. Cynthia Mugo, Dr. Frank Onyambu and Dr. Joan Simam for their endless support and insightful guidance. A special thank you goes to Dr. Suzan Murray, Smithsonian Conservation Biology Institute's Global Health Program Director, for her financial support towards my masters. I am grateful to the Kenya Institute of Primate Research for providing the necessary facilities and support which shaped my interest. I am deeply thankful to my family for their unwavering love, patience, and support.

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ABBREVIATIONS, SYMBOLS, AND ACRONYMS

WHO	World Health Organization
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
HPV	Human papillomavirus
HR	High-risk
LR	Low -risk
MeTRH	Meru Teaching and Referral Hospital
PCR	Polymerase chain reaction
STIs	Sexually Transmittable Infections
CSTs	Community State types
NGS	Next generation sequencing
CCC	Comprehensive Care Clinic
SCC	Squamous cell carcinoma
AC	Adenocarcinoma
ASV	Amplicon sequence variant
CMB	Centre for Molecular Biosciences and Genomics
NSDCC	National Syndemic Diseases Control Council
HSV	Herpes Simplex Virus
LCR	Long control region
VMC	Vaginal microbial community
mNGS	Metagenomic next generation sequencing
SBS	Sequencing by synthesis

RCS	Rolling circle amplification
CPAS	Combinatorial probe-anchor synthesis
NMDS	Non-metric multidimensional scaling
Bps	Base pairs
OTUs	Operational taxonomic units

ABSTRACT

Cervical cancer, caused by Human Papillomavirus (HPV), is a global burden affecting women. The vaginal microbiome is associated with female reproductive health. Immuno-compromised HIV infected women are susceptible to persistent HPV infections. This study sought to characterize the vaginal bacterial communities in HIV-infected women using 16s rRNA sequencing and to investigate their association with HPV status. A cross-sectional study was conducted involving 38 HIV-infected women. The study was carried out at the Meru Teaching and Referral Hospital. Dry Evelyn brush self-sampling kit was used for vaginal swab collection. Genomic DNA extraction and amplification was carried out at the Centre for Molecular Biosciences and Genomics (CMB). Bacterial populations were characterized using the 16S rRNA sequencing on an MGI platform.

We identified 15,774 reads. *Lactobacillus* and *Gardnerella* were the most abundant genera in the study population. Vaginal dysbiosis-associated taxa such as *Shuttleworthia* and *Prevotella* were identified among hr-HPV positive women. Differential abundance analysis revealed an over representation of *Lactobacillus* genus in the hr-HPV positive group. High microbial diversity was observed in both hr-HPV positive and hr-HPV negative women.

In conclusion, this study highlights the bacterial taxa in the vaginal microbiota and the complex relationship between vaginal microbiome and hr-HPV infections in Kenyan women living with HIV. HIV infection is associated with a more diverse vaginal microbiota, however, the overall composition between the hr-HPV-positive and the hr-HPV-negative women is similar. This could be attributed to HIV infection having already distorted the diversity of the bacterial populations.

CHAPTER ONE: INTRODUCTION

Cervical cancer is the fourth most common cancer among women globally. In 2022, approximately 662,301 new cases and 348,874 deaths of cervical cancer were reported globally (Globocan, 2022). Approximately 98% of cervical cancer cases are caused by high-risk human papillomavirus (hr-HPV), with HPV types 16 and 18 causing 70% of the cases (Tesfaye et al., 2024). In Sub-Saharan Africa, cervical cancer is the most common cancer among women. Africa has a high cervical cancer burden with an estimated incidence of approximately 125,699 cases and an approximate mortality of 80,614 (Globocan, 2022). Cervical cancer affects 14% of women in Sub-Saharan Africa, a region which accounts for 18% of cervical cancer-related deaths (Klein et al., 2019). In Kenya, cervical cancer is the second most common cancer among women and the leading cause of cancer-related deaths with an incidence of 5845 and mortality of 3591 (Globocan ,2022).

Kenya has a total of 1,377,784 people living with HIV as per the National Syndemic Diseases Control Council (NSDCC) statistics with a 5.31% prevalence among women (NSDCC 2023). The overall prevalence of HIV in Meru County is 2.5%. The HIV prevalence among women in Meru is 3.7% while HIV prevalence in men stands at 1.3% (NSDCC 2022). The National Syndemic Diseases Control Council estimates that 782 new HIV infections occur in the county annually (NSDCC 2022).

The high prevalence of HIV in Kenya is a contributing factor to the high burden of cervical cancer in the country (Carter et al., 2021; Eastment et al., 2022). There is a substantial correlation between HIV and HPV-associated dysplasia, with women living with HIV having a higher prevalence of hr-HPV genotypes compared to HIV-negative women in sub-Saharan Africa (Castle et al., 2020; Klein et al., 2019; Taku et al., 2020a; Tchouaket et al.,

2023). HPV is a sexually transmitted infection and it is the most common cause of cervical cancer and cervical intraepithelial neoplasia (CIN) (Qingqing et al., 2021). HIV-infected women have a compromised immune system and thus have a higher risk of developing STIs among them, HPV (WHO,2024).

The microbiome refers to a collection of living microbes that live in biological systems. There exist various microorganisms comprising of bacteria, fungi, viruses, archaea on the body surface where the human body supports the life of these microorganisms (Z. Ma et al., 2024; Nieves Delgado & Baedke, 2021) . The human microbiome constitutes a community made up of symbiotic microbes and functions in the human physiology and general body fitness as well as their repetitive genomes (Z. Ma et al., 2024; Y. Wang et al., 2023). A healthy vaginal microbiome is dominated by *Lactobacilli spp*. which modulates the genital health through the production of lactic acid and hydrogen peroxide (Sharifian et al., 2023). A *Lactobacillus*-dominant vaginal microbiome protects women against invading pathogens whereas HPV thrives in a diverse microbiome with an abundance of *Gardnerella vaginalis* and *Atopobium vaginae* and other facultative anaerobes (Santella et al., 2022a; Sharifian et al., 2023).

The immune system status and the vaginal microbiome represent the risk factors for HPV-induced cervical cancer (Aggarwal et al., 2023; Guo et al., 2022; Shen et al., 2024; Zeng et al., 2023). The microbiota and the human host have a homeostatic and mutualistic interaction. This balance, however, can be upset by internal or external factors such as hormonal status, age, and immunological status (Carter et al., 2021). A perturbed microbiota generates DNA damage, which eventually leads to lesions and finally cervical cancer development (Carter et al., 2021). HPV infection is associated with increased microbial

diversity and a lower relative abundance of vaginal commensals including *Lactobacillus*, *Bifidobacterium* and *Atopobium* (Bogale et al., 2020; Zayats et al., 2022). One study found that *Fusobacterium spp.* genera is significantly correlated with HPV infection (Lee et al., 2013). Moreover, hr-HPV is associated with an abundance of anaerobes such as *Prevotella*, *Sneathia* and *Leptotrichia* (Dareng et al., 2016; Wei et al., 2021). Vaginal microbiome composition has also been found to influence HPV clearance, with *Atopobium*-dominated communities showing the slowest rate of remission (Dai et al., 2021).

Few studies describing the vaginal microbiome of HIV infected women have been conducted in Kenya, most of which are cross-sectional. These studies were only confined to major cities including Nairobi, Mombasa and Kisumu (Eastment et al., 2022). The results from these studies were restricted to limited populations such as transactional sex workers and or pregnant women, who are not representative of the entire female population in the country (Carter et al., 2021; Eastment et al., 2022). This study sought to characterize the vaginal microbiome of HIV infected women in Meru County and explored a relationship between HIV, HPV status and the vaginal bacteria microbiota. Performing microbiome analysis for HPV in women living with HIV enhanced our understanding of HIV disease comorbidities, and we present it as a guide to public health policy. We identified interactions between bacterial communities in the vagina and their associations with HPV in patients with immunodeficiency status.

1.2 Problem Statement

Kenya has a total of 1,377,784 people living with HIV with the HIV prevalence standing at 5.31% among women (NSDCC 2023). HIV-infected women have a compromised immune response which puts them at a higher risk of developing STIs among them HPV (Liu et al.,

2018). In 2022, approximately 662,301 new cases of cervical cancer and 348,874 deaths were reported globally (Globocan, 2022). Cervical cancer represents the most common cancer and the leading cause of cancer-related deaths among women in sub-Saharan Africa (Seyoum et al., 2023). HIV-positive women have a six-times higher risk of being infected with HPV, multiple HPV genotypes and HPV persistence than HIV-negative women (WHO 2022). Women living with HIV also report higher rates of high-grade cervical pre-cancer and invasive cervical cancer than HIV-negative women (Sweet et al., 2020).

In Kenya, cervical cancer is the leading cause of cancer-related deaths with cervical cancer representing 12% of the total cancer burden in the country (Gitonga et al., 2022; Mwaliko et al., 2023). Despite a number of studies documenting the HIV-HPV association, a link between HIV, vaginal microbiome and HPV is lacking. Limited number of studies have been conducted in Kenya, most of which are cross-sectional. Alterations in the vaginal microbiome have been linked to a range of cervical and reproductive health issues, including cervical cancer, bacterial vaginosis, preterm birth, and infertility. The high risk of developing cervical cancer in HIV-positive women remains a major concern despite the limited number of studies and the knowledge gaps on the relationship between the vaginal microbiota and HPV in HIV infected women in Kenya.

The availability of scanty resources and infrastructure for conducting microbiome analysis in hospitals across Kenya further exacerbates the knowledge gap of lack of comprehensive understanding of the relationship between the vaginal microbiome and HPV infection in HIV-infected women. Furthermore, the limited number of studies in Kenya hampers our ability to determine the specific alterations in the vaginal microbiome that occur in HIV-infected women with HPV infections as well as identify potential dysbiosis patterns

associated with HPV. Besides, scanty literature exists on defined vaginal microbiota in Kenyan HIV positive women, with available data from cross-sectional studies being geographically limited. This data is unreliable in depicting the vaginal microbiota status in Kenyan HIV positive women since it varies by ethnicity, geographical location and lifestyle. This creates a knowledge gap in the Kenyan population, necessitating further research on this link in various populations and geographical places with high HIV and cervical cancer prevalence throughout Kenya, including Meru County.

In summary, while there is some evidence to suggest that the vaginal microbiome is associated with HPV infection, many questions remain unanswered regarding the precise nature of this relationship and the potential implications for HPV prevention and treatment. It thus calls for further studies to fill these gaps.

1.3 Justification

HIV infection leads to a suppressed immunity which causes a change in the vaginal microbiome leading to a more diverse microbiome. This change increases the risk of persistent HPV infections in the HIV infected women who develop lesions leading to cervical cancer. Kenya has a high HIV prevalence of 5.31% among women who are in the reproductive age. HIV-infected women having a compromised immune system are thus at a higher risk of developing STIs among them HPV. This high HIV rate contributes to the high cervical cancer burden due to a risk of developing STIs and a pathogenic vaginal microbiome. Women living with HIV also have reduced HPV clearance rates and an increased risk of persistent HPV infection compared to HIV-negative women. Studies have shown that HIV-positive women are 1.5 to eight times more likely to have cervical cancer than HIV-negative women (Kudela et al., 2021; Taku et al., 2020b). There is a higher hr-

HPV prevalence, hr-HPV viral load, and cervical lesions among women who are HIV-positive compared to those who are HIV-negative. Studies have also shown that the type of bacteria present within the vaginal microbiome influences the body's ability to combat the HPV infection. However, the precise nature of this relationship is still not well understood and the specific bacterial species or communities strongly associated with increased or decreased risk of HPV infection are not well understood.

By studying the relationship between the vaginal microbiome and HPV status, the study may be able to identify new strategies for diagnosing and preventing hr-HPV and curbing its progression to cervical cancer. Understanding the relationship between the vaginal microbiome and HPV in HIV infection may help identify subgroups of women who are most at risk for cervical cancer, and who may benefit most from targeted prevention or treatment interventions.

1.4 Research Questions

i. What is the bacterial composition of the vaginal bacterial microbiome of HIV-infected women in Meru, Kenya?
ii. What is the relationship between the vaginal bacterial microbiome and hr-HPV status of HIV-infected women in Meru, Kenya
iii. What is the genetic diversity of the vaginal bacterial microbiome in HIV-infected women in Meru-Kenya?

1.5 Research Objectives

1.5.1 Main objective

To investigate the vaginal bacterial microbiome profiles associated with high-risk HPV in women infected with HIV in Meru County, Kenya.

1.5.2 Specific Objectives

i. To characterize the vaginal bacterial microbiome of HIV-infected women using a 16s rRNA metagenomics in Meru, Kenya.

ii. To establish the relationship between vaginal bacterial microbiome and hr-HPV infection status in Meru, Kenya.

iii. To describe the genetic diversity of vaginal bacterial microbiome in HIV-infected women in Meru, Kenya.

1.6 Significance of the Study

This study will generate valuable insights into vaginal microbiome, and high-risk HPV relationship in women living with HIV in Meru County, Kenya. African populations are underrepresented in global studies and having data on this population is indicative of vaginal microbiome status in the county and county. The 16S rRNA sequencing identified the bacterial taxa associated with vaginal health and pathogenic ones associated with disease. The results have clinical and public health practice identifying bacterial taxa linked to dysbiosis and the probiotics that can help. The study findings will contribute to local evidence-based data for the country's effort in cervical cancer prevention and management of those living HIV.

1.7 Limitations of the Study

One of the main limitations of this study was the small sample size, which resulted in reduced statistical power to detect differences in the vaginal microbiome composition and diversity between women with hr-HPV and those without. The second limitation was the absence of an HIV-negative control group. This made it difficult to determine if the observed vaginal microbial dynamics were specifically restricted to HIV-infected women or

could they be extrapolated to the general women population. Another limitation was the inability to assign taxonomy up to the species level due to amplicon fragmentation, which hindered us from assigning community state types (CSTs). Moreover, since this was a nested study, we were unable to collect data on the genital hygiene habits and vaginal dysbiosis indicators such as vaginal pH, clue cells, Nugent score and discharge appearance. Therefore, this limited us from evaluating the relationship between genital hygiene habits as well as vaginal dysbiosis parameters and microbiome composition and alpha diversity. Finally, the self-sampling procedure raised concerns over possible contamination with skin microbiota and sufficient collection of cervicovaginal cells and secretions.

1.8 Delimitations of the Study

This study was delimited to a cohort of 38 HIV positive women attending the critical care clinic at Meru Teaching and Referral Hospital (MeTRH) and who were between 25-59 years of age. Self-sampling technique was employed to obtain the vaginal swabs which allowed study participants to collect their own specimen, ensuring privacy of the participants. The microbiome investigation was solely restricted to bacterial communities in the vaginal microbiome which were analyzed using the 16S rRNA sequencing that targeted the V3-V4 region. The study only focused on the association between vaginal bacterial microbiome and HPV status in HIV infected women living in Meru County. The findings from this study are limited to the HIV positive group in Meru and the results cannot be generalized to HIV negative women or HIV positive women outside the demographic or this geographical region.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Cancer refers to a group of diseases that arise from the unchecked proliferation of abnormal cells in the body (Gitonga et al., 2022; Mutiso, 2023). It is characterized by the inability of the body to control cell growth, cell differentiation, and the normal programmed cell death. This unchecked growth contributes to malignant cancerous cells invading surrounding cells, tissues and eventually other organs. Cervical cancer (CC), refers to a type of cancer that occurs in the cervix cells of a female reproductive system. The cervix plays a vital role in reproductive health and also involved in malignant transformation of cervical epithelial cells giving rise to cervical cancer. Cervical cancer is caused by various strains of the human papillomavirus (HPV) which is a sexually transmitted infection (STI) (Castanheira et al., 2021). As mentioned in the introduction, HPV types 18 and 16 has been reported to be responsible of majority of the cervical cancer cases.

In terms of incidence and mortality, cervical cancer is ranked as the second most common cancer behind breast cancer in lower Human Development Index (HDI) (Gitonga et al., 2022; Wang et al., 2022). Limited access to cervical cancer screening services, societal constraints to HPV vaccination further exacerbates this burden. Cervical cancer is the fourth most common cancer in women worldwide with an estimated prevalence of 662,301 new cases and approximately 348,874 cervical cancer deaths annually as reported in 2022 (Globocan, 2022) . In Kenya, CC ranks second and is the most frequent cancer among women between 15 - 44 years of age (Eastment et al., 2022; Gitonga et al., 2022; Mwaliko et al., 2023). This highlights the disease burden in women navigating reproduction which calls for creating

awareness to cervical cancer and improve diagnostic services in reproductive age women residing in lower income countries.

Cervical cancer is the most common diagnosed cancer in 28 countries and the leading cancer mortality cause in 42 countries most of which are domiciled in Sub-Saharan Africa (Wang et al., 2022). Africa registered a total of 117,316 and 76,745 new cervical cancer cases/deaths respectively (Globocan statistics, 2020). However, North America, Western Asia, Australia and New Zealand reported 7-10 times lower rates than those reported in Sub-Saharan Africa (Wang et al., 2022; Zhang et al., 2021). In Kenya, the annual number of new cervical cancer cases/deaths are 5236 and 3211 respectively (HPV Information Centre 2023). In total, eighty four (84%) of the new CC cases and between eighty seven (87%) and ninety (90%) of the CC deaths occur in low- and middle-income countries (LMICs) (Hull1 et al., 2020). The disproportionate cervical cancer burden reveals the much-needed interventions including routine screening of women in the reproductive age bracket and expanded HPV vaccination of young girls in LMICs.

Cervical cancer can be divided into two histological types; adenocarcinoma (AC) and squamous cell carcinoma (SCC). Squamous cell carcinoma is the most common type of cervical cancer with an occurrence rate of over 70%. It originates from squamous cells lining the outer part of the cervix that opens to the ectocervix while AC originates from columnar glandular cells lining the cervical canal (Hull1 et al., 2020). An understanding of these histological subtypes helps understand the origin, pathogenesis and treatment procedure for a given cervical cancer type. Cervical cancer is mostly caused by the human papillomavirus (HPV) but other risk factors exist which include: reproductive and sexual factors, behavioral factors entailing young age sexual intercourse, multiple sexual partners,

smoking, low socio-economic levels and higher parity (Wang et al., 2022; Zhang et al., 2021).

The microbiome refers to a collection of living microbes that live in biological systems. There exist various microorganisms on the body surface where the human body supports the life of these microorganisms. Bacteria, fungi, viruses, protozoa make up the microbial communities. Humans and microbes coexist in a mutualistic relationship. Humans provide an important habitat for the thriving of microbes while microbes play a crucial role in normal body function development including modulation of the immune system, nutrient absorption and protection of the body from infections (Kombe et al., 2021; Yu et al., 2023). The human microbiome constitutes a community made up of symbiotic microbes and functions in the human physiology and the general body fitness (Chen et al., 2020; Wang et al., 2019; Yu et al., 2023). The female reproductive tract is a dwelling place to many microbes which affect human health and disease. The vaginal microbiome is mostly associated with the bacterial species found in the vagina of the female reproductive system and which function in maintaining the vaginal and cervical health of an individual.

The vaginal microbiome balance is influenced by a number of factors. Epidemiological factors including dietary habits, contraception, smoking and sex life, make up the contributing factors to the dynamics of the vaginal microbiome (Kombe Kombe et al., 2021; Kudela et al., 2021; Santella et al., 2022a). Environmental factors including geographical location, sanitary conditions and socioeconomic status also influence the type of bacterial species present in the vaginal microbiome. These factors influence the susceptibility of different populations to different or similar diseases (Guo et al., 2022; G. Liu et al., 2018; L. Liu et al., 2014; S. Liu et al., 2022; Zhang et al., 2021)

2.2 HIV /AIDS in Sub-Saharan Africa

Human Immunodeficiency Virus (HIV) is a chronic inflammatory disease that leads to the long-term immune dysfunction, which arises from the ability of the virus to deplete CD4+ T-lymphocytes and the normal immune pathways. Moreover, it is further influenced by the microbiome which contributes to systemic and mucosal inflammation (Gosalbes et al., 2022). HIV remains to be a global burden even after major breakthroughs with the anti-retro therapy (ART). Emergence of ART has helped reduce AIDS-related mortality and disease morbidity but the immune function is still affected leading to creation of persistent viral reservoirs.

Sub-Saharan Africa (SSA) still carries the largest HIV burden with 58% of new infections being reported in women in general and 25% among young women (Armstrong et al., 2023; Mtshali et al., 2021). This high incidence in young women of the reproductive age as compared to their male counterparts is associated with factors including but not limited to biological factors such as mucosal microenvironment and mucosal surface viral exposure duration (Mtshali et al., 2021). Women and adolescent girls having a more permeable cervical epithelium correlates with high prevalence of sexually transmitted infections making them more vulnerable.

Increased number of sexual partners, lack of protection measures such as engaging in condomless sex, and engagement of young women in transactional sex have also significantly contributed to the high risk of acquisition of HIV among adolescent girls and young women in sub-Saharan Africa (Floyd et al., 2022). Gender-based violence, intergenerational sexual relationships and socio-economic instability further increases the aforementioned risk. The prevalence and incidence of HIV is not evenly distributed but is

rather distributed spatially across the globe. Regionally, the distribution is also inconsistent with SSA taking the highest burden as compared to the rest of the world. There exist within countries disparities influenced by socio-economic gradients where marginalized communities report higher prevalence rates as a result of structural disadvantages, poverty and limited access to healthcare. Informal settlement areas in urban centers and cities tend to report higher incidences of HIV as compared to affluent settlement patterns. This was revealed by a study conducted in Kenya and Namibia's capital cities; Nairobi and Windhoek respectively; which compared urban slum and non-slum urban residences. The study findings reported a 12% HIV prevalence in informal settlements and a 5% HIV prevalence in affluent settlements (Gibbs et al., 2020). The findings from these studies showcase the interplay between socio-economic factors, environmental differences and vulnerability of communities in HIV transmission dynamics.

Majority of the viral infections, with HIV as an example are initiated at the cell mucosal surfaces of the body. Despite these mucosal tissues serving as physical and immunological barriers, they are points of entry of disease-causing pathogens made possible by large surface area and direct environmental exposure. The female reproductive tract mucosal surface holds a rare viral infection susceptibility to the globally recognized viral pathogens including HIV, Herpes Simplex Virus-1 (HSV-1) and 2 and HPV (Gustin et al., 2021).

The study by Sortino et al 2020 reported that the acute phase of HIV infection disrupts the immune system of the patient's gut which consequentially leads to gut dysbiosis. This gut dysbiosis is characterized by a reduced beneficial bacterial population of *Firmicutes* and *Bacteroidetes* whereas increasing *Proteobacteria* and *Fusobacteria* which are pro-inflammatory taxa. Such microbial shifts resultantly weaken metabolic and immunological

homeostasis. This shifting in microbial taxa resultantly contributes to the chronic inflammation due to activation of the immune system (Sortino et al., 2020). The findings correlate with those of Ishizaka et al, that examined the gut dysbiosis-chronic inflammation relationship in HIV patients on antiretroviral therapy which reported that despite the effectiveness of Antiretroviral Therapy (ART) on suppressing the viral load in HIV patients, gut microbial imbalance still contributes to the persistence of chronic inflammation (Art et al., 2021). The persistent inflammation has been shown to accelerate the ageing process and increasing non-communicable disease infection risk, a phenomenon showing a complex relationship between HIV, ART and the microbiome dynamics of the host.

2.3 Human Papillomavirus

The Human papillomaviruses are double-stranded DNA viruses that belong to the distinct Papillomaviridae family and fall within the *Firstpapillomavirinae* subfamily. This subfamily is adapted to epithelial tissue infections across different bodily anatomical sites. *Firstpapillomavirinae* subfamily is characterized by a 72 capsomer icosahedral capsid which is a highly organized protein shell that plays a role of protecting the viral genome (Sharifian et al., 2023; Tosado-Rodríguez et al., 2024). Human papillomavirus is a small non-enveloped, epitheliotropic icosahedral DNA virus measuring about 50 to 55 nm in diameter. The absence of an envelope enhances its stability during environmental changes and facilitates the virus transmission by enabling its ability to survive on moist surfaces.

There are fourteen (14) HPV species with about two hundred genotypes from which only 40 genotypes are able to infect the epithelial cells of the anogenital tract and other mucosal surfaces (Tosado-Rodríguez et al., 2024). The HPV virion is comprised of a single molecule, which is a histone-bound double-stranded circular DNA that weighs approximately 8kb. The

virion is compactly packaged to facilitate efficient replication and regulate transcription when the virion enters the host cell. HPV has an 8 protein-coding gene that is further organized into 3 regions. These include region one (1) which is a noncoding regulatory long control region (LCR) that contains the promoter, enhancer and silencer sites which are necessary for the timing and level of the viral expression by acting as the central command system.

Region 2 entails an early region which spans from E1 to E7 and its involved in viral replication and cellular transformation. E1 and E2 proteins of region two act as the origin of replication recognizers that facilitate the replication of the viral DNA, whereas the E2 protein also plays a part as a viral gene transcription regulator (Sharifian et al., 2023). E4 and E5 are involved in later replication stages of the viral life cycle by supporting the maturation of the virion, evading the immune surveillance and finally modulating the signaling pathways of the host cells. E6 and E7 proteins are involved in targeting several negative cell regulators that primarily consist of p105Rb and p53 which are regulate apoptosis and thus there is uncontrolled cell proliferation if they are impacted. These early proteins help maintain the stable viral episomal state and stimulate the S phase return of differentiated cells. The last region, region 3, is a late region that comprises of L1-L2 encoding major and minor capsid proteins that are vital for virion assembly (Badial et al., 2018; Niya et al., 2017; Taku et al., 2020a).

HPVs are primarily sexually transmitted where, in most cases the infections generated by the virus result in asymptomatic cases which regress autonomously in a short time (Kombe Kombe et al., 2021; Omame et al., 2021). The human papillomavirus is the most common sexually transmitted infection globally. Majority of HPV infections are always being cleared

quickly by a non-compromised immune system but high-risk human papillomaviruses (hr-HPVs) can persist increasing the likelihood of progression to precancerous lesions and eventually cause cervical cancer development (Shannon et al., 2017). More than one hundred (100) HPV types exist of which, serotypes 16 and 18 alone are related to about 70% of all cervical cancers (CC) and high-grade precancerous cervical lesions (Chen et al., 2022; Santella et al., 2022b). HPV is the most common sexually transmitted virus, with its peak prevalence observed in adolescents and young women, particularly soon after sexual debut but decreasing prevalence with increase in age as the immunity develops (Id et al., 2021; Mbulawa et al., 2018).

The human papillomavirus is divided into 5 genera based on the homology of the DNA sequence in the L1gene. The five genera include the Alpha, Beta, Gamma, Mu and Nu where Alpha papillomaviruses specially infect both the cutaneous and mucosal epithelium while other genera selectively infect the cutaneous epithelium (Sharifian et al., 2023). HPV is further divided into two categories: low-risk HPVs (lr-HPVs) that is responsible for benign anogenital and cutaneous warts, and high-risk HPVs (hr-HPVs) responsible for oropharyngeal (oral, tonsil, and throat areas) cancers and anogenital cancers, which include cervical, anal, vulvar, vaginal and penile cancers. Low risk (LR) HPV types 6 and 11 are major causes of anogenital warts which contribute to psychosocial distress and physical discomfort (Chikandiwa et al., 2018; Van De Wijgert et al., 2020).

Hr-HPVs demonstrate a strong oncogenic potential. These include types 16,18,31,35,39,45,51,52,56,58,59,68,73 and 82 (De Brot et al., 2017). Of these, the most clinically significant high-risk HPV types include type 16,18,31,33,35,39,45 and 53 while type 6, 11,40,42,43,44,54,61 and 70 are considered to be of lower risk (Niya et al., 2017).

Persistent infection with the oncogenic human papillomavirus is the main factor responsible for the progression of cervical lesions to the resultant cervical cancer (Wei et al., 2021). The Human Papillomavirus clears spontaneously on its own in more than 90% of infections in a period between 6 and 8 months. However, about 10% of the total infected women population exhibit the viral persistence which increase for the pathological progression to pre-cervical cancer (Di Paola et al., 2017). The persistence is modulated by the individual immune system, the viral genotype involved, comorbid infections and the bacterial communities present in the female reproductive system.

Papillomaviruses are not only host species-specific, but also tissue-specific, multiplying only in skin epithelial cells or certain mucous membranes. Different HPV types have a preference for different parts of the body (Peter A et al 2020). It is not uncommon for virus autoinoculation to cause new superficial lesions, but systemic dissemination does not occur. In general, HPV types are classified as cutaneous (infecting the skin) or mucosal (infecting the genital tract and occasionally the respiratory tract, oral cavity, or conjunctiva). Furthermore, complete replication and virus particle production occur only in the differentiated superficial layers of squamous epithelium. Warts can live for up to two years in their incubation period. Through an abrasion, the virus enters the skin and infects the basal cell layer (Hatano T et al · 2021). Only early viral genes are expressed in these relatively undifferentiated replicating cells, and limited viral DNA replication keeps the genome stable as a nuclear plasmid (episome).

After several months of incubation, the expression of early viral genes stimulates basal cell proliferation, resulting in acanthosis (thickening) and, in general, a protruding papilloma. Only terminally differentiated keratinocytes present in the outer layers of the epithelium

produce capsid proteins and virions; these cells produce keratin but no longer divide (John E. B 2020). Koilocytes are large cells with perinuclear vacuoles and hyperchromatic, distorted nuclei found in the differentiated layers of the epithelium.

Skin warts are distinguished by hyperkeratosis. Warts usually disappear within a couple of years, usually synchronously. This abrupt regression is not related to antiviral antibody titers and is generally attributed to the T cell-mediated immune response. Indeed, when multiple warts are present, the lesions usually regress at the same time, and removal of one wart can be followed by spontaneous regression of the remaining warts, possibly due to immune stimulation by antigens released after treatment (Christopher J. B 2017).

However, the nature of the mechanism by which T cell immunity is induced, as well as why this response is so delayed, is unknown. In immunocompromised people, more widespread, persistent skin warts can be a problem. The later recurrence of warts after treatment, for example, in the larynx, and the appearance of multiple warts after immunosuppression, however, suggest that long-term latent infection of basal epithelial cells is very common. Although the papillomas caused by HPV on the external genitalia are fundamentally similar to those described previously, lesions around the cervix differ significantly.

The virus enters the body during sexual contact, possibly through a minor abrasion near the squamocolumnar border, where cells are proliferating. A flat condyloma develops after an incubation period of one or more (usually three) months (Kombe A.J et al 2020). Infections with specific HPV types can progress over several years from cervical intraepithelial neoplasia to invasive squamous carcinoma. Cervical dysplasia Papanicolaou smears show the characteristic koilocytes. The HPV genome can survive as a non-integrated nuclear

episome for years, not only within the lesion but also in histologically normal surrounding cells of the mucous membrane for a few centimeters.

Geographic, social, cultural, and genetic factors related to viral genome variability, as well as individual characteristics such as age, gender, anatomic site, and health state, are all connected with hr-HPV morbidity and mortality (Kombe Kombe et al., 2021; Nieves-Ramírez et al., 2021).

Integration of hr-HPV into the host genome exert their oncogenic effects through expression of the HPV E6 and HPV E7 oncoproteins which play a role in inducing HPV-dependent malignant transformation and which are expressed in cervical cancer (Badial et al., 2018). The E6 and E7 oncoproteins target tumor suppressor proteins p53 and retinoblastoma protein (pRb) that induce cell proliferation, lead to inhibition of apoptosis and promote genome instability and evasion of immune system which is worse in already immunocompromised HIV- positive women (Cosper et al., 2021; Szymonowicz & Chen, 2020).

Genetic factors influencing mucosal immunity or metabolic pathways that result in preferential conditions for particular species arise from the ethnic differences which contribute to the disparities in the disease associated with HPV. (Chorna et al., 2020; Taku et al., 2020a). Studies conducted among Black South African women demonstrated a highly diversified cervical microbiota, suggesting that the microbial communities in the vagina and the cervix could be a predisposing factor to high HPV burden in HIV infected black women (Chorna et al., 2020; Taku et al., 2020a). these findings demonstrate a complex relationship between the host immunity, the microbial composition and socio-demographic factors in HPV viral pathogenesis.

2.4 Composition of the Vaginal Microbiome

The human microbiota refers to the complex community of predominantly bacterial microorganisms that reside in various anatomical sites including the skin, the gastrointestinal tract and the oral cavity of a given individual. The microbiota coexist with the host in a mutualistic relationship where it plays a role on physiological processes including protection against invading pathogens, immune modulation and metabolism of several components (Baud et al., 2023; Kho & Lal, 2018). The symbiotic community of microorganisms vary across different regions of the body and between individuals and provide extra metabolic pathways that affect some important physiological functions (Rodríguez et al., 2024). The microbial ecosystem at each anatomical site is shaped by the local biochemical conditions as well as the immune status of the body. The vaginal microbial community (VMC), refers to the collective reference of microorganisms found in the vagina whose composition and function is specific female reproductive tracts (Baud et al., 2023). This microbial community plays a role in modulation of female health throughout their entire reproductive lifespan through influencing the susceptibility to infections.

The female vagina also has glycogen which is deposited there under the influence of estrogen. Glycogen functions as a *Lactobacillus* species substrate that help in the lactic acid production which in turn maintains the low pH in the vagina that play a role in suppressing the thriving of pathogens. It is colonized by a diverse array of microbial communities that then respond to the selective glycogen pressure (Ferlay et al., 2021; Lee et al., 2013).

The vaginal microbiome is a low diversity microbiome compared to the bacterial communities in other anatomical sites and the low diversity status predominantly inhabited

by *Lactobacillus* plays a crucial role in the women health both in reproductive age and general health (De Seta et al., 2022; Vieira-Baptista et al., 2022). Apart from *Lactobacillus* being the most abundant, other genera including *L. iners, Bacteroides, Fusobacterium, Veillonella, Actinomycetes, Bifidobacterium, Peptococcus, Peptostreptococcus, Propionibacterium, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis, Gardnerella vaginalis and Prevotella bivia* exist as part of the vaginal microbiota, although at low proportions (Sharifian et al., 2023). Depletion of *Lactobacilli* and enrichment with obligate anaerobic bacteria of *Gardnerella vaginalis*, *Prevotella spp, Atopobium vaginae* and *Mycoplasma* is characteristic of a dysbiotic vaginal microbiome and bacterial vaginosis (Caselli et al., 2020; Curty et al., 2017; H. Wang et al., 2019). The shift from *Lactobacillus* abundance to pathogenic bacteria has been indicated to contribute to an increase susceptibility to STIs including HIV and HPV.

The presence or absence of certain microbial species is determined by host factors and environmental factors that are varied across regions which include host genetics, immune status, sexual behavior, hygiene practices and nutrition (Mancabelli et al., 2021). The female vagina represents an example of this mutual relationship existence with its resident bacterial species which seem to play a role in wading off infections by maintaining acidic pH and producing antimicrobial metabolites (Sasivimolrattana et al., 2022; Winer & Koutsky, 2004). A *Lactobacillus*abundant microbiota particularly plays a role of protection against invading pathogens whereas HPV thrives in a compromised more diverse microbiota which creates a conducive ecological environment for HPV viral persistence (Santella et al., 2022a). A healthy vaginal environment is characterized by low degree of bacterial diversity and dominance of *Lactobacillus* species that play a role in prevention of exogenous pathogenic

colonization by maintaining a low pH and producing toxic antibacterial metabolites (Caselli et al., 2020).

The female reproductive system comprises its own unique microbiota which undergoes hormonal changes during the menstrual cycle, pregnancy, menopause and other physiological conditions (Reimers et al., 2016; Soleymaninejadian et al., 2022). Vaginal microbiome was first defined by microbial community structure by Rave et al 2011, who classified the vaginal microbiome into 5 community state types (CSTs I - V) (France et al., 2020; Ravel et al., 2011; Shen et al., 2024). CSTs I, II, III and lastly V are dominated by a different *Lactobacillus* species with CST I having *L. crispatus*, CST II *L. gasseri*, CST III *L. iners* and CST V *L. jensenii*. These clusters of *Lactobacillus* dominance are associated with low vaginal pH, reduced risk of infection and a stable vaginal microenvironment.

CST IV was classified to be heterogenous comprising of a diverse array of facultative or strict anaerobic bacteria with *Lactobacillus* being depleted. CST IV has been further subdivided into CST IV-A, IV-B and IV-C. *Candidatus Lachnocurva vaginae* is relatively abundant in CST IV-A while *Gardnerella vaginalis* is the species that dominates CST IV-B (Sharifian et al., 2023; Shen et al., 2024). CSTs IV-A and IV-B both have moderate relative abundances of *Atopobium vaginae* while CST IV-C is further subdivided into 5 sub-CSTs. These include CST IV-C0 dominated by a moderate amount of *Prevotella,* CST IV-C1 dominated by *Streptococcus,* CST IV-C2 dominated by *Enterococcus,* CST IV-C3 dominated by Bifidobacterium and CST IV-C4 dominated by *Staphylococcus* (Dou et al., 2024; Shen et al., 2024). This CST IV subdivisions are associated with vaginal dysbiosis, inflammation as well as being susceptible to the acquisition and persistence of HPV.

The gut microbiota also indirectly affects the composition of the vaginal microbiota through the gut-vaginal axis in addition to the vaginal microbiome (Wessels et al., 2018). A collection of gut bacteria and their estrogen adapted metabolizing genes are referred to as oestrobolome. The gut bacteria influence the vaginal microbiota content by estrogen-mediated machinery (Dou et al., 2024; Wei et al., 2021). The oestrobolome influences the type of vaginal microbiome existing at a certain time point in woman's life. This results to the variability of the vaginal microbiome of an individual woman throughout their lifetime (Irina et al., 2024). The oestrobolome secrete β-glucuronidase and β-glucosidase enzymes. These enzymes lead to deconjugation of hepatically conjugated oestrogen which influences their reabsorption to the circulation henceforth (Sharifian et al., 2023).

The unconjugated free oestrogen is then transported to distal sites including the lower female reproductive system where it binds to its receptors triggering intracellular signaling that eventually increase the production of glycogen as well as other physiological changes like production of mucus and epithelium thickening (H. Wang et al., 2019). Glycogen increase leads to increased *Lactobacillus spp* growth and prevention of HPV entry to host cell thus the speculation that a low gut microbiota diversity could negatively impact the vaginal microbiota composition through the oestrobolome. Altered oestrobolome reduces *Lactobacillus* abundance making women vulnerable to STIs including HPV. *Lactobacillus spp*. limit the other taxa including potential pathogens from colonizing the vagina by maintaining an acidic pH below 4.5 (Baud et al., 2023).

2.5 Association of the Vaginal Microbiome and HPV

The vaginal microbiota is important in HPV infection, persistence and eventual development of cervical cancers as a result of hr-HPV infection. Sexual intercourse has a huge influence

on the vaginal microbiota whereby during sexual activity, microorganisms are temporarily introduced that disturbs the microbial balance which are restored after sometime. Although the vaginal microbiome returns to stable conditions after intercourse, the number of sexual partners and the frequency of sexual intercourse significantly influence the vaginal microbiota stability (Mbulawa et al., 2018). Genital tract infections can be broadly categorized into two; the endogenous and exogenous infections. Excessive proliferation of aerobic or anaerobic bacteria in the female reproductive tract are responsible for endogenous infections (Dou et al., 2024). Exogenous infections comprise of the common sexually transmitted disease pathogens (STDs). These results from specific sexually transmitted pathogens including *Neisseria gonorrhoeae* (*N. gonorrhoeae*), *Chlamydia trachomatis* (*C. trachomatis*), *Ureaplasma urealyticum* (U. urealyticum), *Mycoplasma genitalium* (M. genitalium), Herpes Simplex Virus Type II (HSV II), *Trichomonas vaginalis* (*T. vaginalis*), *Candida albicans* (*C. albicans*), Human Papillomavirus (Dou et al., 2024). The propagation of these pathogens is enabled by a dysbiotic vaginal microbiota which provide a conducive environment for the virus to infect and persist.

Lactobacillus spp. , specifically *L. crispatus*, *L. jensenii*, *L. gasseri* dominates a healthy vaginal microbiota that functions by producing various antibiotic compounds including bacteriocins, reactive oxygen species, hydrogen peroxide and lactic acid against dysbiotic bacteria (Carter et al., 2021; Y. Chen et al., 2020; H. Wang et al., 2019). A healthy vaginal microbiota is defined by the dominance of *Lactobacillus* species bacteria, particularly *L. jensenii*, *L. crispatus*, and *L. gasseri*, with less prevalent *L. iners* (Ntuli et al., 2022; Taku et al., 2022). Hr-HPV microbiota is characterized by a loss or an abrupt drop in overall *Lactobacillus* population and a corresponding considerable increase in anaerobic bacteria

concentration (Carter et al., 2021; Y. Chen et al., 2020; Mitra et al., 2016). Furthermore, an Oxford study found that commensal vaginal *Lactobacillus spp*. aid in the defense against pathogens and sexually transmitted illnesses by producing species-specific metabolites, bacteriocins and mucus adherence, and disrupting biofilms (Mitra et al., 2015, 2016).

The primary mechanism by which the microbiota protects the female reproductive tract is hypothesized to be through production of lactic acid, bacteriocins and reactive oxygen species (ROS) by *Lactobacillus spp*. This happens via the anaerobic metabolisms of host-associated degradation products of glycogen stored in vaginal mucosal cells and maintaining a pH of <4.5 which inhibits growth of other bacterial types but tolerable by *Lactobacillus spp*. (Caselli et al., 2020; Dareng et al., 2016). *Lactobacillus* has been indicated to promote a healthy vaginal environment by lowering the cervical pH which protects one from infections (Sasivimolrattana et al., 2022). *Lactobacilli* also form microcolonies which attach to the vaginal epithelial cells. This prevents adhesion of pathogens and their ability to trigger host defenses (Castanheira et al., 2021). Studies have shown that *Lactobacillus crispatus* dominance with high D-lactic acid levels increase the cervicovaginal mucus viscosity which eventually immobilizes the viral particles. Lactic acid has a contribution to increased interleukin 10 (IL-10) anti-inflammatory cytokine by immune cells , whilst reducing the cytotoxicity of natural killer (NK) cells and interleukin 12 (IL-12) and promotion of proinflammatory cytokine IL-12 synthesis (Szymonowicz & Chen, 2020).

Several clinical studies have been conducted reporting the association between the dysbiotic vaginal microbiome and the risk of developing HPV. A study conducted in Mexican women reported that a decrease in commensal *Lactobacillus spp*. and presence of *Gardnerella*, *Fusobacteria*, *Bacillus cohnii*, *Dialister*, *Prevotella*, *L. iners* and *Mycoplasma* in the vaginal

microbiota have been linked to dysbiosis that generates unstable microenvironment enabling key cervical cancer risk factors (Nieves-Ramírez et al., 2021). A high-risk HPV with *N. gonorrhoeae*, *C. trachomatis*, and HSV-II has been reported to cause an increase in atypical squamous cells of undetermined significance (ASC-US) risk and high-grade squamous intraepithelial lesions (HISIL) (Dou et al., 2024). HPV has been associated with *C. trachomatis* and *U. Urealyticum* where *C. trachomatis* plays a role in the initiation of cellular abnormalities and virus persistence, whereas progression to cervical intraepithelial neoplasia (CIN) happens with an HPV *U.urealyticum* (Dou et al., 2024; Lu et al., 2023).

From a study conducted in a Rwandan population utilizing phylogenetic microarray methods, it was observed that women with a *Lactobacillus crispatus* dominant microbiota were less likely to develop prevalent hr-HPV infection when compared with those with a more diverse microbiota with a mixture of *Atopobium* and *Prevotella* species (Dareng et al., 2016). In contrast, a study conducted in Russia that characterized the vaginal microbiota between HPV Positive and HPV negative pregnant mothers found out that both healthy and HPV infected pregnant women had equally abundant and diverse vaginal microbial communities (Irina et al., 2024). This emphasized variability in host genetics, hormonal status and geographical location.

Despite having studies that link the vaginal bacterial species with HPV status, the precise nature of this relationship is still not well understood and the specific bacterial species or communities strongly associated with increased or decreased risk of HPV infection are not well understood. For instance, there exist conflicting data on the role of *Lactobacillus* species and HPV with some data suggesting that certain *Lactobacillus* species including *L. jensenii*, *L. crispatus*, and *L. gasseri* with no *L. iners* may be protective against HPV

development, while others find no association between *Lactobacillus* abundance and HPV status (Ntuli et al., 2022). *Lactobacillus gasseri* representing CST III has been associated to contribute to a faster acute HPV infection recovery among the HPV-positive women.

Irina et al discovered that the concentration of *Lactobacillus* increased with HPV infection which is contrasting from other study findings which attributed ethnic differences to bring about the changes in the vaginal microbiome. A study which involved transactional sex workers in Nairobi and Kisumu and pregnant women in Mombasa, highlighted the significance of a *Lactobacillus spp*.-dominated cervicovaginal microbiome (Carter et al., 2021; Eastment et al., 2022). The results of this study, as well as others, are restricted to regionally small populations, such as transactional sex workers and pregnant mothers, who are not representative of the women population (Carter et al., 2021; Dareng et al., 2016; Eastment et al., 2022; Mehta et al., 2020; Mitra et al., 2015). These conflicting findings across populations warrant more standardized studies involving HIV-positive women in areas with high proportion of the HPV burden including Africa.

2.6 Relationship between HPV and HIV Status

HIV infection provides an environment where hr-HPV can better establish infection and replicate as a result of changes in the vaginal immune microenvironment as HIV infection progresses (Klein et al., 2019; Taku et al., 2020b). HIV infection leads to an extensive remodeling of the genital mucosa causing an increased immune activation and an increased cytokine production. The epithelial lining is weakened and hence, facilitating the entry, replication and persistence of HPV. Immunocompromised HIV infected women have been found to have reduced HPV clearance rates, an increased HPV infection risk compared to HPV negative women and an increased risk of developing cervical lesions due to a high

likelihood of being infected with hr-HPV (Badial et al., 2018; Taku et al., 2020b). Hr-HPV causes cervical cancer and has a global prevalence of 5% to 35% in women, with Sub-Saharan Africa having a higher incidence than other geographic locations (Carter et al., 2021).

The high HPV burden is sub-Saharan Africa is associated with high HIV prevalence coupled with limited screening, poverty and prevalence of hr-HPV genotypes in the region.

The immune system and the vaginal microbiome represent risk factors for HPV-induced cancer (Kudela et al., 2021). *Lactobacillus* dominant vaginal microbiome is associated with healthy status by maintaining low acidic pH and production of antimicrobial metabolites. In HIV positive women, the balance of *Lactobacillus* is interfered with creating a dysbiotic state with anaerobic bacteria that induce inflammation compromising the vaginal mucosal resilience and supporting the persistence of hr-HPV. High -risk HPV has been discovered to be prevalent in HIV-infected women, implying that it plays a role in up-regulating dysbiotic bacterial dominance including *L. iners*, *Prevotella* and *Gardnerella* species, which subsequently supports the creation of hr-HPV lesions (Badial et al., 2018; Shvartsman et al., 2023).

These bacterial species promote the production of pro-inflammatory cytokines including IL-6, TNF-α and IL-1β that increase inflammation enabling the establishment of hr-HPV. HIV-positive women have a higher risk of being infected with HPV, multiple HPVs and HPV persistence than non- HIV infected women. They also have higher high-grade cervical pre-cancer and invasive cervical cancer rates than non-infected women (Sweet et al., 2020). Studies have shown that HIV-positive women are 1.5 to 8 times more likely to have cervical cancer than HIV-negative women (Whitham et al., 2017). The lower CD4 counts intensifies

uncontrollable proliferation of the HIV virus favouring a pathogenic environment and hr-HPV establishment.

An *L. crispatus* dominated vaginal microbiome is associated with lower STI prevalence and a decreased prevalence of HPV in HIV-infected women while an increased diversity of the vaginal microbiome has a high frequency in HIV-positive women (Curty et al., 2017). This is due to reduced *L. crispatus* and a shift towards a pathogenic microbiome that then increase the oxidative stress, genital inflammation enhancing the penetrative ability of HPV in basal layers and its integration into host genomes. They have a reduced HPV clearance rates and increased risk of persistent HPV infection compared to HIV-negative women with an increased risk of developing cervical cancer (Sweet et al., 2020; Taku et al., 2020a). in HIV positive women, bacterial vaginosis associated microbial communities increase producing enzymes including mucinases and sialidases which subsequently degrade the mucosal barriers increasing hr-HPV susceptibility and establishment. There is higher hr-HPV prevalence, hr-HPV viral load, and cervical lesions among women who are HIV-positive compared to those who are HIV-negative.

HIV infection leads to a significant decline in both the number and function of CD4+ T cells. These cells play a crucial role in orchestrating cellular immunity against invading pathogens and hence their depletion weakens the cytotoxic T-lymphocytes in the host limiting the potential of clearing HPV. This can lead to a high rate of infection with HPV which reduces the chance of their spontaneous elimination (Badial et al., 2018; Chikandiwa et al., 2018). HIV-1 has been associated with induction of innate inflammation that disrupts the mucosal barrier in the female genital tract into the endometrial barrier which predisposes one to STIs (Gholiof et al., 2022; Wessels et al., 2018). HIV is associated with increased progression

risk from sub-clinical to clinical HPV disease. Higher HPV viral loads are linked to increased abnormal cervical cytology risk in HIV infected women but the mechanism for the HIV influence on HPV association is undefined (Klein et al., 2020).

HIV proteins like Tat protein directly enhance the transcription of HPV by increasing of HPV oncogenic proteins E6 and E7. Studies have indicated that HIV influences the cervical microbiota which plays a role in HPV acquisition and progression (Chambuso et al., 2017; Chikandiwa et al., 2018). There is a decreased T-cell surveillance controlling HPV replication with decreasing CD4+ cell count as HIV progresses. There is a shift in HPV genotypes prevalence in HIV infected populations that favor high-risk HPVs. A metagenomics study in Tanzanian women linked HIV infection to increased HPV infection risk and pathogenesis severity (Klein et al., 2019). The combined factors entailing suppression of the immune response, vaginal microbial dysbiosis and chronic inflammation in HIV-infected women increase their susceptibility to hr-HPV.

2.7 Association of Ethnicity and the Cervical Microbiome

The epidemiology of HPV infection distribution and the burden associated with HPV vary significantly across the world (Kombe Kombe et al., 2021; Nieves-Ramírez et al., 2021; Sweet et al., 2020). Geographic location, social, cultural, and genetic factors related to viral genome variability, as well as individual characteristics such as age, gender, anatomic site, and health state, are all connected with hr-HPV morbidity and mortality (Tchouaket et al., 2023). Environmental factors such as socioeconomic status including the income per household and accessibility to healthcare has been linked to influencing the vaginal microbiome of pregnant mothers and contributing to disparities among races (Serrano et al., 2019). Existing research indicates that the vaginal microbiota is vital in the regression or

persistence of the HPV virus and consequent illnesses (Mitra et al., 2015, 2020). Low microbial diversity in the female reproductive tract, as well as a high prevalence of *Lactobacillus* spp. is a sign of good health (Carter et al., 2021). A *Lactobacillus*abundant vaginal bacterial community promotes lowering of the vaginal pH, enhancing the integrity of the vaginal epithelial mucosa and producing antimicrobial metabolites including hydrogen peroxide, bacteriocins and lactic acid which function in inhibiting the infection and pathogenesis of HPV (Petrova et al., 2016).

The CST profiles have been found to vary by ethnicity in women. Ethnicity plays as an important factor influencing variability in the vaginal microbiota composition with studies reporting a higher *Lactobacillus spp.* dominant microbiota prevalence in Caucasian and Asian women as compared to a low prevalence in Black and Hispanic women (Carter et al., 2021; Chorna et al., 2020; Klein et al., 2019; Mehta et al., 2020). The Caucasians exhibit a CST-1 dominant microbiome while African and Hispanic women mostly exhibit a CST-IV dominant microbiome (Reynoso-García et al., 2022). CST-IV bacterial community is dominated by anaerobic bacteria including *Gardnerella*, *Atopobium*, *Megasphaera*, *Sneathia* and *Prevotella* that have been linked to genital inflammation that increase the susceptibility to STI infections and persistence of HPV instead of spontaneous elimination (Molina et al., 2024).

L. iners has been associated with people diagnosed with cervical intraepithelial neoplasia (CIN) and it can be found both in healthy individuals and in those with profound dysbiosis. Moreover, its genome has been found to be relatively smaller when compared to other *Lactobacillus* species, has a polymorphic shape and it also has a complicated nutritional requirement (Chávez-Torres et al., 2023; Moscicki et al., 2020). Unlike *L. crispatus*, *L. iners*

has been found to produce D-lactic acid in very low amounts. It also experiences stability in its genome, express inerolysin which is a pore forming toxin and exhibits flexibility in its metabolism allowing it to survive in very dysbiotic environments. These characteristics have thus linked *L. iners* with HPV persistence and development of genital lesion despite being a *Lactobacillus* species (France et al., 2020).

African American women have been reported to have a decrease in the *Lactobacillus crispatus* and an increase in the *Lactobacillus iners* when compared to the women from the Caucasian ancestry who typically have an increased *L. crispatus* (Paola et al., 2017). This difference has been linked to increased chances of preterm births or experiences of premature birth symptoms among American women of the African ancestry as compared to those of the European ancestry (Serrano et al., 2019). In addition, further evidence has suggested that increased prevalence of *L. iners* and a reduction in *L. crispatus* in women of African descent may be influenced by the host-genetic variations affecting the immunity of the epithelia mucosa. These variations include Toll-like receptor gene polymorphisms and cytokine production pathways that modulate the individual women susceptibility to the colonization patterns of microbial communities. However, more studies are needed to explore this relationship between *L. iners* and a vaginal microbiota dysbiosis in women of African ancestry (Hulgan et al., 2019). Longitudinal studies have shown that African descended women with dominant *L. iners* CST are predisposed to bacterial vaginosis, HPV infection and persistence. These findings have thus linked *L. iners* to a translational vaginal microbiome state to vaginosis (Kwon & Lee, 2022).

In a study conducted by Serrano et al in 2019 involving pregnant women of European, African and Hispanic ancestry, the authors found contrasting results compared with those of

previous metagenomic analysis. The results revealed very minimal disparities in the metabolic potential that is associated with the vaginal microbiomes of non-African women and those of African origin (Serrano et al., 2019). However, despite find the vaginal microbiomes having a similar functional capacity, there were differences in gene expression patterns. This difference in expression pattern may indicate that women from difference ancestry operate under distinct immune interactions, host regulatory environments and metabolic pressures; suggesting that despite the microbiome being similar, clinical disparities cannot be ruled out. Their meta-transcriptome analysis identified pathways that were less prevalent in women of African origin and associated with nucleotide metabolism. Genetic factors influencing mucosal immunity or metabolic pathways that result in preferential conditions for particular species arise from the ethnic differences (Chorna et al., 2020). For instance, variability in the genes that regulate the cervical mucin glycosylation, estrogen metabolism and epithelial receptor expression may also influence the adherence of the bacteria which in turn influence the CST distribution among women of different ethnic origins.

Studies conducted in Black South African women revealed a highly diversified cervical microbiota, suggesting that the vaginal microbiota could be a predisposing factor to a high HPV burden in HIV infected Black women (Taku et al., 2022). The bacterial microbial profiles in these women are characterized by taxa associated with inflammation, reduced stability of *Lactobacillus* and an increase in co-infections with viruses. These factors lead to a vaginal microenvironment that provides easy HPV entry, persistence, reduced clearance and the capability to progress to cervical cancer. HIV infection leads to the suppression of the immune system which influence development of bacterial vaginosis making it a

multifaceted environment (Taku et al., 2022). In summary, the above-mentioned factors underscore the consideration of ethnic differences and genetic predispositions for the cervical and vaginal microbiome in women.

2.8 Hr-HPV Prevention Strategy

The human papillomavirus is a menace that has been implicated in various pathological conditions with cervical cancer being the most life threatening as shown from the above literature. To tackle this menace and allow ladies of the reproductive age to lead a normal life, prevention of the HPV infection is the best solution. There are various vaccines administered to young girls to protect them from HPV infection once they have their sexual debut. Gardasil 9® refers to a prophylactic HPV vaccine that was designed to protect individuals from being infected by nine (9) types among the clinically important HPV subtypes. This vaccine works by targeting HPV 6 and HPV 11 which are two low-risk HPV types that have been shown to be responsible for the majority of the genital warts. The vaccine also targets seven (7) other oncogenic HPV types that include HPV 16,52,45,18,33,31 and HPV 58 that are responsible for the majority of HPV-related cancers (Cheng L,2020). The seven oncogenic HPV types have been shown to be contributing to other types of cancers including vaginal, penile, vulvar, anal, and oropharyngeal cancer as well as being implicated to approximately 90% of all global cervical cancer cases (Garland et al; 2022).

The Centers for Disease Control and Prevention's (CDC) Advisory Committee on Immunization Practices (ACIP) have recommended HPV vaccination to be an essential measure in preventing new HPV infections that would eventually lead to HPV-associated cancers. Strong epidemiological evidence showing a decline in vaccine-type HPV

prevalence, reduced genital warts alongside high-grade cervical lesions in the HPV vaccinated populations further supports the CDC recommendations (Drolet et al., 2019). The CDC recommends routine HPV vaccination from the age of 11 to 12 years but an early vaccination at 9 years would help in the immune response optimization (CDC, 2021). HPV vaccines provide a potent protection against new viral infections but it does not have any therapeutic benefit and thus it is unable in eliminating existing HPV infections or the treatment of already developed HPV related lesions. The HPV vaccine works by generating neutralizing antibodies that in turn block the entry of the virus at the epithelium of the vaginal mucosa. The vaccine is thus disadvantaged in the clearance of viral DNA that had already been integrated inside the host cells (Schiller & Lowy, 2020).

The vaccine response is high when administered before exposure to HPV between age 9 and 12 years because of generating the strongest immune response. Younger adolescents have been found to be capable of mounting higher antibody titers and a longer lasting adaptive immunity as compared to older individuals. The HPV vaccine is capable of preventing approximately 90% of cancers that are related to HPV with long-term data showing protection that is durable for more than 10 years post HPV vaccination (Kjaer et al., 2020). HPV vaccination can be coupled with *Lactobacillus* species rich probiotics where HPV vaccination will ward off new infections by blocking the entry of the virus. A Lactobacillus-rich vaginal and cervical bacterial microbiome enhances the immune system by modulating the cytokine responses. protecting the mucosal barrier and promoting the spontaneous clearance of HPV. This combination is needed to reduce the persistence of the HPV virus and its related carcinogenesis.

2.9 The Metagenomic Sequencing Methods and Platforms for Bacterial Identification

Screening and early detection of hr-HPV and premalignant lesions that might develop to cervical cancer is paramount for curbing the progression to malignant lesions. Visual inspection with acetic acid (VIA) is the commonly used screening method in sub-Saharan Africa ; but its approach is relatively less specific method because it depends on visual recognition of lesions (Seyoum et al., 2023). VIA is an inexpensive method and very feasible in low-resource settings. However, it varies in sensitivity with a sensitivity of 50-90% and an even lower specificity of approximately 49-98%. These variations can lead to false positive as well as false negative results limiting the patient's clinical intervention. The Pap smear identifies abnormalities at cellular level. However, neither VIA or Pap smear methods captures the information about the vaginal or cervical microbiome composition or the dysbiosis of the vaginal microbiome as an indicator for cervical cancer progression (Klein et al., 2020). The cervical cytology does not reveal the alterations in the vaginal microbiome that precedes cellular transformation.

Historically, bacterial culturing, molecular methods and microscopy techniques have shown that the vaginal microbiome communities have a dominance of *Lactobacillus* species (Baud et al., 2023). However, most of the vaginal and cervical bacterial microbiome are fastidious and hence cannot be cultured under the standard laboratory conditions. This limits detection of pathogen bacteria to a small subset which is not a true representation of the entire microbiome. High-throughput sequencing (HTS), a modern molecular technique has illustrated that the vaginal microbiome communities is centered around *Lactobacillus spp.* and other anaerobic bacteria. This expansion has proved essential for defining the community state types and elucidating the role they play in health and disease.

Next-generation sequencing (NGS) has enabled the investigation of microbiota composition, improving discoveries over limited culture approaches (Sharifian et al., 2023). Next generation sequencing refers to a technological genre that makes it possible for a simultaneous and independent sequencing for thousands to billions of DNA fragments (Gu et al., 2019). Allowing parallel sequencing of millions of short DNA fragments has enabled high resolution and deep coverage comprehensively identify microbial taxa taking into account those present at very low abundances (Quince et al;2017). Metagenomic NGS (mNGS) allows for unbiased detection of microbial pathogens including viral, bacterial or fungal. In contrast to 16S rRNA sequencing, mNGS is capable of sequencing the entire nucleic acids present in the sample providing an avenue to identify all microbes from bacteria, fungi, viruses and parasites. High- throughput 16S rRNA gene sequencing has enabled classification of the vaginal microbiota into 5 different community state types (CSTs). These include the *L. crispatus* for CST I, *L. gasseri* for CST II, *L. iners* for CST III, *L. jensenii* for CST V and CST IV which is a heterogenous composition of facultative anaerobes and BV associated *Atopobium vaginae* (Sharifian et al., 2023). Majority of cervical microbiota studies have relied on cultivation-dependent methods for bacterial community assessment but advent of next generation sequencing technologies has enabled rapid and more direct analysis of bacterial taxa (Wei et al., 2021).

There are different sequencing platforms in use from different companies and technologies across the globe. Microbiome research has significantly improved with the advent of sequencing technologies. High-throughput short-read sequencing of the 16S rRNA gene amplicon using the Illumina Miseq 2×300 bp platform (Illumina, USA) specifically targets the V3–V4 hypervariable region among the nine variable regions (Jeong et al., 2023). All

Illumina platforms make use of the bridge amplification strategy where single DNA molecules are attached to a flow cell and then locally amplified to form a clonal cluster. Sequencing by synthesis (SBS) then follows by building complementary DNA and fluorescently labeled nucleotides are determined by an optical readout giving Illumina a high throughput of all market sequencers (Gu et al., 2019). Illumina short reads have a high accuracy of approximately 99.9% making it a gold standard in taxonomic profiling despite limited resolution at species level that requires long-read support. However, the short-read amplicon-based platform is susceptible to identification bias due to potential chimeric sequences generated during PCR amplification for library construction. Additionally, it is restricted to classifying microbes at the genus level according to commonly used 16S rRNA gene-based microbial taxonomy databases.

The conserved nature of the 16S rRNA gene across the bacterial species limits the discrimination of different but related species/ subspecies. This subsequently complicates the identification of commensal bacterial strains versus pathogenic ones. Using the amplicon method for the partial V3–V4 variable region is limited for distinguishing strains with high similarity at the species level (Jeong et al., 2023). The Ion Torrent platform of Thermofisher (Waltham, MA) clones single molecules of DNA on a bead within an emulsion and beads are placed on a semiconductor chip equipped with individual pH sensor grid. Each DNA molecule produces a localized pH change during sequencing which then identifies the sequenced nucleotide. The ION Torrent sequencing technology is cost effective and rapid. However, it is prone to homopolymer errors in repeated nucleotide regions which may impact assigning of taxonomy (Quail et al., 2012).

The BGISEQ platform of BGI technology (Cambridge, MA) locally clones single DNA molecules on a flow cell through clonal DNA nanoballs. Nanoballs undergo SBS producing fluorescence similar to Illumina (Virtanen et al., 2019). BGISEQ produces high quality reads at cost effective prices. This enables sequencing of large cohort studies that require deep sequencing. A BGISEQ platform was used in sequencing DNA in this study.

MinION, GridION and PrometION platforms of Oxford Nanopore Technologies (Oxford, UK) work by guiding single-stranded DNA through protein nanopore grids which gather DNA sequence through electrical current disruptions. This technology is advantaged in that it is faster than sequencing by synthesis and does not necessarily require a prior PCR amplification (Gu et al., 2019). However, this technology has been flawed to experience more sequencing errors than other platforms. The Oxford Nanopore technology provides long-read sequencing of completed genomes or the entire regions of a gene. The advantage of these long-reads is that mobile elements can be identified alongside a high resolution at strain level which are often missed when doing a short-read sequencing. There are ongoing improvements on the sequencing technologies to make hybrid sequencing approaches of both short-reads and long-reads (Wick et al., 2019).

Previous studies have exposed the vaginal microbiome complexity and their influence in health and disease. Studies have shown that Community state types (CSTs) III and IV are linked to a higher HIV, HPV and HSV-2 infection prevalence (Siqueira et al., 2019). Women with a CST-IV vaginal microbiome have been identified to exhibit an increased rate of inflammatory markers which contribute to the disruption of the epithelial barrier, increasing viral susceptibility and persistence. Studies utilizing microbial cultivations enhanced the initial understanding of microbial communities' composition with a limitation

bias making it hard to culture symbiotic bacteria in the laboratory. NGS analyzes the nucleic acid that is directly extracted from samples and hence avoiding biases associated with cloning and library construction and allowing sequencing of DNA regions with secondary structure. NGS thus is credited for identifying novel bacterial strains of clinical importance through its ability to capture low abundant taxa and also bacteria that is uncultured. Most studies have been conducted on the cervical microbiome analysis with little being done on the vaginal microbiome yet the vaginal bacterial communities display fluctuations between community state types (Cervantes & Hong, 2013). Metagenomic NGS (mNGS) offers unbiased sampling enabling a broad identification of already known as well as discovery of novel ones as well as providing a platform for both quantitative and semiquantitative data pertaining organismal concentration in the sample (Gu et al., 2019).

CHAPTER THREE: METHODOLOGY

3.1 Study Site

This study was carried out at the Meru Teaching and Referral Hospital (MeTRH) HIV clinic in Meru County, Kenya. MeTRH is the County's largest and referral hospital. Meru county is located in the Eastern region of Kenya at 0.3557º N, 37.8088ºE at the foot of Mt Kenya and 1579m above sea level. It has a total population of 1,545,714 people of which 2.5% are living with HIV. HIV has an estimated 782 new infections per year as per the National Syndemic Diseases Control Council (NSDCC 2022). Women have a higher HIV prevalence of 3.7% while men are at a 1.3% HIV prevalence. The county has a cancer prevalence of 0.32% making it one of the topmost counties with a high cancer burden in Kenya (Mutiso, 2023). The hospital is a major healthcare hub in Meru County and surrounding counties. It has specialized clinics including oncology, comprehensive care clinic for HIV patients and maternal and baby health. It acts as a referral hospital for the Mt. Kenya East region and thus experiences a heavy patient traffic making it an ideal hospital for this study. Ethical approval for the study was obtained from the Meru University of Science and Technology Institutional Research Ethics Review Committee (MIRERC); approval number MIRERC004/2024. Informed consent was sought from all the study participants.

3.2 Study Design and Population

This was a nested study within a larger cross-sectional study that had recruited a total of 303 HIV-positive women at the Meru Teaching and Referral Hospital (MeTRH) to determine the prevalence and risk factors associated with HPV. The prevalence of hr-HPV in the main study was 60.4%. From this cohort, we selected 38 participants, 20 hr-HPV positive and 18 hr-HPV negative participants to explore the relationship between HPV status and the vaginal

bacterial microbiome. The rationale for the selected number of participants was based on resource constraints and a high hr-HPV positivity rate in the larger study. Semi-structured questionnaires were used to collect data from the participants including socio-demographic and behavioral characteristics. The demographic variables entailed age, education level, marital status, condom use and years on contraceptives.

3.3 Sample Collection

Sample collection was achieved through self-sampling where the participant was provided with a self -sampling kit with instructions as follows; Participating women were instructed to squat, insert the brush gently into the vagina, and pull the plunger down into the clear casing to protect the bristles. They were then be required to push the plunger up to expose the bristles, which was followed by five rotations of the brush in the same direction for the sample collection. Each brush rotation was accompanied by an audible click. Following collection, the brush was returned to its packaging and transported to the laboratory for analysis. Vaginal swabs were collected using the Evalyn brush through a self-sampling procedure and the samples were transported to the Centre for Molecular Biosciences and Genomics (CMB) Nairobi, for laboratory analysis (Rovers® Medical, Netherlands). The chosen study participants represented a balanced distribution of hr-HPV genotypes identified. The negative counterparts were also picked from the same cohort. Collection of socio-demographic characteristics including contraceptive use, age, sexual practices was done because these factors have been shown to influence ethe vaginal and cervical microbial composition.

3.4 Eligibility Criteria

3.4.1 Inclusion criteria

The inclusion criteria for the original study entailed consenting HIV-infected women aged between 25-59 years attending the HIV comprehensive care clinic (CCC) at MeTRH. Women within this age group fall within the recommended range for cervical cancer screening. All participants were required to have been diagnosed of HIV and receiving care at the MeTRH HIV clinic at the point of recruitment.

3.4.2: Exclusion criteria

Participants who were pregnant, had ever been diagnosed with cancer of the cervix, or were below 25 years of age were excluded from the study. Pregnant women were excluded because of the hormonal changes that are associated with pregnancy which have been identified to alter the vaginal microbiome. Women who had previously been diagnosed with cervical cancer were excluded because they might have already undergone changes in the bacterial microbial composition which could have interfered with the findings.

3.5 Sample Processing

3.5.1 DNA extraction and quantification

DNA extraction was done using the Wizard Genomic DNA Purification Kit (Promega Corporation, Wisconsin, USA). Briefly, 1 ml of cervical cell suspension was centrifuged at 16,000 x g for 2 minutes to pellet the cells. The cells were then resuspended in EDTA and lysed with lysozyme to break down Gram-positive bacteria. The samples were lysed further with the nucleic lysis solution and RNA digested using RNase solution. The DNA was precipitated with absolute isopropanol and washed with 70% ethanol. Finally, the DNA was pelleted by centrifugation and eluted in 50 µl of DNA rehydration solution. The Qubit™

dsDNA HS assay kit was used to quantify DNA yield after extraction (Thermofisher Scientific, Massachusetts, USA). Samples with a concentration of ≥1.5 ng/µl were selected for sequencing.

3.5.2 High-risk HPV detection

The GeneProof HPV Screening PCR Kit was used to detect the high-risk HPV genotypes (GeneProof, Brno, Czech Republic). The kit detects 24 high risk HPV types including 16, 18, 26, 30, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82 and 97 with the ability to differentiate HPV types 16, 18 and 45. The total reaction volume was 20 µl. This included 15 µl of the master mix and 5 µl of DNA and the positive and negative controls. The sample DNA and positive controls were put into individual PCR tubes and mixed by pipetting up and down. The PCR tubes were then closed, centrifuged briefly and PCR done on the Bio-Rad CFX-96 real-time PCR platform (Bio-Rad, California, USA). The DNA was amplified starting with UNG decontamination at 37°C for 2 mins followed by initial denaturation at 95°C for 10 mins. The DNA was then denatured at 95°C for 5s, annealing was done at 60°C for 40s and finally extended at 72°C for 20s. FAM, Cy5, HEX, Texas Red and Quasar705 were the fluorescence fluorophores used and the PCR done at 45 cycles.

3.5.3 MGI DNBSEQ-G99 16S rRNA sequencing

The V3-V4 region of the 16S rRNA gene was amplified using the following primer sequences: Forward primer 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3' for reverse primer. Briefly, amplicons were

quantified using a Qubit fluorometer and size validated using gel electrophoresis before libraries were created.

QIAGEN HiFi PCR Master Mix was used for amplification (QIAGEN, Hilden, Germany). Amplicons were cleaned with MGI Clean beads (MGI Tech, Shenzhen, China). Deep sequencing of the V3-V4 region was performed using MGIEasy Fast Library Prep kit (MGI Tech, Shenzhen, China). Amplicons were cleaned, fragmented, end-repaired, ligated with adaptors, barcoded and PCR amplified. Resulting libraries were quantified on a Qubit fluorometer using the Qubit 4.0 dsDNA HS Assay Kit (Life Technologies, USA). Volumes were adjusted based on each library's concentration for pooling. Libraries were circularized to form a single stranded DNA (ssDNA) circular library. This circular library was used as a template to generate a DNA Nanoball (DNB) through the rolling circle amplification (RCA). The DNB was sequenced on the combinatorial probe-anchor synthesis (cPAS)-based DNBSEQ-G99 (MGI Tech, Shenzhen, China) platform using the G99SM FCLPE150 sequencing reagent cartridge at the Makerere University Biomedical Research Centre (MGI Tech, Shenzhen, China).

3.6 Bioinformatic Analysis

The DADA2 and Phyloseq packages were used to perform 16S rRNA sequence data analysis and visualization in R (V4.4.1 https://cran.r-project.org/src/base/R-4). DADA2 was used to generate quality profile plots of the Fastq files which were visualized to assess sequence quality and guide filtering and trimming (V1.32.0 https://benjjneb.github.io/dada2/tutorial.html). In summary, file names were assigned using the list. files function. The files were sorted using the sort function to separate forward reads from reverse reads. Quality plots were created using the plot quality profiles function.

Reads with a quality score of less than 30 were trimmed off. The reads were filter- trimmed to create filtered forward and reverse reads. Forward reads were trimmed at 120 bp while reverse reads trimmed at 100 bp. Errors were identified using learn error rates and errors plotted. Sequence table was made and chimeras removed. A sample inference algorithm was used to determine the number of unique sequences per sample. The denoised reads were then merged and amplicon sequence variants (ASVs) were assigned into an ASV table. PCR chimeras were removed prior to taxonomic assignment using the Silva database (https://www.arb-silva.de/).

Phyloseq was used to calculate diversity metrics and generate data visualizations (V1.48.0, https://bioconductor.org/packages/release/bioc/html/phyloseq.html). Alpha diversity was calculated using Shannon's diversity index and observational richness. The statistical significance of alpha diversity was calculated using t-test. Beta diversity was determined by Bray-Curtis's dissimilarity distances which were plotted by non-metric multidimensional scaling (NMDS). The vegan package was used to determine the statistical significance in beta diversity (V2.6.6.1, https://cran.r-project.org/web/packages/vegan/index.html). DESeq2 was used to perform differential abundance analysis to identify both underrepresented and overrepresented taxa based on HPV status (V1.44.0, https://bioconductor.org/packages/release/bioc/html/DESeq2.html).

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Demographic Characteristics of the Study Population

The study recruited a total of 38 participants. Twenty (52.63%) participants were hr-HPV-positive, and 18 (47.37%) were hr-HPV-negative. Seven (39%) were hr-HPV-negative, and 9 (50%) were hr-HPV-positive in the age group of 25-35 years. Five (28%) were hr-HPV-negative, and 8 (44%) were hr-HPV-positive in the 36-45 years age group while the 46-55 years age group had six (33%) hr-HPV-negative participants, and 3 (7.9%) of the participants was hr-HPV-positive. On the condom use demographic, 4 (22%) hr-HPV-negative and 5 (28%) hr-HPV-positive participants reported to always have been using a condom. Moreover, another four (22%) hr-HPV-negative and 4 (22%) hr-HPV-positive participants reported to have never used a condom, while 10 (55.5%) hr-HPV-negative and 11 (56%) hr-HPV-positive participants reported to have been using a condom sometimes. Regarding the use of contraceptives, five (28%) from the hr-HPV-negative group and 4 (20%) from the hr-HPV-positive group reported to have used contraceptives for a period between 2-4 years.

Eight (44%) participants from the hr-HPV-negative group and 6 (30%) participants of the hr-HPV-positive participants had used contraceptives for more than 5 years. Ten (50%) hr-HPV-positive participants and five (28%) hr-HPV-negative participants to have never used or been on contraceptives. On matters education, one participant from the hr-HPV negative group and one (5%) hr-HPV positive participant reported to have reached college level. Eleven (61%) of the selected hr-HPV negative women and 5 (28%) hr-HPV positive women reached primary school level. Those who reached secondary school education level included 12(61%) hr-HPV positive women and 6 (33%) hr-HPV negative women. Two participants

from the hr-HPV positive group reported to never have attended school. Concerning the marital status, eight hr-HPV negative women and eight hr-HPV positive women reported to being married. Twelve (61%) hr-HPV positive study participants and eight (44%) hr-HPV negative women reported to be single. Finally, one hr-HPV negative study participant was widowed while one was divorced. These socio-demographic characteristics revealed no statistical significance to the HPV status as shown in **Table 1**.

Table 1

Demographic Characteristics of both Hr-HPV Positive and Hr-HPV Negative Study Participants.

Demographic characteristic	**Variable**	**Hr-HPV positive**	**Hr-HPV negative**
Age in years	25-35	9	7
	36-45	8	5
	46-55	3	6
Total		20	18
Condom use	Always	5	4
	Never used	4	4
	Sometimes	11	10
Total		20	18
Contraceptive use duration	Between 2-5 years	4	5
	More than 5 years	6	8
	Never used	10	5

Total	condom	20	18
Level of education	College	1	1
	Secondary school	12	6
	Primary school	5	11
	Never attended school	2	
Total		20	18
Marital status	Married	8	8
	Single	12	8
	Widowed		1
	Divorced		1
Total		20	18

Source: (Researcher, 2023)

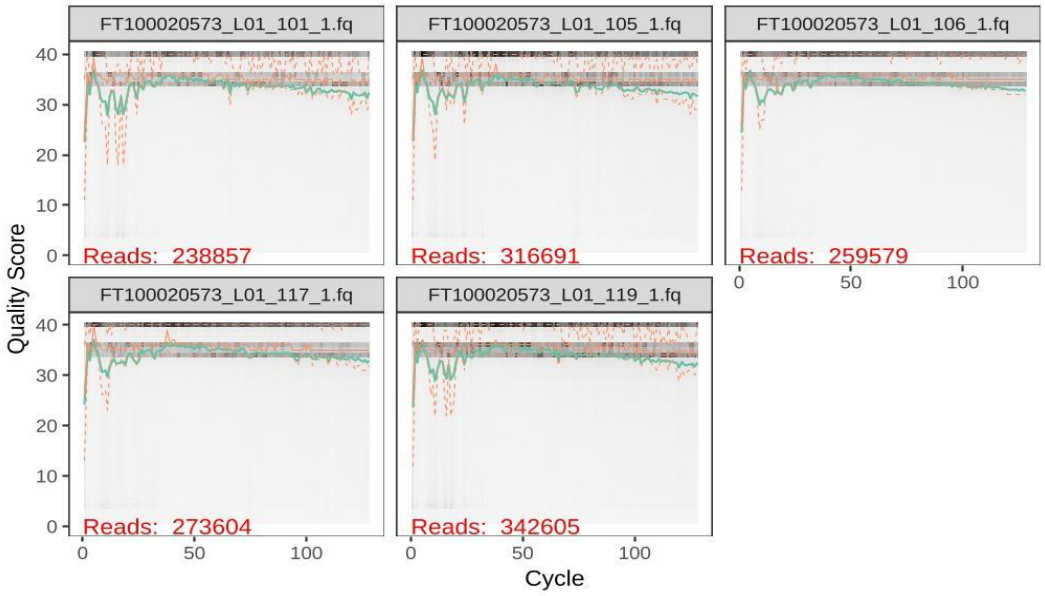
4.3 Read Quality and Counts

MGI DNB sequencing is a high-fidelity, highly accurate next-generation sequencing platform capable of producing paired-end read lengths of 150bp and generating up to 800 million reads per sequencing. This technology utilizes the DNA nanoball chemistry, that enhances signal clarity whereas minimizing amplification errors. The total read count ranged widely with 342605 reads for the highest and 238857 reads for the lowest read count per sample. The read count range falls within the acceptable metagenomic microbiome thresholds making it possible for the identification of both dominant and lowly expressed taxa. The sequencing run was successful and of high quality as exhibited from the high Phred quality scores of above 30-40 in Figures 1 & 2. A Phred score that is above 30 and

ranging between 30-40 shows that the sequencing was robust with a high base-calling accuracy of approximately 99%. This score illustrates that the sequencing encountered very low errors validating the reliability of the resultant data. The sequencing quality tended to drop at the 120 cycle for the forward reads and 100 cycle for the reverse reads. The drop in quality is normal and it is attributed to the accumulation of biochemical limitations that affect the functioning of the Taq polymerase during next generation sequencing. The drop in quality at the beginning is attributed to random primer binding bias due to cluster instability while the drop towards the end is due to polymerase degradation and the reduced ability/accuracy to add correct bases after many sequencing cycles. The sequenced forward reads were trimmed at 110bp while reverse reads were trimmed at 90bp. The quality plots are as shown in Figure 1 and Figure 2.

Figure 1

Quality profile plots for forward reads showing the quality scores

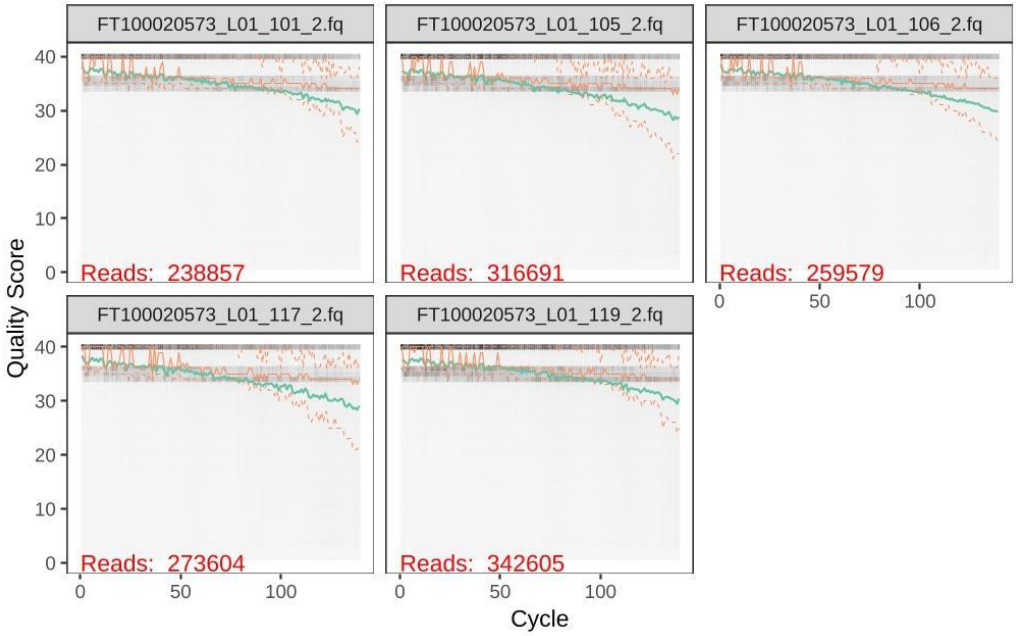


Source: (Researcher, 2024)

Note. The x-axis represents the sequencing cycle while the y-axis represents the quality scores on a Phred's scale. All plots have quality scores of above 30 indicating robust sequencing.

Figure 2

Quality profile plots for reverse reads showing the quality scores after sequencing



Source: (Researcher, 2024)

Note. The x-axis represents the sequencing cycles while the y-axis represents the quality scores on a Phred's scale. All plots have quality scores of above 30 indicating robust sequencing.

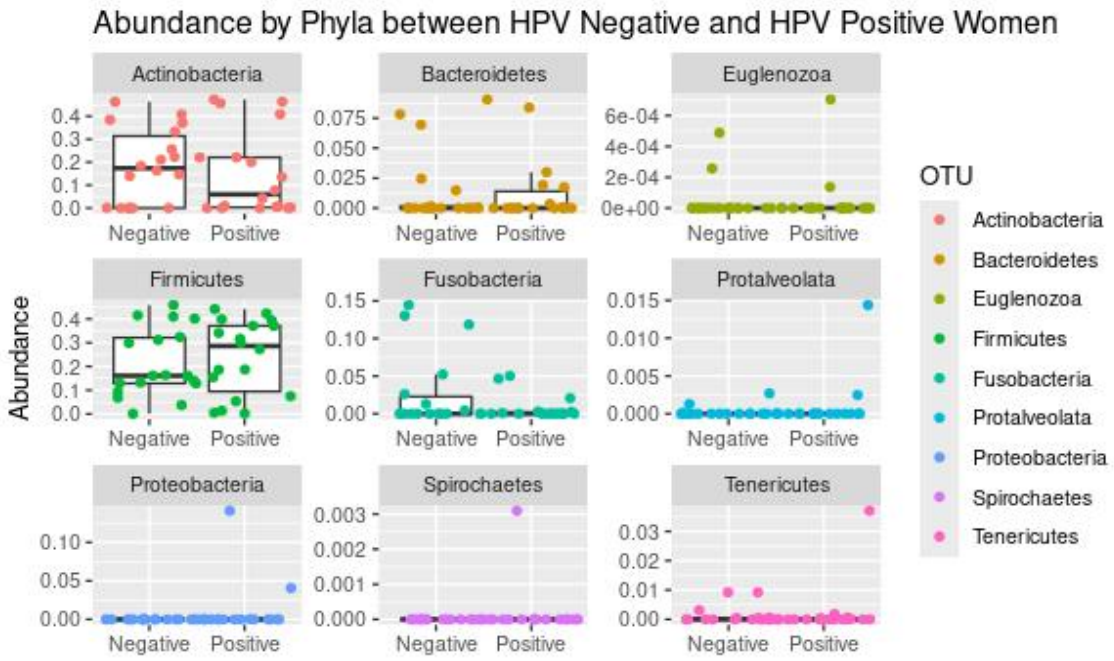
4.4 Vaginal bacterial microbiota composition and relative abundance in hr-HPV positive and hr-HPV negative groups

From the analysis, 15,774 operational taxonomic units (OTUs) were identified. Firmicutes and Actinobacteria, were the most abundant phyla in both the hr-HPV positive and hr-HPV negative groups. However, *Proteobacteria* and *Spirochaetes* were only present in the hr-HPV positive group as displayed in Figure 1. Overall, *Lactobacillus, Gardnerella, Atopobium, Sneathia* and *Streptococcus* were the most common genera in our study population. The hr-HPV negative group had a higher abundance of *Lactobacillus*,

Gardnerella, *Sneathia, Bifidobacterium* in comparison to the hr-HPV positive group as shown in Figure 2. This showed that women living with HIV have a highly diverse vaginal microbiome despite their HPV status. *Atopobium, Streptococcus, Shuttleworthia, Anaerococcus* and *Prevotella* were more frequent in the hr-HPV positive group Figure 2. *Escherichia/Shigella* was solely present in the hr-HPV positive group as displayed in Figure 2. Generally, *Gardnerella* was the most abundant genus of the vaginal microbiome followed by *Lactobacillus*, *Sneathia*, *Prevotella*, *Atopobium* and *Streptococcus*. *Gardnerella* being the most abundant genera conforms with the dysbiotic nature of the vaginal microbiome in people living with HIV. Other genera that were identified included: *Bifidobacterium*, *Peptoniphilus*, *Anaerococcus*, *Parvimonas*, *Megasphaera*, *Veillonella*, *Fastidiosipila*, *Shuttleworthia*, *Porphyromonas, Dialister* and *Mobiluncus* in the respective abundance. *Haemophilus*, *Escherichia/Shigella* and *Actinomyces* were among interesting genera also identified as part of vaginal microbiota. Three STI-associated genera were identified. These included: *Treponema, Ureaplasma* and *Mycoplasma.*

Figure 3

Abundance of phyla between HPV negative and HPV positive women

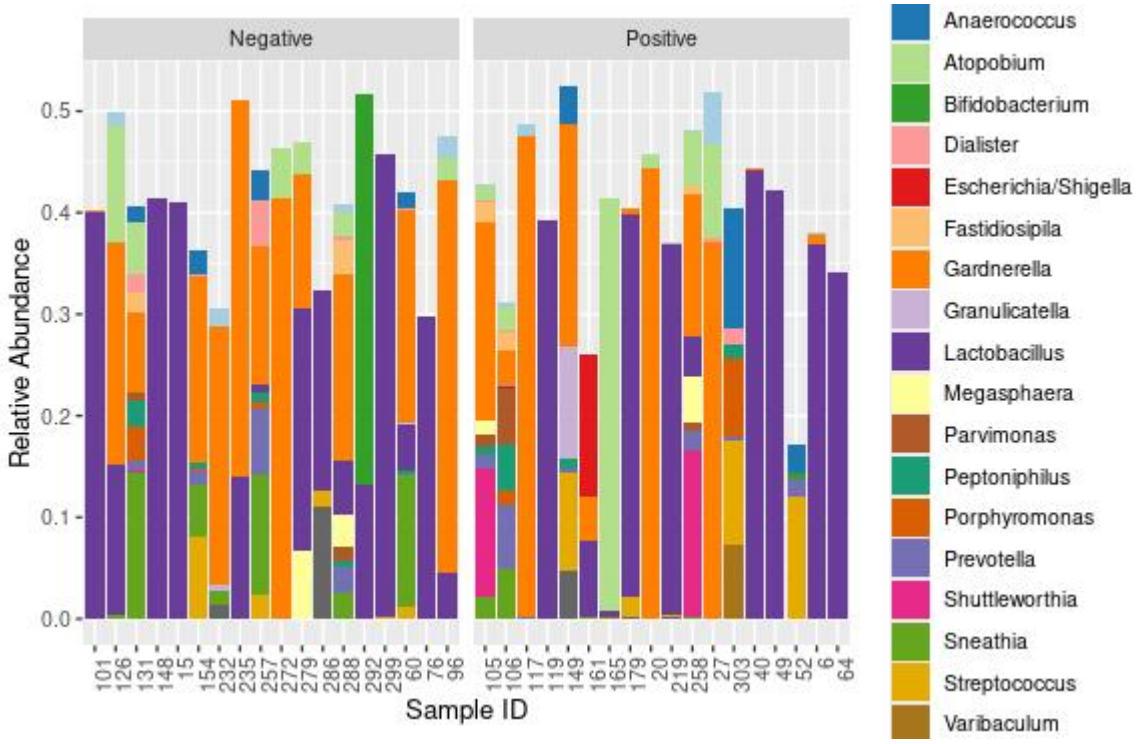


Source: (Researcher, 2024)

Note. Box plots displaying the vaginal microbiome phyla relative abundances of both hr-HPV positive and hr-HPV negative women living with HIV. X-axis represents the HPV status while the y-axis represents the relative abundances ranging from 0 to 0.4. Firmicutes, actinobacteria and bacteroidetes dominance is seen across the two groups. From the box plot, the two groups seem to portray similar phyla in the vaginal microbiome.

Figure 4

Vaginal microbiome genera between hr-HPV positive and hr-HPV negative women



Source: (Researcher, 2024)

Note. Bar plot displaying the vaginal microbiome genera differences between hr-HPV positive and hr-HPV negative women living with HIV. Lactobacillus and Gardnerella can be seen as the predominant genera. The y axis represents the relative abundances of the phyla ranging from 0 to 0.5 while the x axis represents the sample ID of participants in each group. Prevotella, Atopobium and Escherichia coli can be seen more abundant in the hr-HPV positive group than the negative group. Different colours represent each bacterial genus present in the vaginal microbiome of the study participants.

4.5 Diversity Analysis; Alpha and Beta Diversity

Alpha diversity measures the within sample/group microbiome differences to identify the evenness and richness of the microbiome. The alpha diversity of the vaginal microbiome

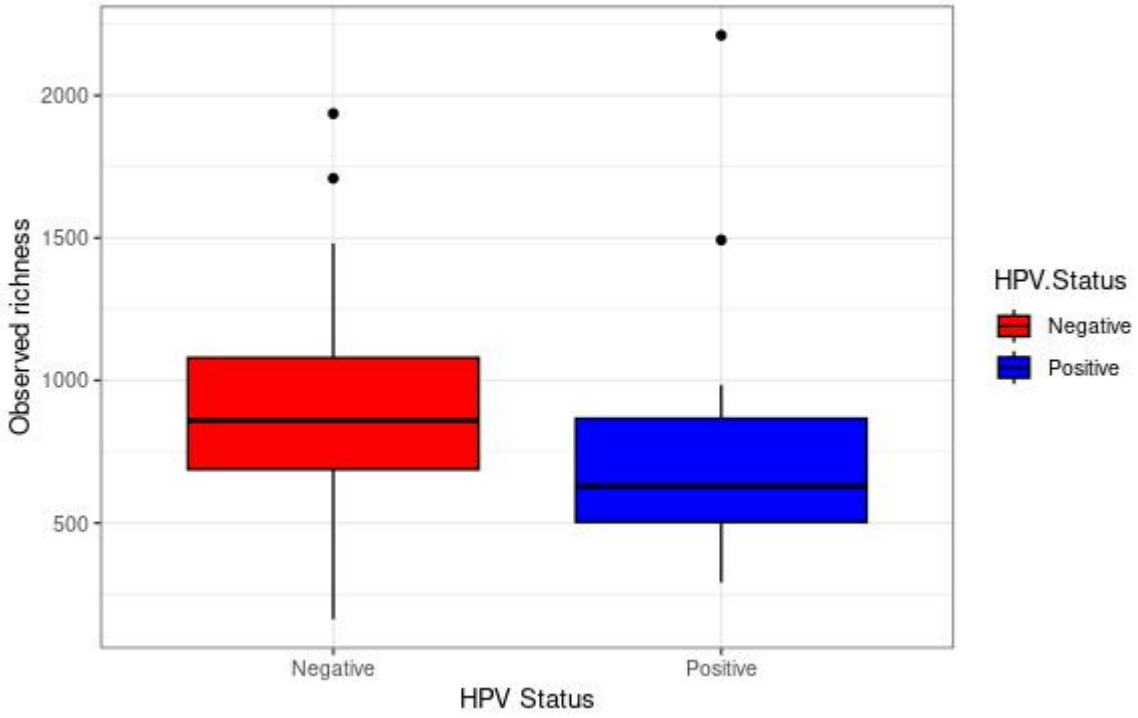
was measured using the observed OTU richness as well as Simpson's and Shannon's diversity indices. The observed richness ranged from 162 OTUs to 2,211 OTUs with a mean and median of 841.8 and 768.5, respectively. The hr-HPV negative group had a higher median alpha diversity in comparison to the hr-HPV positive group as illustrated in figures 3,4 and 5. This showed that the hr-HPV negative group had a more diverse vaginal microbiome. However, Wilcoxon rank sum test generated a p-value of 0.15 >0.05. Therefore, the observed bacterial richness did not show a significant relationship between the hr-HPV positive and hr-HPV negative women living with HIV in Meru. Overall, Shannon's diversity index ranged from 4.51 to 6.99 with a mean of 5.91 and a median of 5.73. The hr-HPV negative group had a mean and median Shannon's diversity index of 5.99 and 6.01 while the hr-HPV positive group had a mean and median of 5.83 and 5.66, respectively. The t-test was used to test the statistical significance of Shannon's diversity index between both groups, which produced a p-value of 0.45 (>0.05). Simpson's index ranged from 0.9854 to 0.9991 with a mean of 0.9939 and a median of 0.9940. Wilcoxon rank sum test was used to test the statistical significance of the Simpson's index between the hr-HPV positive and hr-HPV negative groups, which generated a p-value of 0.32 (>0.05). Thus, the richness and evenness of the vaginal microbiome was not statistically different between the two groups.

Beta diversity refers to the measure of change in taxa between groups, communities, habitats or ecosystems. In this case, it is the measure of change/ difference in bacterial taxa between hr-HPV positive and hr-HPV negative groups. Bray-Curtis dissimilarity distances was used to calculate beta diversity of both hr-HPV-positive and hr-HPV-negative groups. PERMANOVA was used to evaluate the statistical significance of beta diversity between the two groups. The p-value = 0.328 (>0.05), indicating that the vaginal microbiome

compositions of HPV-positive and HPV-negative groups were similar. Bray-Curtis dissimilarity distances were plotted using non-metric multidimensional scaling (NMDS) as displayed in Figure. 6. NMDS was used to visualize the similarity or dissimilarity of the bacterial community composition between the hr-HPV positive and hr-HPV negative groups. Each point on the graph represented a single sample and the overall microbial composition is reflected by each point's position. There was no clear distinction between the microbial communities of both the hr-HPV positive and hr-HPV negative groups as indicated by the overlap of the red and blue dots. There was no statistically significant difference in the microbial diversity between hr-HPV positive and negative women as shown with the lack of statistical significance in both alpha and beta diversity.

Figure 5

Observed OTU richness between hr-HPV positive and hr-HPV negative women

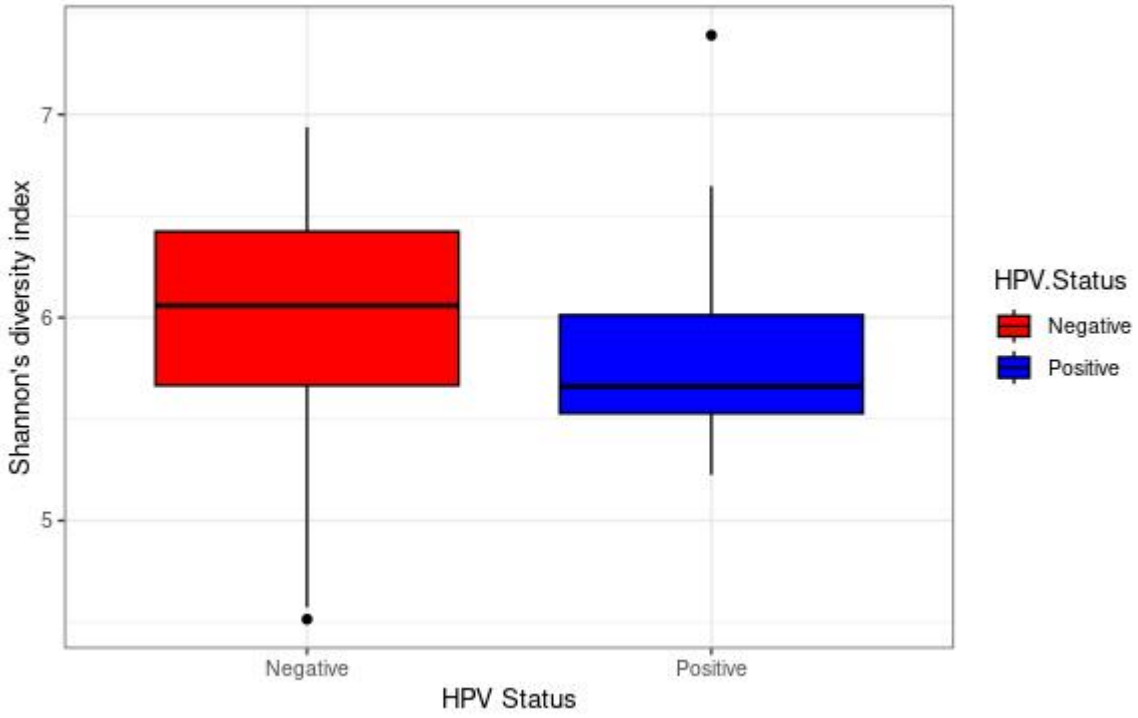


Source: (Researcher, 2024)

Note. Alpha diversity matrix. Box plot of observed richness, the x-axis represents the HPV status of both groups while the y-axis represents the observed richness. Hr-HPV negative group is observed to have a slightly higher median OTU richness than the hr- HPV positive group from the box plot. Red colour represent the hr-HPV negative while blue colour represent the hr-HPV positive individuals.

Figure 6

Shannon's diversity index between hr-HPV positive and negative women

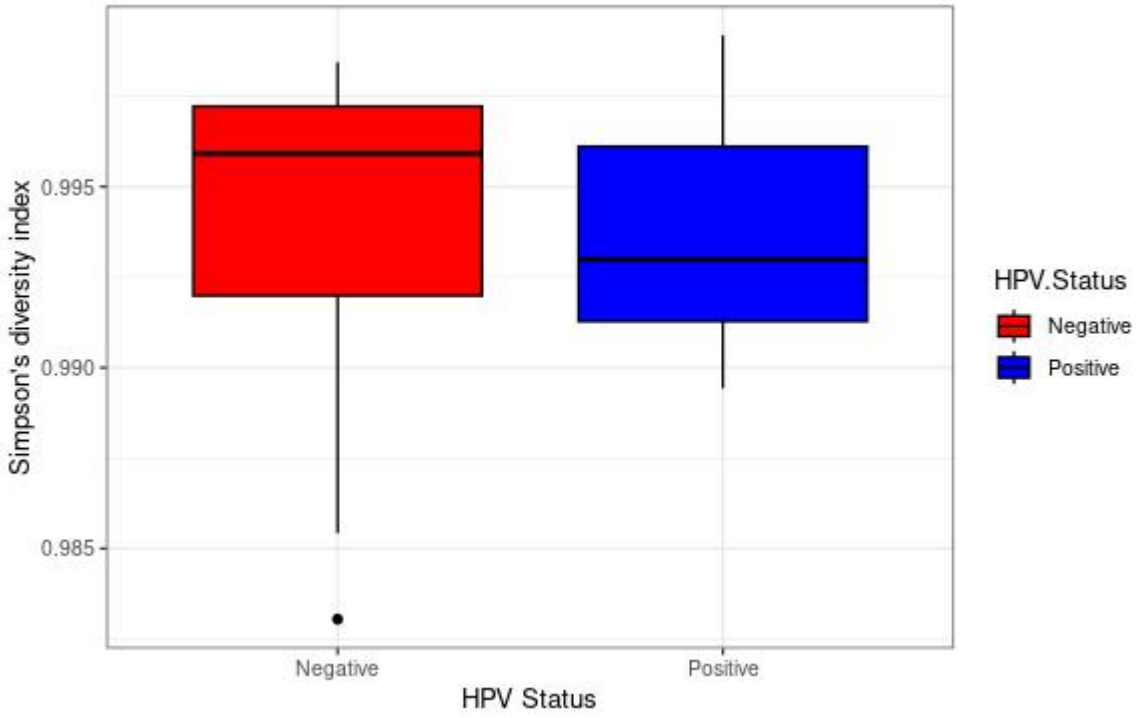


Source: (Researcher, 2024)

Note. Alpha diversity matrix. A box plot displaying the Shannon's diversity indices with hr-HPV negative group showing a higher median Shannon's index than the hr-HPV positive group. the x-axis represents the HPV status of participants while the y-axis represents the Shannon's index. Red colour represents the hr-HPV negative while blue colour represents the hr-HPV positive individuals.

Figure 7

Simpson's diversity indices of hr-HPV positive and negative women



Source: (Researcher, 2024)

Note. A box plot showing the Simpson's alpha diversity indices indices with hr-HPV negative group showing a higher median Simpson's index than the hr-HPV positive group. The x-axis represents the HPV status of participants while the y-axis represents the Simpson's index. Red colour represent the hr-HPV negative while blue colour represent the hr-HPV positive individuals.

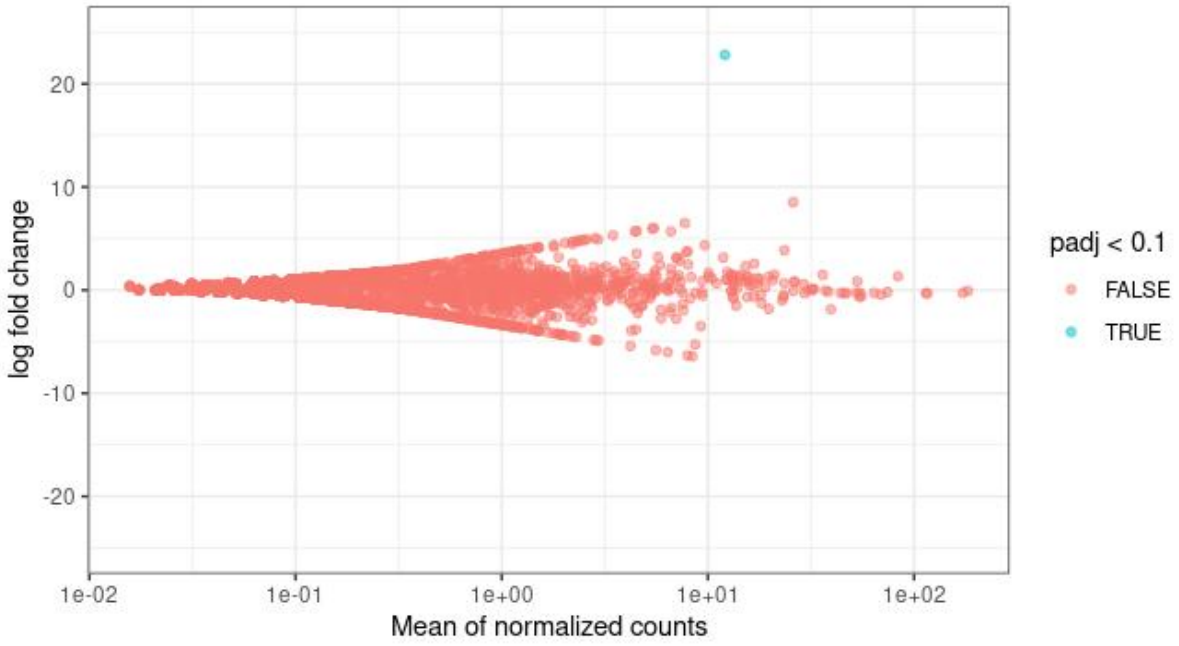
4.6 Differential Abundance Analysis

Differential abundance measures the distribution of OTUs between groups, communities or ecosystems. It was used to identify if there are OTUs that were more abundant between the hr-HPV-negative and hr-HPV-positive groups' vaginal microbiome. Differential abundance analysis was performed on 5,166 bacteria with nonzero total read count. *Lactobacillus*was

significantly upregulated in hr-HPV-positive women with a log2 fold change of 22.80 and an adjusted p-value of $3.77e^{-11}$ (padj<0.1), indicating a statistically significant upregulation. A MA plot was generated to visualise the differential abundance as displayed in Fig. 6.

Figure 8

Differential abundance of taxa between hr-HPV positive and hr-HPV negative women



Source: (Researcher, 2024)

Note. MA plot representing differential abundance of bacterial taxa highlighting the distribution of log2 fold changes across varying abundances, with the blue point representing *Lactobacillus*.

There is inadequate research on the composition of the vaginal bacterial microbiome in HIV-infected women with hr-HPV co-infection in Kenya. Existing data is primarily from cross-sectional studies conducted across the country, most of which focused on commercial sex workers in major cities with little being done on HIV infected women. This left a significant knowledge gap about HIV infected women who might be facing differences in

behaviour, socioeconomic factors and varied biological risk profiles. In this study, we investigated the relationship between the vaginal microbiome and hr-HPV infection in a cohort of HIV-infected women in Meru County, one of the 47 counties in Kenya having a high cancer prevalence (Mutiso, 2023). An understanding of the vaginal bacterial microbial landscape within the HIV-infected women population is important in depicting the vaginal status of a rural population.

After characterizing the vaginal bacterial microbiome**,** *Lactobacillus genus* was the most abundant genus in both hr-HPV positive and hr-HPV negative women followed by *Gardnerella, Atopobium, Sneathia* and *Streptococcus genera*. Abundance of *Lactobacillus* genus, which is typically the most dominant genus in a healthy vaginal microbiome, illustrates the typical vaginal microenvironment.(Ntuli et al., 2022; Reimers et al., 2016). *Lactobacillus* dominance is associated with aa healthy vaginal status. It primarily plays a role in lowering and maintaining an acidic vaginal pH through the production of lactic acid that consequently suppress the thriving of pathogenic organisms. This finding shows that despite the diversity and presence of pathogenic bacterial genera; the vaginal microbiome strives to maintain an abundant Lactobacillus. However, the high abundance of dysbiosis-related bacteria genera such as *Gardnerella*, *Sneathia* and *Streptococcus* could be attributed to the participants' HIV status. Antimicrobial peptides and vaginal microbiota with lactobacilli as the primary bacteria, make key defense mechanisms relied upon by the cervicovaginal mucosa (Zeber-Lubecka et al., 2024). The healthy vaginal microbiota is a low diversity microbial community dominated by *Lactobacilli*, with an average Shannon's diversity index of 1.2±0.8 (Baud et al., 2023; Freitas et al., 2018).

Bifidobacterium genus, which is also thought to exert a protective role similar to that of

Lactobacillus since it is also a lactic acid-producing bacterium was present in vaginal bacterial microbiota (Chávez-Torres et al., 2023). *Bifidobacterium* presence in hr-HPV positive women may ensure a vaginal eubiosis through production of lactic acid with absence of *Lactobacillus* or its availability in low numbers (Irina et al., 2024). Presence of Bifidobacterium may be indicative of a compensatory mechanism to stabilize the vaginal microenvironment as a result of immunological alterations that interfere with *Lactobacillus* abundance. Presence of *Bifidobacterium* in our hr-HPV positive women may suggest a concerted effort between *bifidobacterium* and *Lactobacillus* in maintaining a steady lactic acid production. *Atopobium, Streptococcus, Staphylococcus, Megasphaera* and *Leptotrichia* have also been reported to possess the capability for homolactic or heterolactic acid fermentations (López-Filloy et al., 2022; B. Ma et al., 2012).

The abundance of *Escherichia/Shigella* may be attributed to poor hygiene since these are commensal gut bacteria that have access to the gut-vaginal axis. Ththis may be indicative of individual hygiene habits or determinants of behaviour that end up transferring bacterial from the gastrointestinal tract to the reproductive system. The presence of *Treponema* may have indicated a co-infection with syphilis, which is a well-known risk factor of HPV infection due to genital ulceration it causes (Mbulawa et al., 2018; Omame et al., 2021). A study conducted in Mexican women also reported that an increase in *Gardnerella*, *Fusobacteria*, *Dialister*, *Prevotella*, *Bifidobacterium* and *Mycoplasma* in the vaginal microbiota was linked to a dysbiotic milieu; our study population reported having these bacterial genera (Chávez-Torres et al., 2023; Mancabelli et al., 2021; Nieves-Ramírez et al., 2021)

The vaginal bacterial microbiome composition in our study population was similar between

the hr-HPV positive and hr-HPV negative groups, with no differentially abundant taxa. The findings reveals no direct relationship between the vaginal bacterial microbiome and HPV status. However, the absence of significant relationship might be due to the HIV status and the small sample size for our study population which hindered assigning taxonomy to the species level. The chronic inflammation as a result of HIV infection might play a role in disturbing the vaginal microbial homeostasis and interfering with the reproductive system capability to discriminate bacterial microbial taxa associated with HPV. Our findings corroborate a study done on Nigerian women which showed that there were no distinct bacterial taxa between the vaginal microbiomes of HIV-positive women with persistent hr-HPV infection and those without HPV (Dareng et al., 2016). HIV infection has been associated with the proliferation of dysbiotic bacteria including *Lactobacillus iners*, *Prevotella bivia*, *Atopobium*, and *Gardnerella*, thereby increasing susceptibility to hr-HPV (Badial et al., 2018; Klein et al., 2019, 2020; Shvartsman et al., 2023).

HIV also alters the cervicovaginal immune microenvironment and epithelial lining, creating favourable conditions for hr-HPV infection and replication (Mtshali et al., 2021; Ntuli et al., 2022; Van De Wijgert et al., 2020). Moreover, HIV infection leads to a decline in both the number and function of CD4+ T cells which results in an increased susceptibility to infections including HPV (Badial et al., 2018; Chikandiwa et al., 2018). HIV-1 has been associated with induction of innate inflammation that disrupts the mucosal barrier in the female genital tract into the endometrial barrier which predisposes one to STIs (Gholiof et al., 2022; Wessels et al., 2018). HIV is associated with increased progression risk from sub-clinical to clinical HPV disease which could not be confirmed because of the small sample size. This suppression of the immune system may also interfere with ability of the vaginal

microbiome in distinguishing the microbial status of hr-HPV positive women and those without hr-HPV.

In describing the genetic diversity of the vaginal bacterial microbiome, the hr-HPV negative group reported a higher alpha diversity than the hr-HPV positive group, however, this was not statistically significant. These findings contrast those from previous studies that have reported higher alpha diversity in hr-HPV positive women than in hr-HPV negative women, suggesting an even distribution of bacterial species and decreased *Lactobacillus* abundance (S. Liu et al., 2022; Zeber-Lubecka et al., 2022) (S. Liu et al., 2022; Zeber-Lubecka et al., 2024). The high alpha diversity in our study population could be also attributed to the participants' HIV status and the small sample size (Chambuso et al., 2017; Reimers et al., 2016; Whitham et al., 2017; Zayats et al., 2022). Additionally, ethnicity could also explain the high alpha diversity observed in our study. The vaginal microbiome composition in a majority of women of Black descent is described as being highly diverse as characterized by Ravel et al; 2011, with a low relative abundance of *Lactobacillus* and a high abundance of *Atopobium*, *Gardnerella*, *Sneathia* and *Prevotella* (Guo et al., 2022; Ravel et al., 2011; Serrano et al., 2019; Taku et al., 2022). A study conducted in Black South African women also reported a high vaginal microbial diversity, suggesting that the diversity in vaginal microbiota could be a predisposing factor to the high burden of HPV African women living with HIV (Chorna et al., 2020). However, ethnicity or genetics alone cannot be used to explain the variability considering the many factors that contribute to the vaginal microbiome.

Beta diversity analysis revealed that the vaginal microbiome composition in our study population was similar regardless of hr-HPV status. Our findings corroborate those from a

study done in Nigerian women which showed that there was no distinct difference in vaginal microbiome composition between HIV-positive women with persistent hr-HPV infection and those without HPV (Dareng et al., 2016). In contrast, a study conducted in Chinese women as well as one on Russian women found significant differences in the vaginal microbiome composition between women with HPV and those without the infection (Guo et al., 2022; Irina et al., 2024). The contrasting findings from these studies demonstrate how complex the vaginal bacterial microbiome can be and how susceptibility varies depending on the biological factors, the geographic location and cultural set up.

In our study, differential abundance analysis revealed that *Lactobacillus* was significantly upregulated in women with hr-HPV in comparison to those without. This may suggest that despite the overall bacterial community being similar in the hr-HPV-positive and the hr-HPV-negative counterparts, the vaginal microenvironment may be shaped by specific taxa like *Lactobacillus* which might be working harder to lower the vaginal pH. A possible explanation for this maybe that *Lactobacillus* species abundance is increased upon detection of pathogenic disturbance and the increase acts as a compensatory mechanism working towards restoring the vaginal homeostasis. However, several studies have reported that HPV infection results in alterations to the vaginal microbiome, resulting in the depletion of *Lactobacillus spp* (S. Liu et al., 2022; Mao et al., 2023)**.** A decrease in the abundance of *Lactobacilli* promotes the growth of pathogenic bacteria resulting in increased susceptibility to viral infections such as HPV (Zeber-Lubecka et al., 2022, 2024).

Overall, ethnicity, age and hygiene habits are among the several factors that affect the structure and composition of the vaginal microbiome (Guo et al., 2022). Environmental factors such as socioeconomic status including the income per household and accessibility to

healthcare has been linked to influencing the vaginal microbiome of pregnant mothers and contributing to disparities among races (Serrano et al., 2019). Our study participants having been black and with lower socio-economic statuses, and HIV positive, might explain the pathogenic vaginal bacterial microbiome dynamics recorded. The inconsistent findings of the various cross-sectional vaginal microbiome analysis studies across the world have been attributed to these factors (Guo et al., 2022; Irina et al., 2024; Serrano et al., 2019). Our study population having been black African women in Kenya, a sub-Saharan country, may correlates with the aforementioned vaginal microbiome inclination. Lifestyle factors including personal hygiene, diet, stress levels and sexual behaviour cannot be overlooked as they may contribute to the vaginal bacterial microbiome shift.

In contrast to this study, most studies conducted on the vaginal microbiome, cervical microbiome or cervicovaginal microbiome reported differences in the microbiota composition between the HPV negative and HPV positive women. They have also shown how longitudinal follow up reveals about the cervicovaginal microbiota impact on HPV existence (Guo et al., 2022; Irina et al., 2024). A study conducted by Guo et al in Chinese women found significant differences between the HPV positive and HPV negative women's vaginal microbiome (Guo et al., 2022). However, our study population being already immune-compromised, might have led to the similarity in the vaginal microbiome. Genetic factors influencing mucosal immunity or metabolic pathways that result in preferential conditions for particular species arise from the ethnic differences (Chorna et al., 2020). Studies conducted in Black South African women resulted in diversified vaginal microbiota suggesting that the vaginal microbiota could be a predisposing factor to a high HPV burden in HIV infected black women (Chorna et al., 2020).

CHAPTER FIVE: CONCLUSION, RECOMMENDATIONS AND PUBLICATION

5.1 Introduction

In this chapter, the conclusion and recommendations in relation to the study objectives are given.

5.2 Conclusion

The vaginal bacterial microbiome of women living with HIV in Meru is composed of *Lactobacillus, Gardnerella, Atopobium, Sneathia* and *Streptococcus genera* in both hr-HPV positive and hr-HPV negative women. The findings report no significant differences in the vaginal microbiome of both hr- HPV-positive and hr-HPV-negative women living with HIV in Meru County, Kenya.

Both groups reported high alpha and beta diversity with abundance of *Lactobacillus*and *Gardnerella*. To our knowledge, this could be attributed to HIV infection having already distorted the diversity of the microbial species due to the compromised immune system favoring the thriving of dysbiotic bacterial species.

This study also conform to the HIV-HPV comorbidities regarding the vaginal microbiota.

The high differential abundance of *Lactobacillus*in hr-HPV-positive might be a compensatory mechanism to lower the vaginal pH despite the dysbiosis.

This study concludes that HIV has a highly statistically significant association with a dysbiotic vaginal bacterial microbiome. However, a follow up study with a higher sample size is warranted to support this conclusion.

5.3 Recommendations

A longitudinal study with healthy HIV negative women who are negative of HPV virus to be included as controls is recommended.

A study involving HIV infected participants should be divided into groups depending on their viral loads to determine at what level of immune suppression is one at risk of hr-HPV infection. A subsequent study should try to assign taxonomies up to the species level to enable classification of the vaginal bacterial microbiome by community state types (CSTs) in the Kenyan population.

Finally, subsequent studies should recruit more participants to minimize the bias that might arise with small sample sizes.

5.4 Publication

Mutoro, T. A., Mogere, S., Kemunto, C., Simam, J., Mwenda, C. N. M., & Onyambu, F. . G. (2025). Vaginal bacterial microbiome composition in women living with HIV in Meru, Kenya. *African Journal of Science, Technology and Social Sciences*, *4*(2), PAS 104–107. https://doi.org/10.58506/ajstss.v4i2.340

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APPENDICES

Appendix A: Publication

AFRICAN JOURNAL OF SCIENCE, TECHNOLOGY AND SOCIAL SCIENCES. 4 (2) 2025, PAS 104-107 OPEN ACCESS

AFRICAN JOURNAL OF SCIENCE, TECHNOLOGY AND SOCIAL SCIENCES

Journal website: https://journals.must.ac.ke

MERU UNIVERSITY OF SCIENCE AND TECHNOLOGY

A publication of Meru University of Science and Technology

Vaginal bacterial microbiome composition in women living with HIV in Meru, Kenya

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ARTICLE INFO

Keywords

Vaginal microbiome,
16SrRNA,
Vaginal dysplasia,
Dysbiosis,
HIV.

ABSTRACT

The immune system status and the vaginal microbiome represent risk factors for hrHPV and women living with HIV are susceptible to persistent HPV infections. This study characterized the vaginal bacterial communities in women living with HIV in Meru. A cross-sectional study was conducted involving 38 women living with HIV at the Meru Teaching and Referral Hospital. Genomic DNA extraction and 16S rRNA amplification was carried out. Bioinformatic analysis was performed using R and Cutadapt was used to perform sequence quality control. We identified 6,481 bacterial taxa. Lactobacillus and Gardnerella were the most abundant genera in the study population. In conclusion, this study shows the status of vaginal microbiome in Kenyan women living with HIV. Follow up studies are recommended to include control group of HIV-negative women.

Introduction

Kenya has a total of 1,377,784 people living with HIV as per the National Syndemic Diseases Control Council (NSDCC) statistics, with a 5.31% prevalence among women (NSDCC 2023). Women living with HIV are six times more likely to develop cervical cancer in comparison to those without HIV, due to their immunocompromised status (WHO, 2024). In Sub-Saharan Africa, there is a substantial correlation between HIV and HPV-associated dysplasia, with HIV-infected women having a higher prevalence of hrHPV genotypes compared to HIV-negative women (*Castle et al.*, 2020; *Klein et al.*, 2019, 2020; *Taku et al.*,2020; *Tchouaket et al.*, 2023).

The human microbiome constitutes a community made up of symbiotic microbes which function in the maintenance of human physiology and general body fitness (*Ma et al.*, 2024; Nieves Delgado & Baedke, 2021). A healthy vaginal microbiome is dominated by Lactobacilli which modulate genital health through the production of lactic acid and hydrogen peroxide (*Sharifian et al.*, 2023). A Lactobacillus-dominant vaginal microbiome protects women against invading pathogens whereas hrHPV thrives in a diverse microbiome with an abundance of Gardnerella vaginalis and Atopobium vaginae (*Santella et al.*, 2022; *Sharifian et al.*, 2023).

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https://doi.org/10.58506/ajstss.v3i2.340

AFRICAN JOURNAL OF SCIENCE, TECHNOLOGY AND SOCIAL SCIENCES. ISSN:2958:0560

Appendix B: Questionnaire

Questionnaire for Participants

This questionnaire is developed to obtain the socio-demographic information only. Carefully read the questions below and provide the responses to the best of your knowledge. You have the freedom to ask for clarification where you do not understand. Kindly note that the information given will be treated with highest confidentiality and no one will find out about your participation.

Do not write your name or any identification details anywhere on this questionnaire.

Section A: Background Information (Tick where Appropriate and provide an answer where applicable)

1. What is your age range? 25-30 () 31-35 () 36-40 () 41-45 () 46- 50 ()

2. Which year were you born? _____

3. What is your nationality? Kenyan () Other () Mixed ()

4. In which county were you born? _____

5. What is your education level?

Primary school () Secondary school () College /University () Never attended ()

6. Are you employed? Yes () No ()

7. If yes, which occupation? _____

8. What is your monthly income? 0-10,000ksh () 11,000-20,000 () 21,000- 50,000 () 51000 and above ()

9. What is your marital status?

Married/ Cohabiting () Single () Divorced/ Widowed ()

10. If married, do you have a cowife? Yes () No () Don't know ()

11. If yes, does she know her HIV status? Yes () No () Don't know ()

12. How many years have you lived with your current partner? 0-3yrs () 4-9yrs ()

10-19yrs () 20 and more ()

13. Do you smoke? Yes () No ()

14. Do you take alcohol? Yes () No ()

15. How many times have you given birth? 0 () 1-2 () 3-4 () 5 or more ()

16. Do you use any family planning method? Yes () No ()

17. At what age did you become sexually active? 9-15yrs () 16-19 () 20

and above ()

18. How many sexual partners have you had in the past year? 0 () 1-3() 4-6() 7- 10 () more

than 10 ()

19. How many lifetime sex partners have you had? 0 () 1-5 () 6-10 () 11- 15 () more than 15()

Section B: HIV status

1. What year did you find out about your HIV status? _____

How many years have you lived since you were diagnosed with HPV? _____

2. Which year did you start on your HIV treatment? _____-

3. Have you ever skipped on your medication? Yes () No ()

Section C: Knowledge about HPV and Cervical cancer

1. Have you ever heard of the human papillomavirus (HPV)? Yes () No ()

2. Have you ever heard of cervical cancer? Yes () No () Don't know ()

3. Is there a history of cervical cancer in your family? Yes () No () Don't know ()

4. Do you think HPV can cause cervical cancer? Yes () No () Do not

Know () Maybe ()

5. Have you ever heard of HPV testing? Yes () No ()

6. If yes, where did you hear? Health Facility () Radio () TV () Community Health Volunteer () Don't remember ()

7. Have you ever had any HPV Test? Yes () No ()

8. If yes please specify which one. Pap smear () Via Villi () HPV test () Pap smear & Via Villi ()

9. How long ago did you have your most recent HPV test? 1-12months ago () 13-24months ago () 25-36months ago () more than 36 months ago () Never ()

Appendix C: Consent Form

1. General Information

Study title: Vaginal Bacterial Microbiome Profiles associated with high-risk HPV in Women infected with HIV in Meru County, Kenya.

Principal Investigator: Thomas Atenya, MSc student, MUST.

Faculty Advisor: Dr. Cynthia Mugo, Department of Biological Sciences

Funder: Dr. Frank Onyambu, Department of Medical Laboratory Science.

Supervisor 2: Dr. Joan Simam.

The study is open to your participation. You should be able to make a decision about participating in the study using the information on this form.

Key Information

- The study aims to characterize the cervical microbiota of HIV-infected female patients visiting Meru Teaching and Referral Hospital, Comprehensive Care Center.
- You will need to sign this form if you choose to partake, fill in the questionnaire and agree to giving your cervical sample for characterizing your cervical microbiome. You will be given a self -sample collection kit and you will be required to collect the sample at the comfort of your home and return the kit to us for analysis in the laboratory.
- The direct benefits of your participation are knowing your HPV status and access to further treatment of HPV types likely to lead to cervical cancer.

It is absolutely optional to take part in this study. You are not obligated to participate, and you are free to do so whenever you like. Please take the time to read this entire form and ask any questions before deciding whether or not to take part in this research study.

2. Aim of this Research

Women infected with HIV are at a greater risk of developing HPV with some of the subtypes likely to lead to cervical cancer because of the suppressed immune system. Cervical microbiome contributes to the development of immunity. Immunocompromised HIV infected women are susceptible to persistent HPV infections. The relationship between the cervical microbiome, HPV, and HIV statuses is not well understood. This study aims to characterize cervical bacterial communities in HIV-infected women using 16s rRNA metagenomics and to investigate their relationship with an individual's HPV status.

3. Who is eligible to become a participant

3.1 Who can become a study participant?

HIV positive women aged 25-59 years visiting Meru Level 5 Teaching and Referral Hospital Comprehensive Care center are eligible to take part in this study. Women who at the time are experiencing active vaginal bleeding, are pregnant and have been diagnosed with cervical cancer are excluded from this study.

3.2 What is the total number of people expected to partake in the study?303 participants are expected to enroll.

4. Information on Participation in the Study

4.1 What will happen to the participant in the study?

Enrollment will take place at Meru Level 5 Teaching and Referral Hospital. You will be required to fill in a questionnaire on socio-demographic parameters such as age, number of children marital status, sexual partners and answer a few questions that will help us in reporting the results.

Training will then be done on how to use the self-sample collection kit to collect cervical specimen for analysis. When comfortable, you will then be given the sample collection kit to collect the specimen at the comfort of your home. We will then organize on how the sample will get to the lab where DNA will be extracted and analysis done using PCR.

4.2 How much time will you spend in the study?

Once you're enrolled, you will need to fill out the questionnaire which should take approximately 30 mins long and interview lasting about 15 mins long. Training on how to use the kit will take 20 mins then you will be allowed to leave. The next time we interact it will be to deliver the sample collected and results.

5. Study Benefits and Risks

5.1 What are the risks associated with this study? What protection measures are in place?

Breach of personal information is a possible risk. Researchers will try to lower the risk by using password encrypted files to store the data in a computer. Physical files will be kept under lock and key to prevent access to the confidential information.

For the questionnaire and interviews; you can answer the questions you are comfortable with. Your confidentially being compromised is the main risk associated with this research because it gathers information about you. See Section 8 of this document for more details on how the research team will protect your privacy and confidentiality.

5.2 What are the benefits for me and others in enrolling in this study?

The benefit entail knowing if you have a pathogenic cervical microbiome that predisposes you to high-risk HPV subtype that could lead to cervical cancer. If this is the case, we will link you to the right clinicians for treatment to avoid the risk of developing cervical cancer.

The study results will also be beneficial to relevant stakeholders in incorporating the bacterial species in the cervical microbiome for HPV screening methods.

6. Halting the Study

6.1 What happens if I no longer want to participate?

You can leave the study whenever you choose. There won't be any consequences for you if you pull out. Be sure to inform people listed in Section 9 regarding exiting the study before completion. Your reasons for leaving the study may be recorded in the study file if you decide to share them with the researchers. Unless you request that we remove it from our records, the researchers will maintain the data they have acquired about you for the research. It won't be possible to have your information deleted if the information has been utilized in the research.

7. Information sharing and protection measures

7.1 How will my information be protected by the researchers?

Your personal information will be handled with strict confidentiality. Physical files will be kept secured in safe cabinets and password encrypted files will be used for soft copy data storage. All the researchers and interviewers with access to your information will be required to sign confidentiality agreement. Breach of agreement will lead to serious consequences.

7.2 How will the data be kept?

The information we collect about you throughout the research will be kept on file for use in further research endeavors, for monitoring the study, and to request actions from the Ministry of Health. We will keep the information we collected about you for research

purposes distinct and secret from your name and any other information that can be used to identify you specifically.

However, there won't be any information in the study's findings that could be used to personally identify you in a publication or presentation.

7.4 In future research, will my information be used or shared?

We may share or utilize your study data in upcoming initiatives. We will only share de-identified information about you with other researchers; we won't reveal your name or any other information that can be used to personally identify you. The results of the study could be totally different from those of this study. We won't need your additional informed consent for these trials.

8. Contact Information

8.1 Who are the contact persons for this study?

To receive the below information, get in touch with the researchers below;

- Gather additional information
- Follow up questions on the study procedures
- Report any injury, illnesses or other hitches
- Pull out from the study before completion
- If you have any other concerns

Principal Investigator: Thomas Atenya

Email: thomasatenya43@gmail.com

Phone:0115607754

Departmental Supervisor: Dr. Cynthia Mugo

Email: cmugo@must.ac.ke

Phone: 0706755928

Study Coordinator: Dr. Frank Onyambu

Email: fonyambu@must.ac.ke

Phone:0722585337

Supervisor 2: Dr. Joan Simam

Email: jsimam@must.ac.ke

Phone: 0720260159

9. Your Consent

9.1 Consent to participate in the research

By signing this document, you give your agreement to take part in this study. Make sure you understand the goal of the study before signing. You will receive a copy of this document for your records, and a second copy will be stored with the study records. If you have any questions about the study after you sign this document, you can contact the study staff using the information in Section 9 provided above.

My *inquiries have thus far been satisfactorily addressed, and I am aware of the study's purpose. Therefore, I'm willing to participate in this research study.*

Name: _____

Sign: _____

Date (dd/mm/yy): _____

10. Optional Approval

10.1 Giving permission for future research to use and/or disclose your personal information

Your personally identifiable information will be used by the researchers for future studies that could be related to this one or entirely unrelated to it. If the data is identifiable, it will have details that can be used to specifically identify you. You won't be contacted by the study team to provide additional permission for this ongoing investigation. We might also give other researchers access to your personal information. You can ask us to cease using your information at any time by getting in touch with us. However, if your data has already been used by research projects, we won't be able to retrieve it.

_____ I agree that the researcher(s) may use and share my personally identifiable information for future study.

_____ I do not agree that the researcher(s) may use and share my personally identifiable information for future study.

Name: _____

Date: _____

Date (dd/mm/yy): _____

10.2 Acceptance of Contact for Potential Future Research Participation

Researchers may want to keep your contact information on file in order to encourage you to take part in upcoming studies that could be entirely unconnected to the current study project or entirely comparable to it.

_____ I consent to being contacted by researchers about potential future study initiatives.

_____ I do not consent to being contacted by researchers about potential future study initiatives.

Appendix D: Ethical approval

MERU UNIVERSITY INSTITUTIONAL RESEARCH & ETHICS REVIEW COMMITTEE
(MIRERC)
Email: mirerc@must.ac.ke ***Website:*** ***https://research.must.ac.ke/research-ethics/***

REF: MU/1/39/28 Vol.3 (017) Date: 2nd April, 2024

TO: Thomas Atenya (MSc. Molecular Biology-MUST)- SC410/202592/22
Dr. Cynthia N. Mugo (Supervisor)

Dear Sir/madam

RE: Investigating The Relationship Between the Cervical Microbiome and HPV Status in HIV-Infected Women in Meru County, Kenya

This is to inform you that ***MIRERC*** has reviewed and approved your above research proposal. Your application approval number is ***MIRERC004/2024.*** The approval period is ***2nd April, 2024– 1st April, 2025.***

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by ***MIRERC***.
iii. Death and life-threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to ***MIRERC*** within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to ***MIRERC*** within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to ***MIRERC***.

You may also be required to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI), visit: https://research-portal.nacosti.go.ke and also obtain any other clearances needed for your study.

Yours sincerely

Prof. Peter Masinde, Ph.D.
Chair, MIRERC