IMPACT OF DAILY ACETYLSALICYLIC ACID INTAKE ON COMMENSAL VAGINAL BACTERIA AND YEAST

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DECLARATION

I hereby declare that this research project is my original work and has not been presented for the award of a degree or any other award in any university.

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DEDICATION

This work is dedicated to my daughters, Achieng and Akinyi.

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ABBREVIATIONS AND ACRONYMS

ASA	Acetylsalicylic Acid
BV	Bacterial Vaginosis
CMC	Cervical Mononuclear cells
COX	Cyclo-oxygenase enzyme
FGT	Female Genital Tract
FSW	Female Sex Workers
HCQ	Hydroxychloroquine
HIV	Human Immunodeficiency Virus
HESN	Human Immunodeficiency Virus-Exposed Seronegative
MSM	Men who have Sex with Men
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
PBMC	Peripheral Blood Mononuclear cells
PEP	Post-exposure Prophylaxis
PrEP	Pre-exposure Prophylaxis
PWID	People Who Inject Drugs
SIV	Simian Immunodeficiency Virus
SWOP	Sex Workers Outreach Program
UNITID	University of Nairobi Institute of Tropical and Infectious Diseases

ABSTRACT

Genital inflammation plays a crucial role in the acquisition of Human Immunodeficiency Virus (HIV) as it leads to the migration of susceptible HIV target cells to the genital mucosa. Mucosal surfaces, such as the female genital tract, are critical in understanding HIV transmission since they form the first point of contact for HIV during sexual transmission. Certain factors like bacterial vaginosis and yeast infection trigger inflammation at the mucosal barrier. The reduction in the levels of *Lactobacillus* spp. and increased diversity of microbial communities such as *Gardnerella vaginalis, Prevotella bivia* and other anaerobes have been associated with genital inflammation and increased risk of HIV infection by enhancing high infiltration of immune cells that are susceptible target cells for HIV infection and disruption of the epithelial barriers. Bacterial vaginosis (BV) is a prevalent condition among women in Kenya, particularly among high-risk populations such as female sex workers. Some studies reported a BV prevalence of 38% among female sex workers in Nairobi, indicating a significant public health challenge that could facilitate increased susceptibility to HIV and other sexually transmitted infections (STIs). These findings underscore the urgent need for targeted interventions to address BV and its implications for sexual health in this vulnerable population. Since women are more vulnerable and at higher risk of acquisition of HIV and other STIs, various interventions, including use of pharmacological drugs, have been rolled out as prevention tools. The purpose of this study was, therefore, to evaluate the impact of the daily acetylsalicylic acid (ASA) intake on the commensal vaginal bacteria. This study was designed as a prospective longitudinal study nested in a randomized open label clinical study. The study hypothesized that daily intake of acetylsalicylic acid would not alter commensal vaginal bacteria in the genital tract. The study involved 100 HIV seronegative female sex workers recruited from the Sex Workers Outreach Program (SWOP) Majengo clinic in , Nairobi. The participants were randomized to three arms (no drug, 81mg or 325 mg). Vaginal swabs were collected from consenting participants and smears were made and Gram stained for microscopy. Gram staining and the standard Nugent score system were used to measure the abundance of *Lactobacillus* spp. ,*Gardnerella vaginalis* morphotypes and yeast cells, thereby diagnosing and quantifying BV at baseline, 3 months and 6 months of (ASA) intake. Statistical analysis was conducted using ANOVA to compare differences in BV prevalence and bacterial populations across the treatment arms. Out of 100 women, only 64 had complete BV results at all time points in the study. In these 64 sex worker women, no significant differences were observed regarding the, proportion of women having bacterial vaginosis, across the drug arms at any point during drug intake. Arm D (p=0.1836), Arm K (p=0.0898), and Arm H (p=0.1181). Additionally, there were no significant differences observed in bacterial populations, *Lactobacillus* spp. median scores for Arm D were 2 (IQR 0-4), 2 (IQR 0-2), and 1 (IQR 0-3) at v1,v3 and v6 respectively. Arm K had median scores of 2 (IQR 0-4), 2 (IQR 0-3), and 0 (IQR 0-3) at v1,v3 and v6 respectively and Arm H had scores of 2 (IQR 0-3), 2 (IQR 0-3), and 1 (IQR 0-2) with corresponding p-values indicating no significant differences, Arm D (p > 0.9999), Arm K (p=0.2918), Arm H (p=0.4966). For *Gardnerella vaginalis*, median scores for Arm D were 0 (IQR 0-1), 0 (IQR 0-2), and 0 (IQR 0-0) for v1, v3 and v6 respectively. Arm D (p>0.9999), Arm K (p>0.9999), Arm H (p=0.8949). The median score for yeast cells was 0 for all arms and no significant difference was observed in the yeast cells scores Arm D (p>0.9999), Arm K (p>0.9999), Arm H (p>0.9999). Overall, the findings of this study suggested that acetylsalicylic acid (ASA) had no impact on the commensal vaginal bacteria associated with BV over the course of the study. The conclusion of the study is that taking ASA daily does not alter the vaginal microbiome and therefore does not put women at additional risk of HIV. The findings from this study can inform future research to evaluate the impact of ASA and other anti-inflammatory drugs on the vaginal microbiota.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The HIV pandemic continues to pose a significant challenge in global public health. Currently, 39 million HIV infected people are living with the disease globally and about 53% of these are women and girls (UNAIDS, 2023). Sub-Saharan Africa accounts for almost two thirds (53%) of HIV infection with women twice likely to acquire HIV than men (UNAIDS, 2023). In Kenya, the national HIV prevalence is 3.7%, and women had a prevalence 4.9% - twice that in men (UNAIDS, 2023). In Kenya, the HIV epidemic continues to be concentrated among vulnerable populations such as female sex workers (FSW). Female sex workers are 26 times at higher risk of getting infected with HIV than persons from the general population (Baral *et al.*, 2012).

The higher risk of HIV infection among FSW is multifactorial that is due to a combination of behavioral, biological, and socioeconomic factors. One of the primary reasons is the engagement of FSWs with multiple sexual partners. One study reported that 65% of FSWs had more than five sexual partners in the past month (Bitty-Anderson *et al.*, 2022). This high number of concurrent partners increases the likelihood of exposure to HIV, as each additional partner presents a potential risk.

The transient nature of these relationships often limits the opportunity for establishing consistent safer sex practices, thereby heightening the risk of HIV transmission. Inconsistent

condom use among FSWs further exacerbates their vulnerability to HIV. In another study, only 48% of FSWs reported using condoms consistently with all clients despite their availability (Bitty-Anderson *et al*, 2022). Factors contributing to this inconsistency include client refusal to use condoms, negotiation challenges, and the desire to maintain favorable relationships with clients. Such inconsistent use significantly increases the risk of HIV transmission, particularly when FSWs engage in unprotected sex with HIV-positive clients, which can lead to new infections (Bitty-Anderson *et al*, 2022).

Sexually transmitted infections (STIs) and bacterial vaginosis (BV) are significant biological factors that contribute to increased HIV rates among female sex workers. The presence of STIs can facilitate the transmission of HIV by creating mucosal lesions and inflammation, which provide easier access for the virus to enter the bloodstream, for example, one study found that women with untreated STIs had a higher risk of acquiring HIV, with the risk increasing significantly in the presence of multiple STIs (Cohen, Craig R *et al.*, 1995).

Limited access to healthcare services also plays a significant role in the high rates of HIV among FSWs. Some studies have by indicated that only 30% of FSWs in Kenya had accessed HIV testing and counseling services in the past year (Baral *et al*, 2012). Stigma and discrimination often prevent FSWs from seeking necessary healthcare, resulting in delayed diagnosis and treatment of HIV. This lack of access to preventive measures, such as pre-exposure prophylaxis (PrEP), further perpetuates the cycle of transmission as untreated HIV-positive FSWs continue to infect their clients and partners.

Substance abuse is another contributing factor to the high HIV rates among FSWs. Many FSWs engage in alcohol and drug use, which can impair judgment and lead to risky sexual behaviors. One study found that 58% of FSWs reported using alcohol before engaging in sexual activities, which was associated with a higher likelihood of unprotected sex (Strathdee *et al.*, 2015). Substance abuse reduces the ability to negotiate condom use and increases vulnerability to violence, further exacerbating the risk of HIV transmission. Lastly, socioeconomic factors significantly influence the health outcomes of FSWs. Economic instability often drives women into sex work, with financial pressures leading to riskier behaviors. Another study has shown that 70% of FSWs cited financial necessity as their primary reason for entering the profession (Agot *et al.*, 2025) . This economic dependency on clients can limit their ability to insist on safer sex practices, as immediate financial gain often takes precedence over long-term health considerations. Thus, socioeconomic vulnerability plays a crucial role in perpetuating the cycle of HIV transmission among FSWs.

Additionally, the structure of the female genitalia also makes women more susceptible to HIV infection during unprotected sexual intercourse, as the vaginal epithelium and cervix have a larger surface area exposed to HIV compared to the penis during heterosexual intercourse. The cervicovaginal surfaces can also develop microtears and abrasions during coitus, providing a portal of entry for HIV. The combination of these factors contributes to the significantly higher rates of HIV infection among female sex workers compared to the general population (Baral *et al*, 2012).

Nevertheless, younger women, due to their underdeveloped cervix (cervical ectopy) and low vaginal mucus production, coupled with other factors like concurrent sexually transmitted infections (STIs), have an even much greater risk of HIV infection (UNAIDS, 2023).

The disruption of the vaginal microbiome and associated inflammation caused by bacterial vaginosis and yeast infections can compromise the protective barrier of the female genital tract, making it more vulnerable to HIV transmission (Gosmann *et al.*, 2017). Therefore, the complex interplay between vaginal infections, can significantly influence a woman's risk of contracting HIV.

Current biomedical HIV prevention methods have focused on directly blocking the virus or specific viral entry pathways. These methods include, the use of antiretrovirals (ARVs) for pre-exposure prophylaxis (PrEP) and post exposure prophylaxis (PEP) and condoms. Antiretrovirals (ARVs) are essential in preventing HIV infection through various mechanisms that inhibit the virus's replication and entry into host cells. One key mechanism is the inhibition of reverse transcriptase, an enzyme critical for converting HIV RNA into DNA. Nucleoside and non-nucleoside reverse transcriptase inhibitors (NRTIs and NNRTIs) effectively block this process. A study has shown that tenofovir disoproxil fumarate (TDF) used as pre-exposure prophylaxis (PrEP) reduced the risk of HIV infection by 86% among men who have sex with men (MSM) who adhered to the regimen (Baeten *et al.*, 2012).

Additionally, integrase inhibitors, such as dolutegravir, prevent the integration of viral DNA into the host genome. Early ART initiation with integrase inhibitors significantly lowered viral loads, correlating with reduced transmission rates. Entry inhibitors prevent HIV from entering host cells by blocking viral fusion with the cell membrane or preventing binding to essential co-receptors. They include, Enfuvirtide, Fostemsavir and Ibalizumab. Enfuvirtide is a fusion inhibitor that stops HIV from fusing with the cell membrane. It's used in Kenya and also included in the guidelines for ARV drug therapy in Kenya. Fostemsavir a prodrug that is converted into a molecule that blocks the HIV envelope glycoprotein 120 (gp120), preventing its binding to CD4 cells. Additionally, Ibalizumab is a monoclonal antibody that targets the CD4 protein on immune cells, effectively blocking HIV entry. (Gandhi *et al.,* 2025).

Another important HIV prevention tool is condoms which serve as a physical barrier that prevents the transmission of HIV and other sexually transmitted infections (STIs) during sexual intercourse. They effectively block the exchange of bodily fluids, which is a primary mode of HIV transmission. According to a systematic review, consistent condom use can reduce the risk of HIV transmission by up to 85%. The protective effect of condoms is enhanced when used in conjunction with other preventive measures, such as ARVs, creating a multifaceted approach to HIV prevention (Weller *et al.*, 2002).

While these strategies have been important, they do not address the underlying host immune factors that influence susceptibility to HIV infection. Despite the availability of these prevention tools, some approaches, such as microbicides, have shown limited effectiveness, potentially due to factors related to immune activation and inflammation within the genital

tract. For example, a study in a South African cohort highlighted the role of vaginal inflammation in reducing the efficacy of some microbicide products (Naranbhai *et al.*, 2012). The study found that inflammatory conditions alter the vaginal microenvironment, including changes in pH and disruption of healthy microbial flora. These alterations compromised the vaginal epithelium's protective barrier. The study indicated that women with higher levels of inflammation exhibited increased concentrations of pro-inflammatory cytokines, which further diminish the effectiveness of microbicides. This reduction in microbicide levels was associated with a compromised barrier function, which not only diminished the protective effects of the microbicides but also potentially increased susceptibility to HIV infection.

To address the limitations of existing biomedical prevention methods, new approaches that target host factors and modulate the immune system are being explored and developed. Targeting host factors and modulating the immune system represents a promising avenue for enhancing HIV prevention strategies. Traditional methods, such as antiretroviral therapy and microbicides, primarily focus on directly inhibiting the virus or preventing its entry into cells. However, these approaches often face limitations, including issues related to adherence, resistance, and the impact of coexisting conditions, such as vaginal inflammation. By shifting the focus to host factors, researchers aim to bolster the body's natural defenses against HIV, potentially leading to more effective and sustainable prevention strategies.

One promising strategy is the use of non-steroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid (ASA, also known as aspirin). Acetylsalicylic acid (ASA) has been shown

to potentially reduce the proportions of HIV target cells (activated CD4+ T cells) within the female genital tract (Lajoie, J *et al.*, 2018). This suggests that modulating inflammation and immune activation through the use of NSAIDs, like ASA, may represent a novel approach to enhancing the host's resistance to HIV infection. The advantage of using acetylsalicylic acid (ASA) is its well-established safety profile and widespread accessibility, making it a potentially feasible option for broader implementation in HIV prevention efforts, especially in resource-limited settings.

One of the primary mechanisms by which ASA works is through the reduction of inflammation. Inflammatory responses in the genital tract often lead to an increase in activated CD4+ T cells, which are the main targets for HIV infection (Passmore *et al.*, 2016). Inflammation is typically driven by cytokines and chemokines released in response to infections or irritants. ASA inhibits cyclooxygenase (COX) enzymes responsible for converting arachidonic acid into prostaglandins-lipid compounds that promote inflammation. This inhibition of the production of pro-inflammatory mediators, can potentially decrease overall inflammation in the genital tract. Indeed, previous research from our lab has shown that ASA significantly reduces the levels of pro-inflammatory cytokines, such as IL-6 and TNF-α, which are associated with increased activation of CD4+ T cells (Lajoie, J *et al*, 2018).

Specifically, the study reported a 30% reduction in activated CD4+ T cells in participants treated with ASA compared to the control group. This reduction in inflammation is critical for lowering the risk of HIV transmission. In recent studies examining the effects of ASA

treatment on inflammatory pathways, findings from a Kenyan cohort of HIV uninfected non-sex worker women demonstrated that six weeks of ASA treatment significantly reduced metabolites from both the lipoxygenase and cyclooxygenase pathways in plasma. While ASA directly inhibits COX function, it does not directly affect lipoxygenase (LOX). However, previous studies have shown that inflammation is linked to increased LOX activity, particularly n-6 oxylipins. (Kowatsch *et al.*, 2024).

In addition to reducing inflammation, ASA contributes to a decrease in the activation status of CD4+ T cells (Lajoie, Julie *et al.*, 2021). Activated CD4+ T cells are more susceptible to HIV infection, and ASA's anti-inflammatory effects help reduce the activation of these immune cells. When inflammation is diminished, there is less stimulation of the immune system, resulting in a lower proportion of CD4+ T cells being activated (Lajoie, Julie *et al*, 2021) Understanding the role of inflammation in this context is crucial, particularly as conditions like bacterial vaginosis (BV) can exacerbate immune activation.

Bacterial Vaginosis (BV) is a prevalent vaginal condition characterized by an imbalance in the normal bacterial flora of the vagina. The etiology of BV is multifactorial, with several key contributors identified in the literature. One of the primary causes is the disruption of the vaginal microbiome, particularly a decrease in *Lactobacillus* species, which are essential for maintaining a healthy vaginal environment. This imbalance allows for the overgrowth of other bacteria, such as *Gardnerella vaginalis* and *Mobiluncus* species, leading to the clinical manifestations of BV (Sobel, J. D., 2000).

Sexual activity is another significant factor associated with the development of BV. Although BV is not classified as a sexually transmitted infection, certain sexual behaviors can increase the risk of its occurrence. Studies have shown that women with multiple sexual partners or new sexual partners are at a higher risk of developing BV. A study found that the incidence of BV was significantly higher among women attending STI clinics, with an annual incidence rate of approximately 30% (McClelland *et al.*, 2008). Additionally, practices such as douching have been implicated in the disruption of the vaginal flora, further contributing to the risk of BV. Douching can alter the natural balance of bacteria, leading to a higher likelihood of developing this condition (Martino *et al.*, 2002).

Hormonal changes also play a crucial role in the development of BV. Fluctuations in hormone levels, particularly during menstruation, pregnancy, and menopause, can affect the vaginal microflora and contribute to an increased risk of BV. (Mirmonsef *et al.*, 2011). Furthermore, the use of antibiotics has been shown to disrupt the balance of beneficial bacteria in the vagina, allowing harmful bacteria to proliferate. (Belay *et al.*, 2024).

Globally the prevalence of BV is estimated to range from 10% to 50%. The variation in prevalence is influenced by geographic location and population characteristics (Kenyon *et al.*, 2013). In Sub-Saharan Africa, the prevalence of BV is notably high, with studies indicating rates between 30% and 50% among women attending reproductive health clinics. A systematic review reported a prevalence of approximately 42% in this region, highlighting the significant

burden of BV among women in Sub-Saharan Africa (Nyemba *et al.*, 2022). Specifically in Kenya, research shows that the prevalence of BV among women of reproductive age is around 30% to 40% (Bukusi *et al.*, 2006).

The interrelationship between bacterial vaginosis (BV) and HIV has been extensively documented, highlighting the increased susceptibility to HIV infection among women with BV. A systematic review and meta-analysis found that women with BV are approximately 1.5 to 2 times more likely to acquire HIV compared to those without the condition (Atashili *et al.*, 2008). This heightened risk is attributed to the disruption of the vaginal microbiome and the inflammation caused by BV, which can facilitate the entry of the virus into the bloodstream. Additionally, BV has been associated with adverse effects on HIV progression. A study in South Africa showed that HIV-positive women with BV exhibited higher levels of viral load, with BV linked to a 2.5-fold increase in the likelihood of having a detectable viral load (Asare *et al.*, 2023). This suggests that BV may not only increase susceptibility to HIV but also accelerate the progression of the disease.

Vaginal inflammation is not solely attributed to BV; it can arise from various other conditions that also influence the risk of HIV transmission. One common cause of vaginal inflammation is yeast infections, specifically candidiasis, which is often due to an overgrowth of *Candida albicans*. Approximately 20% to 25% of women experience recurrent yeast infections, leading to significant inflammation characterized by itching, burning, and discharge. This

inflammatory response can disrupt the vaginal epithelium, potentially increasing susceptibility to HIV by compromising the mucosal barrier (Faustino *et al.*, 2025).

Sexually transmitted infections (STIs) are another major contributor to vaginal inflammation. Infections such as chlamydia, gonorrhea, and trichomoniasis can cause significant mucosal inflammation. The World Health Organization (WHO) estimates that there are about 376 million new STI infections globally each year (WHO, 2018). The inflammation associated with these infections can elevate the concentration of HIV target cells, such as CD4+ T cells, in the vaginal mucosa, further enhancing the risk of HIV transmission (Mwatelah *et al.*, 2019).

Allergic reactions and irritants also play a role in causing vaginal inflammation. Products such as soaps, douches, and contraceptives can lead to localized inflammation in some women. Some studies reported that up to 10% of women may experience allergic reactions resulting in vaginal irritation. While these irritants do not directly increase the risk of HIV transmission, they can compromise the integrity of the vaginal mucosa, making it easier for viral entry (McClelland *et al.*, 2006). Additionally, hormonal changes, particularly during menopause, can lead to vaginal atrophy due to decreased estrogen levels. During the postmenopausal phase women experience symptoms related to vaginal atrophy, which can weaken the protective barrier of the vaginal mucosa, increasing susceptibility to infections, including HIV (Alvisi *et al.*, 2019).

Despite the complex etiology of female genital inflammation, targeted strategies to control inflammation are needed to reduce the risk of HIV acquisition in women. Pharmacological

approaches using licensed drugs can be one such approach. A pilot study conducted by our group explored the effectiveness of safe, affordable, and accessible non-steroidal anti-inflammatory drugs (NSAIDs) in decreasing the number of HIV target cells in both the bloodstream and the female genital tract . The pilot study involved a cohort of 91 HIV-negative women from the general population, who were randomly assigned to receive either NSAIDs either HCQ or ASA for six weeks. The results demonstrated a statistically significant reduction in HIV target cells in the blood, with a decrease of 35% of CD4+T cells and Th17 cells by 28% in the ASA group. In contrast, the HCQ group did not show any significant impact on mucosal levels of HIV target cells, indicating that while ASA effectively reduced these cells in the bloodstream, HCQ did not influence their presence in mucosal tissues. These findings suggest that ASA may offer a novel approach to reducing HIV susceptibility and highlight the need for further research in this area (Lajoie, J *et al*, 2018).

Both the pilot study and my research examine the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on the female genital tract, though they focus on different endpoints and participant groups. The pilot study evaluated the impact of NSAIDs on reducing HIV target cells in a cohort of 91 HIV-negative women from the general population, demonstrating significant reductions in both blood and genital tract target cells after six weeks of treatment. Only half (n=39) of this HIV-negative women were on ASA. In contrast, my study investigates the effects of daily acetylsalicylic acid intake on commensal vaginal bacteria and yeast, utilizing a cohort of 100 HIV-seronegative female sex workers. My study focused on female sex workers because they are at greater risk of acquiring HIV, would likely be the population to use this intervention and their mucosal immunology and microbiome is different from

women in the general population who are non-sex workers. Secondly, FSW exhibit distinct differences in their vaginal microbiome and mucosal immunology due to their high-risk sexual behavior compared to non-sex worker women in the general population (Wessels *et al.*, 2017). One primary factor contributing to these differences is the increased overall bacterial diversity found in their vaginal microbiomes. FSWs often lack the beneficial Lactobacillus-dominant communities typical of healthy vaginal microbiomes, instead displaying a higher abundance of bacteria associated with BV) such as *Gardnerella* spp. .This shift towards a non-Lactobacillus-dominant microbiome is associated with an elevated risk of acquiring sexually transmitted infections STIs and HIV (Wessels *et al*, 2017).

Moreover, the implications of this altered microbiome extend to mucosal immunology. The increased microbial diversity correlates with heightened levels of local mucosal inflammation, which can compromise the epithelial barrier. A less stable microbial community, particularly one enriched with BV-associated bacteria like *Gardnerella* spp. can degrade the protective epithelial glycan coat, weakening the genital tract's barrier function. Additionally, this altered microbiome can influence the local immune environment, potentially fostering a pro-inflammatory state that increases vulnerability to infections (Sivro *et al.*, 2020).

While the pilot study emphasized the implications for HIV susceptibility, the focus of my study was to ensure that ASA did not create a microbial environment which placed women at greater risk of acquiring HIV. While research conducted in the pilot study, found no harmful alterations in the vaginal flora after six weeks of ASA use, there have been no studies

demonstrating the impact of longer term ASA use on commensal genital flora across different dosages of the drug (Lajoie, J *et al*, 2018). Together, both studies highlight the importance of exploring the broader implications of NSAID use in the female genital tract. Therefore, maintaining a healthy genital microbiome is important when evaluating new HIV prevention strategies.

Further, no studies have explored the effect of ASA on incidence of vaginal candidiasis. Therefore, to address these gaps in knowledge, this thesis describes a follow-up study aimed at evaluating the impact of the daily ASA (no drug, 81 mg or 325 mg) intake on the commensal vaginal bacteria and yeast. As HIV rates are highest among female sex workers, we wanted to conduct the study in a cohort that may benefit by adding another HIV prevention tool to their prevention toolbox. HIV negative female sex workers were recruited from the Sex Workers Outreach Program (SWOP) clinic in Majengo, Nairobi. Vaginal swabs were collected from consenting participants, smears were made and Gram stained to assess the microbiome. The standard Nugent score (Nugent *et al.*, 1991) was used with microscopy for BV diagnosis and bacterial morphotypes quantification at baseline, 2 months and 5months of (ASA) intake because it is the standard technique commonly used in clinical settings for assessing the vaginal microbiome.

1.2 Statement of Problem

Women who experience elevated levels of basal genital inflammation face a significantly increased risk of contracting HIV. This is particularly evident among female sex workers (FSWs), in whom the prevalence of BV can be as high as 60% to 70% (Wessels *et al*, 2017). Elevated genital inflammation, often associated with BV and a range of sexually transmitted infections (STIs), has been shown to

increase susceptibility to HIV infection (Masson *et al.*, 2015). This heightened risk is compounded by the fact that individuals living with HIV often experience higher rates (2-10 times) of genital inflammation, further exacerbating their vulnerability to STIs and increasing the likelihood of HIV transmission to others (Cohen, Craig R. *et al.*, 2012). Bacterial vaginosis, characterized by an imbalance in the normal vaginal flora, has also been associated with increased susceptibility to HIV. A meta-analysis demonstrated that women with BV were approximately 1.5 times more likely to acquire HIV compared to those with a healthy vaginal microbiota. This increased risk is likely due to the inflammatory response associated with BV, which can lead to heightened genital tract inflammation, which enhances the likelihood of HIV transmission during sexual intercourse. Furthermore, the altered microbial environment may compromise the protective mechanisms of the vaginal epithelium, making it easier for the virus to enter the bloodstream (McClelland *et al*, 2008). As established by prior research and corroborated by my study, female sex workers (FSW) represent a particularly vulnerable key population, with a higher prevalence of STIs and BV than that of women in the general population due to factors such as inconsistent condom use and multiple sexual partners (McClelland *et al*, 2008).

Research has demonstrated the crucial role that BV-associated bacteria play in regulating genital tract inflammation. These bacteria can disrupt the normal vaginal flora, leading to an inflammatory response that enhances the risk of HIV acquisition (Naranbhai *et al*, 2012). Current intervention strategies aimed at reducing inflammation, such as the use of probiotics, antimicrobial treatments, and behavioral interventions, have shown some efficacy in lowering the risk of HIV transmission. Pre-exposure prophylaxis (PrEP) has also been effective in

reducing HIV transmission rates by up to 99% when taken consistently (Abdool Karim *et al.*, 2010). However, in the presence of genital inflammation the protective effect of the anti-retroviral was completely negated, thereby suggesting that genital inflammation needs to be controlled in order for the anti-retroviral to be effective.

Despite the promise of these interventions, there is a critical lack of longitudinal research evaluating the long-term effects of anti-inflammatory strategies on the vaginal commensal bacteria in the genital tract. Understanding how these interventions impact the vaginal microbiome over time is essential for developing effective prevention strategies against HIV. Future research should focus on examining the relationship between changes in bacterial composition, levels of genital inflammation, and the risk of HIV acquisition. By elucidating these dynamics, researchers can better inform effective intervention strategies that not only reduce inflammation but also enhance overall vaginal microbiome health, ultimately contributing to HIV prevention efforts.

1.3 Hypothesis

Overall, this study hypothesized that the daily intake of ASA will not alter commensal vaginal bacteria and yeast in the genital tract, thereby not increasing the risk of HIV acquisition.

1.4 Objectives

1.4.1 General objective

To characterize effect of daily ASA intake on the commensal vaginal microbiome.

1.4.2 Specific objectives

i) To determine the changes in *Lactobacillus* spp. populations in the female genital tract before and after use of 81mg, 325mg of ASA and no drug.

ii) To determine changes in the populations of *Gardnerella vaginalis* and other anaerobes in the female genital tract before and after use of 81 mg, 325 mg of ASA and no drug.

iii) To evaluate the effect of ASA on yeast cell populations in the female genital tract before and after use of 81 mg, 325 mg of ASA and no drug.

iv) To determine changes in the expression of activation markers CD25 and CD69

1.5 Justification and Significance of the Study

Inflammation in the female genital tract may provide a conducive environment for HIV acquisition. Understanding various causes of genital inflammation and their link to risk of HIV acquisition is critical in determining effective interventions. Thus, understanding the effect of daily anti-inflammatory drug intake on the vaginal flora will be important in determining the safety of pharmacological approaches to controlling genital inflammation.

Furthermore, the safety and efficacy of daily anti-inflammatory drug intake in this context have not been thoroughly investigated. While anti-inflammatory medications may reduce genital inflammation and potentially lower HIV risk, their impact on the composition of the vaginal microbiome remains largely unexplored. A balanced microbiome is essential for protecting

against infections, and any negative alterations could inadvertently increase the risk of HIV acquisition.

The need for this study arises from the complex interplay between genital inflammation, the vaginal microbiome, and HIV susceptibility, which remains inadequately explored in current literature. While existing research has established associations between genital inflammation and increased HIV risk, there is a critical need to investigate how anti-inflammatory interventions could potentially mitigate these risks.

The findings of this study aim to fill this gap by examining the effects of anti-inflammatory strategies on both one aspect of inflammation and the vaginal microbiome, thereby contributing to a more nuanced understanding of their roles in HIV transmission dynamics. The significance of this research lies in its potential to establish a foundational understanding that will inform future research initiatives focused on optimizing interventions targeting both inflammatory conditions and the balance of the vaginal microbiome.

This study was conducted in the context of a larger clinical study whose goal was to evaluate the impact of ASA on HIV target cell numbers and immunology among FSW. This analysis is comprehensive and is currently ongoing. Therefore, this thesis will not address those primary questions about HIV target cells and inflammation, rather, it was conducted to specifically

assess whether any of the interventions altered the vaginal microbiome as a change to a pro-inflammatory microbiome could increase the risk of HIV infection.

CHAPTER TWO

LITERATURE REVIEW

2.1 Immunology of the Female Genital Tract

The female genital tract (FGT) is an immunologically active site with a unique mucosal immune system that differs from the systemic immune system (Hickey *et al.*, 2011). This mucosal immunity is regulated by sex hormones, such as estradiol (E2) and progesterone (P4), and consists of both innate and adaptive components (Hickey *et al*, 2011). The innate components include; the epithelium and mucosal lining which form a physical barrier that acts as the first line of defense against pathogenic microorganisms, such as bacteria and HIV (Mwatelah *et al*, 2019). Presence of commensal bacteria such as *Lactobacillus spp* on the vaginal mucosa helps maintain a healthy vaginal environment. These commensal bacteria produce antimicrobial agents and lactic acid, which lowers the pH of the vagina. This acidic environment helps prevent the invasion and growth of pathogenic microorganisms (Rose Ii *et al.*, 2012). Additionally, macrophages, dendritic cells, and Langerhans cells, play crucial roles in pathogen neutralization through phagocytosis, enzyme digestion, and antigen presentation (Rosales, 2018). Macrophages also make a significant contribution to the genital tract's pathogen defense mechanism. These phagocytic cells are capable of engulfing and destroying invading microbes directly. Furthermore, macrophages can release inflammatory signaling molecules that help recruit and activate other critical immune components to the site of infection (Kaushic *et al.*, 2010).

Macrophages play a crucial role in the immune system by orchestrating inflammatory responses through various cytokines and chemokines (Masson *et al*, 2015). Key pro-

inflammatory cytokines produced by activated macrophages include Tumor Necrosis Factor-alpha (TNF-α), which enhances immune cell recruitment and is associated with conditions like rheumatoid arthritis and sepsis, as well as increased HIV replication. Interleukin-1 beta (IL-1β) also mediates inflammation, inducing fever and other cytokines, with elevated levels linked to cardiovascular disease and heightened HIV transmission. Interleukin-6 (IL-6) exhibits both pro- and anti-inflammatory properties, contributing to chronic conditions such as obesity and metabolic syndrome while also promoting HIV replication (Arnold *et al.*, 2016). Additionally, chemokines like CCL2 and CCL5 are vital for recruiting immune cells to inflammation sites, with high levels correlating with diseases like atherosclerosis and chronic obstructive pulmonary disease (COPD),and CCL2 facilitating HIV entry into target cells (Borges *et al.*, 2015).

The cumulative effect of these inflammatory signals produced by macrophages leads to significant impacts on immune responses and disease outcomes. The interplay of pro-inflammatory cytokines and chemokines can drive chronic inflammation, contributing to the pathogenesis of various infections such as HIV (Passmore *et al*, 2016). Understanding these inflammatory pathways is crucial for developing targeted therapeutic strategies to mitigate inflammation and its associated risks.

The adaptive arm of the immune system is essential for safeguarding the genital tract against pathogenic invasion. It comprises two cellular components, B lymphocytes and T lymphocytes cells (Machado, J. R. *et al.*, 2014). B cells are responsible for stimulating the production of

secretory IgA antibodies, which line the genital mucosal surfaces. These antibodies can neutralize or opsonize invading microbes, effectively preventing their entry and subsequent establishment within the genital tract. Whereas cells play a crucial coordinative role in the defense against genital pathogens (Machado, J. R. *et al*, 2014) .

CD4+ T helper cells have the capacity to activate and stimulate the function of other key immune effector cells, such B cells, to actively participate in the clearance of invading microbes. Conversely, CD8+ cytotoxic T cells possess the specialized ability to directly recognize and eliminate host cells that have been compromised by pathogen infection through the granzyme-perforin system (Hickey *et al*, 2011). The adaptive immune system's defense against pathogens in the genital tract relies on the coordinated and complementary functions of B cells and T cells.

The expression of activation markers CD25 and CD69 on CD4+ T cells is critical in understanding immune responses, particularly in high-risk populations such as female sex workers (FSWs) who are disproportionately affected by HIV (Omollo *et al.*, 2021). CD25, the alpha chain of the interleukin-2 receptor, is upregulated upon T cell activation and is associated with T regulatory cells (Tregs), which play a dual role in promoting immune tolerance and regulating inflammation. CD69, an early activation marker, is rapidly expressed following T cell activation and is involved in the retention of T cells in inflamed tissues (Ssemaganda *et al.*, 2021). In the context of HIV susceptibility, studies have demonstrated that increased

proportions of activated CD4+ T cells in the genital mucosa may correlate with enhanced risk of infection (Fontenot *et al.*, 2005).

Recent studies have shown that the genital mucosa of FSWs exhibited significantly higher proportions of CD25+ and CD69+ T cells compared to peripheral blood, emphasizing the importance of localized immune responses in the genital mucosa of FSWs. For example, in one study, researchers demonstrated that the proportion of activated CD4+ T cells expressing CD25 and CD69 was markedly elevated in the genital mucosa, with CD25+ T cells reaching up to 45% and CD69+ T cells at 65%, compared to lower levels in systemic circulation (Hladik *et al.*, 2009). This suggests that the genital mucosa serves as a hotspot for immune activation and inflammation, which may contribute to increased susceptibility to HIV infection. Additionally, the presence of CD69+ T cells in the genital mucosa has been associated with ongoing inflammation due to STIs, which can further enhance HIV transmission risk (Ssemaganda *et al*, 2021).

Despite these robust immune responses, genital inflammation triggered by factors such as bacterial vaginosis and sexually transmitted infections can enhance HIV susceptibility by increasing the recruitment of HIV target cells to the FGT (Masson *et al.*, 2014). Inflammation is defined as a localized protective immune response that occurs as a result of tissue injury, infection, or irritation within a specific anatomical site. When pathogens invade the mucosal surfaces in the female genital tract, the immune system is activated.

2.2 Genital Inflammation and HIV Risk

The main goal of genital inflammation is to neutralize invading pathogens, limit the spread of infection, and facilitate the healing and repair of the damaged tissue. When the genital mucosa is compromised by the presence of harmful microbes, the immune system becomes activated. This activation initiates an inflammatory response, which is characterized by increased blood flow, swelling, redness, and the recruitment of various immune cells to the affected site. The inflammatory process in the genital tract involves the release of chemical mediators, such as cytokines and chemokines, which help coordinate the mobilization and targeted activity of immune effector cells, including neutrophils, macrophages, and lymphocytes (Mtshali *et al.*, 2021).

Genital inflammation significantly enhances susceptibility to HIV infection through several interconnected mechanisms. One primary factor is the disruption of the epithelial barrier in the genital tract, which serves as a crucial defense against pathogens. Inflammatory conditions, such as bacterial vaginosis (BV) or sexually transmitted infections (STIs), lead to increased levels of inflammatory cytokines like Interleukin-1 beta (IL-1β) and Tumor Necrosis Factor-alpha (TNF-α). These cytokines can cause epithelial cell apoptosis and increase permeability, allowing HIV easier access to underlying immune cells, particularly CD4+ T cells, which are the primary targets for the virus (Alcaide *et al.*, 2017).

Additionally, genital inflammation results in the recruitment of various immune cells to the inflamed tissue, including CD4+ T cells, macrophages, and dendritic cells. Chemokines such

as CCL2 (MCP-1) and CCL5 (RANTES) attract these immune cells through a chemotactic gradient, increasing the presence of CD4+ T cells in the genital mucosa and thereby enhancing the potential for HIV transmission. The inflammatory environment also alters the activation state of these immune cells, leading to an upregulation of HIV co-receptors on CD4+ T cells, making them more susceptible to infection (Appay *et al.*, 2008) Chronic inflammation can create a microenvironment that favors HIV replication, characterized by high levels of cytokines that promote viral entry and replication (Passmore *et al*, 2016).

Moreover, genital inflammation is associated with elevated viral loads in genital secretions, increasing the risk of transmission during sexual contact. Studies indicate that individuals with genital inflammation often have higher concentrations of HIV in their genital tracts, compounding the risk of infection (Masson *et al.*, 2016).

Inflammation has also been associated with damage to the epithelial barrier due to elevated levels of inflammatory cytokines in the genital mucosa associated with altered expression of proteases which degrade the tight junctions of the epithelia, therefore increasing permeability of the virus toward the susceptible target cells that lie beneath the epithelia (Arnold *et al*, 2016).

Among the various cytokines involved, Interleukin-6 (IL-6) stands out due to its role in promoting the acute phase response and influencing the differentiation of B and T cells. Elevated levels of IL-6 have been linked to increased HIV replication and heightened

susceptibility to HIV infection. The inflammatory environment created by IL-6 can facilitate the entry of HIV into target cells, particularly CD4+ T cells, thereby exacerbating the risk of HIV transmission (Catalfamo *et al.*, 2012).

Another critical chemokine involved in genital inflammatory responses is Interleukin-8 (IL-8). This chemokine primarily attracts neutrophils to sites of inflammation and is produced by epithelial cells and macrophages in response to infection. High levels of IL-8 not only enhance neutrophil recruitment but also create a more permissive environment for HIV infection. Neutrophils can release enzymes and reactive oxygen species that further promote viral entry and replication, contributing to the overall inflammatory response (Masson *et al*, 2014).

Tumor Necrosis Factor-alpha (TNF-α) is another key player in genital inflammation. As a potent pro-inflammatory cytokine, TNF-α activates endothelial cells and recruits immune cells to sites of infection. Its elevated levels have been shown to enhance HIV replication in infected cells and increase the expression of HIV co-receptors on target cells, facilitating viral entry. Similarly, Interleukin-1 beta (IL-1β) is crucial for regulating immune and inflammatory responses. Increased levels of IL-1β can enhance HIV replication and contribute to an inflammatory milieu that raises susceptibility to HIV infection (Masson *et al*, 2016).

In addition to these cytokines, chemokines such as CCL2 (MCP-1) and CCL5 (RANTES) play significant roles in genital inflammation. CCL2 and CCL5 attract monocytes and T cells to

sites of inflammation, promoting immune cell migration. Their presence is implicated in HIV pathogenesis, as CCL2 can enhance HIV entry into target cells by facilitating the migration of CD4+ T cells to the site of infection. Meanwhile, CCL5 has been shown to assist in HIV entry into T cells, further complicating the inflammatory landscape (Silva *et al.*, 2023).

Increased risk of inflammation is potentially mediated by biological factors which include sexually transmitted diseases, bacterial vaginosis, hormonal contraceptive use, multiple sexual partners and infrequent condom use (Masson *et al*, 2015). STIs such as, *Haemophilus ducreyi*, herpes and syphilis are known to cause damage on the mucosa by forming lesions and ulcers and this damage leads to inflammation (Mwatelah *et al*, 2019).

Conversely, individuals with a quiescent immune state and reduced genital inflammation may have fewer available HIV target cells, potentially conferring some degree of protection against HIV infection, as observed in studies of HIV-exposed seronegative individuals (HESN). Some research studies have shown that a state of reduced genital inflammation and immune activation may confer protection against HIV infection. Low levels of pro-inflammatory cytokines, indicative of low inflammation, have been associated with a decreased risk of HIV seroconversion (Naranbhai *et al*, 2012). Therefore, while increased immune activation has been identified as a risk factor for HIV acquisition, "immune quiescence," a phenotype characterized by reduced systemic and mucosal immune activation, has been proposed as a potential model of protection against HIV infection Consistent with this, studies have found that HESN individuals such as female sex workers, exhibit a distinct genital cytokine and

chemokine profile that is associated with reduced susceptibility to HIV-1 (Lajoie, J *et al.*, 2012). HIV- exposed seronegative FSW show a quiescent phenotype in the CD4+ T cell compartment.

and reduced expression of HIV-dependent host factors (McLaren *et al.*, 2010). This decreased immune activation and resistance to HIV-1 infection is associated with an elevated frequency of $CD4^{+}CD25^{+}FOXP3^{+}$ regulatory T cells (Card *et al.*, 2009). Overall, the interplay between HIV target cells and genital inflammation is a critical factor in understanding the dynamics of HIV acquisition and transmission, with important implications for HIV prevention and treatment strategies.

2.3 Bacterial Vaginosis

Bacterial vaginosis (BV) is the most frequent type of vaginal condition in women of reproductive age (Atashili *et al*, 2008). The abnormal shift from a Lactobacilli-Dominant (LD) microflora to a non-LD microflora (which is a diverse microbial community) due to depletion of the *Lactobacillus* spp. and overgrowth of bacterial anaerobes as *Gardnerella vaginalis*, *Mobiluncus* and other anaerobes induces inflammatory responses. This disruption of the normal vaginal microflora has also been noted to influence vaginal inflammation (Atashili *et al*, 2008).

A normal vaginal microflora also known as a healthy vaginal microbiome is predominantly characterized by the dominance of *Lactobacillus* spp. which produce lactic acid to lower the vaginal pH and create a protective environment against harmful pathogens by producing

antimicrobial products such as hydrogen peroxide (Petrova *et al.*, 2015). This community of bacteria plays a crucial role in safeguarding the vaginal microbiome by preventing the overgrowth of harmful anaerobic bacteria such as *Gardnerella vaginalis*, thereby protecting against infections like bacterial vaginosis (BV) (Chee *et al.*, 2020). *Lactobacillus* spp. in the female genital tract are Gram-positive rods that can be found singly, in pairs, or in short chains. Morphologically, they are non-spore forming and can vary in length, appearing as either short, plump rods or long, slender rods. When subjected to Gram staining, these bacteria typically appear as blue/purple thick rods (Money, 2005). *Lactobacillus* spp. are considered a normal component of the vaginal flora and serve as important biomarkers due to their crucial role in maintaining vaginal microbiome(Chen *et al.*, 2021).

The inhabitance of other anaerobic bacteria such as, *Gardnerella vaginalis, Mobiluncus* spp. and *Atopobium vaginae* in the vaginal area is facilitated by their ability to adhere to host tissues, utilize available nutrients, evade the immune response, and tolerate environmental variations (Smritee *et al.*, 2021). These factors collectively contribute to their dominance in the vaginal microbiome during bacterial vaginosis. *Gardnerella vaginalis* is particularly adept at adhering to vaginal epithelial cells, which is critical for its colonization. Studies have shown that this bacterium expresses surface proteins such as adhesins, sialic acid-binding proteins, lipopolysaccharides, hemolysins, and surface layer proteins that facilitate attachment to host tissues, allowing it to establish a foothold in the vaginal microbiome (Hardy *et al.*, 2015). *Gardnerella vaginalis* employs various mechanisms for adhesion, adhesins bind to host epithelial cell receptors, sialic acid-binding proteins attach to sialic acid on host cells to enhance colonization lipopolysaccharides (LPS) facilitate adhesion and trigger inflammatory

responses; hemolysins lyse host cells to release nutrients and promote adherence and surface layer proteins (S-layers) provide protection and enhance stability while aiding in attachment (Srinivasan *et al.*, 2009) The ability to form biofilms further enhances its capacity to persist in the vaginal environment, as biofilms provide protection from host immune responses and antimicrobial agents.*Atopobium vaginae* also demonstrates strong adhesion capabilities, which are essential for its survival in the competitive vaginal milieu. Its adherence to epithelial cells allows it to maintain a stable population even when *Lactobacillus* species are present, contributing to the dysbiotic state associated with BV (Smritee *et al*, 2021).

The vaginal environment presents unique nutritional challenges, and the ability of these microorganisms to utilize available resources is crucial for their survival. *Mobiluncus* spp. are known to ferment a variety of carbohydrates and produce short-chain fatty acids, which can create an acidic environment that may inhibit the growth of *Lactobacillus* species. This metabolic flexibility allows them to thrive in conditions where other organisms may struggle.

Prevotella spp. are also capable of utilizing a range of substrates, including proteins and carbohydrates, allowing them to flourish in the nutrient-rich environment of the vaginal microbiome (Larsen, 2017). Their ability to metabolize different nutrients enhances their competitive advantage over *Lactobacillus* species, which prefer a more specific set of substrates.

The ability to evade the host immune response is another critical factor that enables these BV-associated microorganisms to inhabit the vaginal area. *Gardnerella vaginalis* can produce enzymes that degrade host mucins, disrupting the protective mucus layer and facilitating bacterial invasion (Machado, A. *et al.*, 2015). This enzymatic activity not only aids in colonization but also compromises the host's first line of defense. *Atopobium vaginae* has been shown to modulate the immune response, allowing it to persist in the presence of immune cells. By evading detection and destruction, *Atopobium vaginae* can maintain its population within the vaginal microbiome (Macklaim *et al.*, 2013).

The vaginal environment is dynamic, with variations in pH, moisture, and temperature. BV-associated microorganisms exhibit a degree of tolerance to these fluctuations, for example, *Mobiluncus* spp. thrive in low-oxygen conditions, which are typical in the anaerobic environment of the vagina during BV. This tolerance to anaerobic conditions allows them to outcompete *Lactobacillus* species, which prefer aerobic environments (Srinivasan *et al*, 2009).

BV can lead to or increase the risk of developing certain gynecological conditions such as pre-term births and pelvic inflammatory disease. A prospective study among Danish pregnant women observed an association between BV and low birth weights as well as pre-term births (Svare *et al.*, 2006). The researchers found that colonization of the endometrium by BV infection triggered production of inflammatory cytokines that affected the placental function and initiation of labor (Svare *et al*, 2006). The association of BV and HIV or how BV increases HIV acquisition has not been well understood, but it’s thought that BV plus other STIs are major

drivers of genital tract inflammation therefore increasing risk of acquiring HIV (Cohen, Craig R. *et al*, 2012).

Cytokines and chemokines are immune mediators that play a critical role in the host's response to infection, but they can also contribute to tissue damage and chronic inflammation. The presence of bacterial vaginosis (BV)-associated bacteria triggers the host immune system, leading to the release of various pro-inflammatory cytokines and chemokines, for example, *Gardnerella vaginalis* has been shown to stimulate the production of interleukin-6 (IL-6) and interleukin-8 (IL-8) in vaginal epithelial cells, which are crucial for recruiting immune cells to the site of infection and enhancing the inflammatory response (Brotman, 2011). Elevated levels of IL-8, in particular, are associated with increased neutrophil recruitment, exacerbating inflammation and tissue damage.

Similarly, *Atopobium vaginae* can induce the secretion of tumor necrosis factor-alpha (TNF-α) and other inflammatory mediators in response to bacterial components, with the activation of Toll-like receptors (TLRs) on host immune cells leading to the upregulation of cytokines such as IL-1β and IL-6, further amplifying the inflammatory response (Macklaim *et al*, 2013). Additionally, *Mobiluncus* spp. can stimulate the production of chemokines like CCL2 (MCP-1) and CCL5 (RANTES), which are critical for the recruitment of monocytes and T cells to the site of infection. This recruitment not only enhances the inflammatory response but also contributes to the chronicity of inflammation associated with BV (Srinivasan *et al*, 2009). The increased production of inflammatory cytokines and chemokines in response to BV associated

bacteria has significant implications for local immunity; the heightened inflammatory state can disrupt the vaginal microbiome, creating a feedback loop that favors the persistence of pathogenic bacteria while inhibiting beneficial *Lactobacillus* species. This dysregulation can lead to an increased susceptibility to secondary infections, including sexually transmitted infections (STIs) (Brotman, 2011). Furthermore, the inflammatory environment created by BV has been linked to an increased risk of HIV acquisition, as the presence of inflammatory cytokines may facilitate the entry of the virus into target cells, compounding the public health implications of BV (Srinivasan *et al*, 2009).

BV is also known to have frequent recurrence despite its treatment with Metronidazole and Clindamycin (Aroutcheva *et al.*, 2001). Resistance to the drugs has been linked to the overgrowth and colonization of the diverse microbial communities such as *Gardnerella vaginalis*, and *Mobiluncus* spp. that take time to clear up even after treatment (Aroutcheva *et al*, 2001).

BV associated pathogens possess antibiotic resistance genes and components that enable them to resist the effects of antibiotics. These mechanisms include the presence of antibiotic resistance genes (ARGs), biofilm formation, enzymatic degradation, capsule production, and altered target sites. Such traits complicate treatment strategies and contribute to the persistence of these microorganisms in the vaginal microbiome.

The presence of ARGs in BV-associated pathogens poses a significant challenge to treatment.. *Gardnerella vaginalis* has been identified to harbor various ARGs in some strains, which confer resistance to commonly used antibiotics, including tetracyclines, macrolides,lincosamides and metronidazole. These strains include tetracycline resistance genes such as tet(M), tet(L), and tet(W). Additionally, they harbor macrolide resistance genes, including erm(B) and erm(F), as well as the lincosamide resistance gene lsaC. In metronidazole resistance, some *Gardnerella vaginalis* strains carry genes like YadH and Mc(r)A, which are associated with resistance to this particular drug. These genetic traits enable *Gardnerella vaginalis* to withstand the effects of these commonly used antibiotics, complicating treatment options (Castellano *et al.*, 2025). The genetic mechanisms underlying this resistance often involve plasmids or transposons that facilitate horizontal gene transfer among bacterial populations.

Similarly, *Atopobium vaginae* has been found to contain resistance genes that enable it to withstand antibiotic treatments. Specific genes conferring resistance in *Atopobium vaginae* are not extensively documented in the available research; however, it is known to exhibit high resistance to nalidixic acid and colistin. Most studies have focused on the phenotypic resistance profile rather than identifying specific resistance genes. For example, *A. vaginae* demonstrates significant resistance to metronidazole, which is associated with the presence of nim genes that encode nitroimidazole reductase enzymes capable of detoxifying the drug. Generally, *A. vaginae* is susceptible to clindamycin, penicillin, and linezolid, although instances of resistance can occur (De Backer *et al.*, 2006). Additionally, some strains of *Mobiluncus* spp. have been reported to carry ARGs that provide resistance to multiple classes of antibiotics. Specific

antibiotic resistance genes identified in *Mobiluncus* species include erm(X), which confers macrolide resistance (particularly to clindamycin), and tet(O) and tet(W), which are associated with tetracycline resistance. These genes are frequently found on transposable elements, such as the composite transposon Tn5432, facilitating their horizontal transfer. This mechanism has been linked to high levels of resistance observed in strains like *M. curtisii (Zhang et al., 2020)* complicating treatment options for infections associated with these bacteria (Smritee *et al*, 2021).

Bacterial vaginosis is linked to the formation of adherent polymicrobial biofilms on the vaginal epithelium, primarily composed of *Gardnerella vaginalis*. A biofilm is a structured community of microbes attached to a surface, embedded in a self-produced polymeric matrix made of carbohydrates, proteins, and nucleic acids. The biofilm formation process is complex and dynamic, involving motile planktonic microbes and microbial aggregates. Various bacterial and fungal species, including *Gardnerella spp.* and *Candida* spp., can contribute to biofilm formation. These biofilms provide effective protection against host immune responses and antimicrobials (Machado, A. *et al*, 2015).

The polymicrobial biofilms on the vaginal epithelium are crucial to the pathogenesis of BV, with *G. vaginalis* acting as the primary colonizer that facilitates the attachment of other BV-associated microbes. *Atopobium vaginae*, is a notable secondary colonizer that is strongly associated with BV. Biofilms formed by *G. vaginalis* exhibit greater tolerance to lactic acid and hydrogen peroxide than planktonic cells, which may help shield *Gardnerella spp* and other BV-associated microbes from hostile environments (Swidsinski *et al.*, 2023).

Atopobium vaginae has been shown to produce enzymes that can degrade certain antibiotics, rendering them ineffective. This enzymatic activity is a common mechanism of resistance in various bacteria and can significantly hinder treatment efforts (Hardy *et al*, 2015). *Mobiluncus* spp. may produce a capsule that provides a physical barrier against phagocytosis and antibiotic action. This structural feature enhances the bacteria's survival in the presence of antimicrobial agents and contributes to their persistence in the vaginal environment (Belay *et al*, 2024). In the altered target sites mechanism, mutations in target sites of antibiotics can occur in BV-associated bacteria, leading to reduced binding affinity for the drugs. This mechanism is often observed in bacteria that develop resistance to macrolides and other antibiotics (Belay *et al*, 2024).

Several studies have demonstrated the role of bacterial vaginosis (BV)-associated bacteria in modulating genital tract inflammation. For example, a study by by some reseearchers showed that BV-associated bacteria, such as *Gardnerella vaginalis* and *Prevotella bivia*, were linked to increased inflammatory cytokines and chemokines in the genital tract (Gosmann *et al*, 2017). A meta-analysis by (Torrone *et al.*, 2018) concluded that BV is associated with an increased risk of HIV acquisition among women, potentially due to the inflammatory changes induced by BV-associated bacteria (Torrone *et al*, 2018). Women with persistent BV had a higher incidence of HIV infection compared to those without BV, emphasizing the importance of maintaining a healthy vaginal microbiome (Eastment *et al.*, 2018).

Bacterial vaginosis (BV) can be characterized by both the standard Nugent score and molecular methods e.g.; deep sequencing of the 16S rRNA gene (Seq BV) and quantitative PCR (qPCR-BV) (McKinnon *et al.*, 2019). The Nugent score is a diagnostic tool that incorporates Gram staining and microscopy to the vaginal bacterial composition by assessing three key morphotypes, *Lactobacillus* spp. (large, Gram-positive rods), *Gardnerella vaginalis* (small, Gram-variable rods) and *Mobiluncus* spp. (curved, Gram-variable rods). Each morphotype is scored from 0 to 4, based on the number of bacterial cells observed per oil immersion field. The scores for the three morphotypes are then summed to obtain the final Nugent score, which ranges from 0 to 10. The interpretation of the Nugent score is as follows: (0 to 3) Normal vaginal flora, (4 to 6) Intermediate vaginal flora and (7 to 10) Bacterial vaginosis. Both Nugent score method and molecular methods can categorize bacterial vaginosis (BV) as symptomatic or asymptomatic. The vaginal microbial communities characterized by molecular methods have been collectively referred to as Molecular-BV (McKinnon *et al*, 2019). The Molecular-BV communities are depleted of *Lactobacillus spp* and a diverse abundance of anaerobes such as *Gardnerella vaginalis*. The Seq-BV molecular method has categorized the microbial communities as cervicotypes 3 and type 4 that (CT3) and (CT4) respectively. The (Seq-BV) method has been shown to correlate with Nugent score method (McKinnon *et al*, 2019).

2.4 Bacterial Vaginosis Associated Bacteria

The primary pathogens associated with BV include *Gardnerella vaginalis, Atopobium vaginae, Mobiluncus* spp. and *Prevotella* spp. Each of these pathogens exhibits unique characteristics, virulence traits, and pathogenic mechanisms that contribute to the development and persistence of BV.

2.2.1 General Characteristics

Gardnerella vaginalis is a facultative anaerobic, gram-variable (a mix of both gram positive and gram negative) bacterium recognized as one of the most commonly identified pathogens in bacterial vaginosis (BV). This organism is characterized by its small, non-spore-forming rod shape and its ability to exist in both planktonic and biofilm states. While *Gardnerella vaginalis* is often found as part of the normal vaginal flora in women, its overgrowth is closely associated with the development of BV. A critical aspect of its pathogenicity is its ability to adhere to vaginal epithelial cells, which is essential for its colonization and persistence in the vaginal environment (Ferris *et al.*, 2004).

Atopobium vaginae is a gram-positive, anaerobic bacterium frequently isolated from women diagnosed with BV. This non-motile organism typically forms part of the complex microbial community within the vagina. Studies indicate that *Atopobium vaginae* is often present in higher concentrations in women with BV, suggesting its significant role in the pathogenesis of this condition. Its presence is indicative of a dysbiotic state, contributing to the overall microbial imbalance associated with BV (Smritee *et al*, 2021).

Mobiluncus spp. are curved, gram-negative anaerobic rods. These bacteria are motile due to their flagella, which enables them to navigate effectively through the vaginal environment. *Mobiluncus* spp. are typically found in higher concentrations in women diagnosed with BV, underscoring their association with this condition (Alcaide *et al*, 2017). Their motility and ability to thrive in anaerobic conditions contribute to their pathogenic potential in the context

of BV. *Prevotella* spp. are gram-negative anaerobic bacteria commonly isolated from women with BV. These bacteria are non-motile and are typically found within polymicrobial communities in the vagina. *Prevotella* spp. are known for their ability to thrive in low-oxygen environments, which is characteristic of the vaginal microbiome in cases of BV. Their presence further complicates the microbial landscape of the vagina and contributes to the dysbiosis observed in BV.

2.2.2 Virulence traits

The mechanisms employed by BV pathogens to induce and maintain bacterial vaginosis are multifaceted. Through adhesion, biofilm formation, production of virulence factors, tissue degradation, and modulation of the host immune response, these bacteria disrupt the normal vaginal microbiome, leading to the clinical manifestations of BV. *Gardnerella vaginalis* possesses several virulence factors that enhance its pathogenic potential. One notable trait is its ability to form biofilms, which provide a protective environment for the bacteria and facilitate their colonization (Machado, A. *et al*, 2015).

The production of sialidase is another critical virulence factor; this enzyme breaks down sialic acid in mucins, compromising the vaginal epithelial barrier and promoting inflammation. Additionally, *Gardnerella vaginalis* can produce various metabolites, such as amines and fatty acids, which can alter the local pH and contribute to dysbiosis. These factors collectively enable *Gardnerella vaginalis* to evade host immune responses and establish infection. *Gardnerella vaginalis* possesses several virulence factors that enhance its pathogenic potential.

Additionally, *Gardnerella vaginalis* can produce various metabolites, such as amines and fatty acids, which can alter the local pH and contribute to dysbiosis. These factors collectively enable *Gardnerella vaginalis* to evade host immune responses and establish infection (Shvartsman *et al.*, 2023) .

Atopobium vaginae exhibits several virulence traits that enhance its pathogenic potential. One significant trait is its ability to adhere to vaginal epithelial cells, which facilitates colonization. The bacterium produces short-chain fatty acids, such as propionic and acetic acids, which can lower the pH of the vaginal environment and contribute to local inflammation (Vaseruk *et al.*, 2025).Furthermore, *Atopobium vaginae* can modulate the host immune response, allowing it to persist in the vaginal environment despite immune challenges. This immune modulation may involve the secretion of factors that inhibit the activity of immune cells, thereby promoting its survival (Chen *et al*, 2021).

Mobiluncus spp. possess several virulence traits that enhance their pathogenic potential. They produce proteolytic enzymes, such as collagenase and gelatinase, which can damage host tissues and disrupt the epithelial barrier (Smritee *et al*, 2021). This tissue damage can facilitate the invasion of other pathogens and contribute to the inflammatory response. Additionally, their motility allows *Mobiluncus* spp. to migrate through vaginal secretions, increasing their chances of establishing infection. The presence of these bacteria is often correlated with increased inflammatory markers, such as C-reactive protein (CRP) and cytokines, further complicating the clinical picture of BV (McKenzie *et al.*, 2021).

Prevotella spp. possess several virulence factors that enhance their pathogenic potential. One significant trait is the production of lipopolysaccharides (LPS), which can trigger strong inflammatory responses in the host (Larsen, 2017). The presence of LPS can activate immune cells, leading to the release of pro-inflammatory cytokines and contributing to the inflammatory state associated with BV. Additionally, *Prevotella* spp. can form biofilms, which enhance their survival and resistance to antimicrobial agents. Their metabolic byproducts, including short-chain fatty acids, can further disrupt the vaginal environment, promoting dysbiosis and inflammation.

2.2.3 Pathogenicity

The pathogenicity of BV pathogens is characterized by their ability to adhere to and colonize vaginal epithelial cells, produce virulence factors that disrupt host tissues, and elicit inflammatory responses. *Gardnerella vaginalis* is primarily linked to its role in the inflammatory response associated with BV. The presence of this bacterium is often correlated with elevated levels of pro-inflammatory cytokines, such as interleukin-1 (IL-1) and interleukin-6 (IL-6), indicating an inflammatory response in the vaginal environment (Witkin *et al.*, 2007). This inflammation can lead to symptoms such as abnormal discharge, odor, and irritation, which are characteristic of BV. By disrupting the normal vaginal microbiome and eliciting an immune response, *Gardnerella vaginalis* significantly contributes to the clinical manifestations of BV. The pathogenicity of *Atopobium vaginae* is closely linked to its interactions with other BV-associated bacteria, particularly *Gardnerella vaginalis*. The co-colonization of these organisms can exacerbate inflammation and dysbiosis, leading to more severe BV symptoms. Studies have indicated that *Atopobium vaginae* is often present alongside

other anaerobic bacteria, contributing to a polymicrobial environment that complicates the clinical picture of BV. The ability of *Atopobium vaginae* to persist and proliferate in this environment underscores its role in the pathogenesis of BV (Smritee *et al*, 2021).

Mobiluncus spp. is primarily associated with their contribution to the inflammatory milieu in BV. Their presence is often linked to increased levels of pro-inflammatory cytokines, which can exacerbate symptoms such as abnormal discharge and odor. By disrupting the normal vaginal flora and promoting an inflammatory response, *Mobiluncus* spp. play a significant role in the pathogenesis of BV. Their interactions with other anaerobic bacteria in the vaginal environment can lead to a synergistic effect, further promoting dysbiosis and inflammation. *Prevotella* spp. is closely linked to their role in the inflammatory response associated with BV. Their presence has been shown to correlate with increased levels of inflammatory cytokines, such as IL-6 and tumor necrosis factor-alpha (TNF-α), which can exacerbate the symptoms of BV and increase the risk of secondary infections, such as sexually transmitted infections (STIs) (Pérez-Ibave *et al.*, 2025).

2.5 Yeast Infection and Genital Inflammation

Vulvovaginal candidiasis, commonly known as a yeast infection, is a prevalent fungal condition that affects women of reproductive age. The primary causative agent is Candida species, with *Candida albicans* being the most common (Fidel Jr, 2002). Clinically, yeast infections typically present with a thick, white, "cottage cheese-like" vaginal discharge that has a characteristic yeast-like odor (Smritee *et al*, 2021). Diagnosis of vulvovaginal candidiasis can

be made through direct observation of the clinical symptoms, as well as confirmatory laboratory tests. These include a potassium hydroxide (KOH) wet mount examination, which reveals the presence of budding yeast cells, pseudo-hyphae, or true hyphae (Singh *et al.*, 2022). Gram staining can also be used to visualize the fungal elements. The standard treatment for vulvovaginal candidiasis involves the use of antifungal medications, which can be administered topically as creams, ointments, or vaginal suppositories, or systemically as oral tablets (Singh *et al*, 2022). Common antifungal agents used include miconazole, clotrimazole, and fluconazole (Singh *et al*, 2022).

Yeast infections can lead to inflammation in the genital tract through several mechanisms. Overgrowth of yeast in the vagina, triggers an immune response that recognizes the yeast cells as foreign and responds by activating inflammatory pathways. This leads to the release of inflammatory cytokines and chemokines, which recruit immune cells to the site of infection. These yeast cells can adhere to and penetrate the vaginal epithelial cells, releases enzymes and toxins that contribute to tissue irritation and causing direct tissue damage which further stimulates the immune system and leads to inflammation (Naglik *et al.*, 2011).

Yeast infections can also alter the balance of the vaginal microbiome, which plays a crucial role in maintaining a healthy, protective environment. *Lactobacillus* bacteria typically dominate the vaginal microbiome and therefore inflammation caused by yeast overgrowth alters the vaginal pH and creates an environment more favorable for anaerobic bacterial growth (Witkin *et al*, 2007).

The inflammation caused by the yeast infection can create a pathway for the acquisition and transmission of other sexually transmitted infections (STIs), including HIV. Yeast cells, particularly *Candida albicans*, play a significant role in activating inflammatory pathways within the host immune system. This activation occurs primarily through the recognition of yeast cell wall components, such as β-glucans and mannans, by pattern recognition receptors (PRRs) on immune cells. Notably, Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are crucial in this process. For instance, TLR2 and TLR4 have been shown to recognize β-glucans, while Dectin-1, a CLR, specifically binds to these components on the yeast surface (Marakalala *et al.*, 2011).

Upon binding, these receptors initiate intracellular signaling cascades that activate nuclear factor kappa B (NF-κB) and other transcription factors, leading to the expression of pro-inflammatory cytokines and chemokines. The recognition of *Candida albicans* by immune cells results in the release of several key inflammatory cytokines and chemokines. Tumor necrosis factor-alpha (TNF-α) is one of the most critical cytokines produced during this response, promoting inflammation and recruiting immune cells to the site of infection (Jang *et al.*, 2021). Interleukin-1 beta (IL-1β) is another important cytokine that enhances the inflammatory response and activates T cells and macrophages. Additionally, interleukin-6 (IL-6) plays a role in the acute phase response and is involved in the differentiation of B cells and T cells (Kerkis *et al.*, 2024). Interleukin-8 (IL-8), a chemokine, is particularly noteworthy for its ability to attract neutrophils to the site of infection, thereby amplifying the inflammatory response (Wang *et al.*, 2014).

These inflammatory signals significantly enhance the activities of immune cells, for example, TNF-α and IL-1β stimulate the proliferation and activation of T cells, thereby strengthening the adaptive immune response (Mantovani *et al.*, 2019). IL-8 is crucial for recruiting neutrophils to the infection site, where they can phagocytose *Candida* cells and produce reactive oxygen species (ROS) to eliminate the pathogen (Zhu *et al.*, 2023). Furthermore, the production of these inflammatory mediators influences the polarization of macrophages, promoting a pro-inflammatory M1 phenotype that is effective in combating fungal infections. This polarization is vital for orchestrating a robust immune response against *Candida* (Cicuéndez *et al.*, 2021).

The inflamed and damaged tissues are more vulnerable to infection, and the resultant immune activation can also increase the risk of HIV acquisition and transmission. This interconnected chain of events highlights the importance of addressing yeast infections promptly and effectively. Proper treatment and management of yeast infections can help reduce the associated inflammation, restore the vaginal microbiome, and potentially lower the risk of acquiring or transmitting other STIs, including HIV. The relationship between yeast infections and bacterial vaginosis (BV) has been well-documented, with studies showing that these conditions often co-occur, as an overgrowth of yeast can disrupt the normal balance of vaginal bacteria (Bradshaw *et al.*, 2015).

2.6 Use of anti-inflammatory drugs in HIV prevention

Given the intricate relationship between HIV and inflammation, there is growing interest in the use of anti-inflammatory drugs for HIV prevention which potentially reduce inflammation and immune activation at the genital tract. The most commonly used anti-inflammatory drugs are known as non-steroidal anti-inflammatory drugs (NSAIDs) which include; acetylsalicylic acid (ASA), Ibuprofen, and diclofenac (Cromarty *et al.*, 2021).

2.6.1 Acetylsalicylic Acid Use

Acetylsalicylic acid is an NSAID. Oral ASA is in a tablet form and due to its ability to inhibit platelets aggregation, it is used as a blood thinner to prevent blood clots from forming in the arteries for those at risk of heart attack or stroke. It is also has been indicated for use as an antipyretic to reduce fever and pain (Vane *et al.*, 2003).

2.6.2 Mode of Action

Acetylsalicylic acid (ASA) primary mechanism of action involves inhibiting the activity of cyclooxygenase (COX) enzymes, which convert arachidonic acid into prostaglandins. In turn, prostaglandins perform several functions, including causing and resolving inflammation (Vane *et al*, 2003).

2.6.3 Impact of ASA on Bacterial Populations

Encouraging results from our own research group have shown that taking ASA was associated with reductions of inflammation and HIV target cells at the genital mucosa of low-risk women (Lajoie, Julie *et al.*, 2018). The study showed that a 6-week course of low-dose ASA (81 mg/day) decreased CD4+CCR5+ T cells, which are HIV target cells, at the genital mucosa by up to 35% (Lajoie, Julie *et al*, 2018). This suggests that use of ASA impacted the immune response in a way that is potentially beneficial for the prevention of HIV. Therefore, this study offered new insights into the immunological effects of ASA at the genital tract. Use of anti-inflammatory agents, including NSAIDs and corticosteroids, was effective in reducing inflammatory cytokines and dampening T cell activation within the genital mucosa. These findings provide additional evidence that targeting inflammation through the use of anti-inflammatory drugs could be a viable approach to address the heightened HIV susceptibility linked to genital inflammation. Collectively, previous studies have indicated that various anti-inflammatory drugs have been evaluated and found to be effective in controlling genital inflammation and attenuating T cell activation, which are crucial considerations in understanding and potentially reducing the risk of HIV transmission in the female genital tract (Cromarty *et al.*, 2020).

While ASA primarily acts through systemic mechanisms, the indirect effects of ASA on the microbiome have been noted in some gut studies (Rogers *et al.*, 2016). A recent study found that aspirin reduced colorectal tumor development in mice, but the gut microbiome reduced aspirin's bioavailability and chemopreventive effects. Specifically, the study demonstrated that the gut microbiome can metabolize and inactivate aspirin, diminishing its ability to suppress

colorectal tumor growth (Wu *et al.*, 2023). Another review suggests that aspirin and other non-antibiotic drugs can significantly alter the composition and diversity of the human gut microbiome. These drug-induced changes to the gut microbiome were found to have important implications for the drugs' therapeutic efficacy (Le Bastard *et al.*, 2021). In another study, findings showed that daily oral administration of 325mg aspirin significantly altered the composition and diversity of the gut microbiome compared to placebo. The researchers observed that aspirin intake was associated with changes in the abundance of bacterial taxa that have been previously linked to increased colorectal cancer risk (Prizment *et al.*, 2020). Additionally, a study in America found that aspirin modulated the abundance of the colorectal cancer-associated bacterium *Fusobacterium nucleatum* in the gut. This suggested that aspirin's effects on the gut microbiome composition may have contributed to its chemopreventive properties against colorectal cancer (Brennan *et al.*, 2021).

Acetylsalicylic acid (ASA) is primarily absorbed in the gastrointestinal tract where it is also metabolized before being distributed throughout the body, including the genital area. Although ASA’s primary mechanism of action is not directly linked to the genital microbiome, its systemic distribution and potential influences on the gut microbiome could indirectly affect the genital microbiome the area (Vane, 1971). However, the concentration of acetylsalicylic acid and its metabolites in the genital area may be likely low compared to other parts of the body. The specific effects of ASA on the genital microbiome are not well-established, and the concentrations necessary to have significant impact on the genital microbiome remain unclear. The potential effects, if any, may be indirect and depend on various factors, such as the

medication's distribution, concentration, and interactions with the host's physiology (Vane, 1971).

The genital microbiome analysis on the ASA arm which showed two major microbial communities among the women: Lactobacillus dominant (LD) communities which were less common (n= 22, 33%) while non-Lactobacillus dominant (non-LD) was more common (n= 45, 67%). The non-Lactobacillus dominant taxa had high proportions of diverse microbial communities mostly made up of *Gardnerella vaginalis*, *Prevotella* or *Atopobium vaginae*. Interestingly, majority of the women (n= 24, 77.4%) on the ASA arm sustained an unchanged microbial diversity over time, whereas four women (12.9%) changed from non-*LD* to *LD* and three women (9.7%) changed from Lactobacillus dominant to non-Lactobacillus dominant post treatment and there was no significant change over time with use of ASA (Lajoie, Julie *et al*, 2018). According to these findings, in the majority of women, ASA use did not significantly alter the microbial diversity of the genital tract, which remained unchanged. However, a small proportion of women experienced a shift from a non-LD state to a LD state.

Lactobacillus-dominant strains, such as *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus gasseri*, are predominant in a healthy vaginal microbiome, maintaining a low pH (3.8-4.5) through lactic acid production, which inhibits pathogenic bacteria and yeast, thereby protecting against infections like bacterial vaginosis (BV) and vulvovaginal candidiasis (VVC) (Smritee *et al*, 2021). These strains also enhance local immunity and stabilize the microbiota, making it less susceptible to disruptions. In contrast, non-Lactobacillus-dominant strains,

including *Gardnerella vaginalis* and various anaerobes, are associated with dysbiosis, leading to a higher vaginal pH (>4.5) that fosters the overgrowth of pathogens, increasing the risk of infections and sexually transmitted infections (STIs) (Masson *et al.*, 2019).This imbalance can trigger inflammatory responses, resulting in symptoms like itching and abnormal discharge, further compromising the vaginal mucosal barrier. Thus, the presence of *Lactobacillus* is crucial for maintaining vaginal health, while a non-Lactobacillus dominance is linked to increased infection susceptibility and inflammation. The pilot study found that there were no changes in the microbiome (Lajoie, Julie *et al*, 2018).

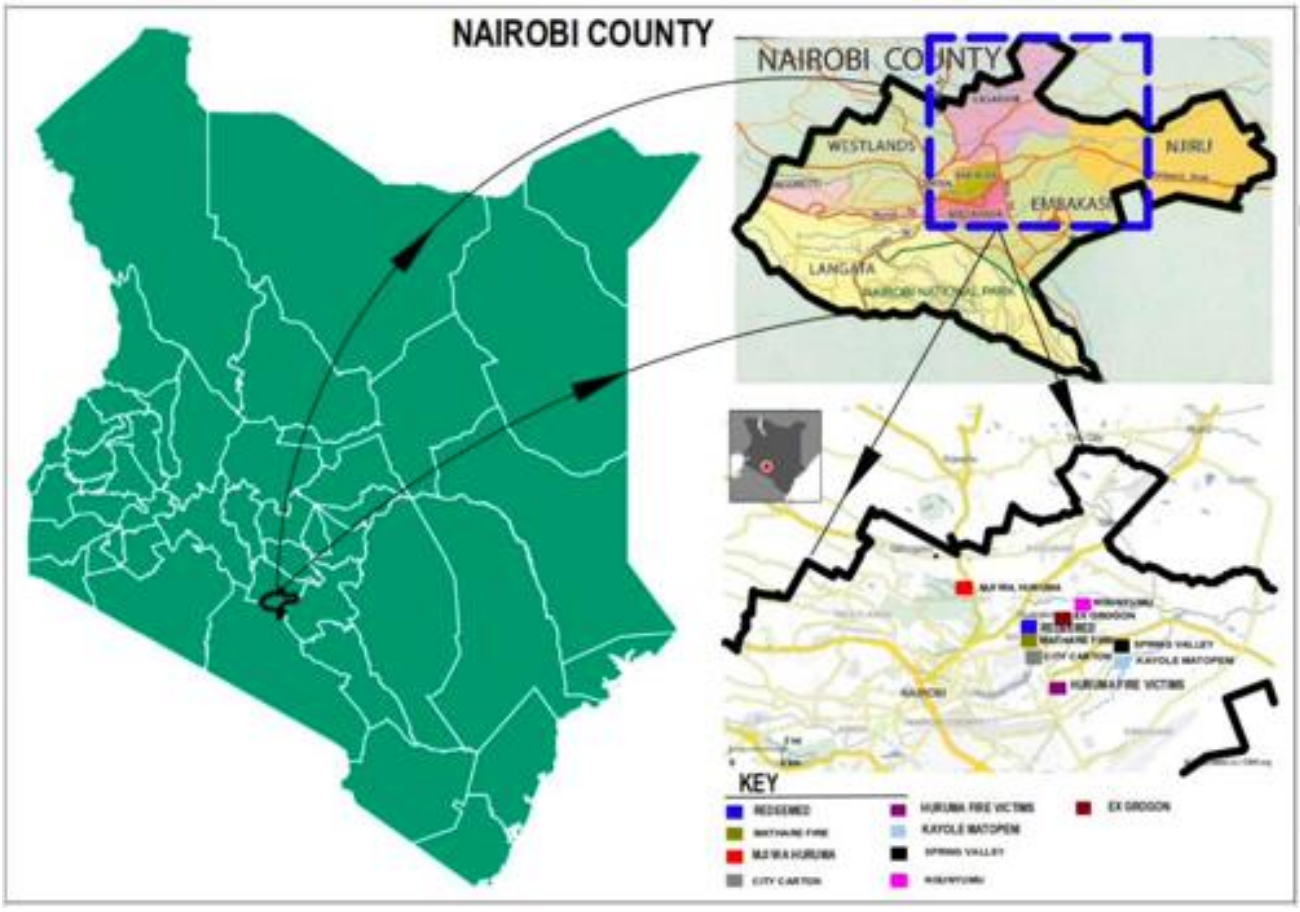
Overall, ASA did not significantly change the microbiome of either the LD or the non-LD groups. However, it remains unknown whether a longer duration of ASA use would significantly impact genital microbiome leading to bacterial vaginosis. Changes in the vaginal microbiome induced by anti-inflammatory interventions like ASA could potentially predict the success or failure of these strategies as HIV prevention tools. Understanding their impact on the vaginal microbiome is crucial, as it can provide insights into the suitability and effectiveness of these anti-inflammatory approaches for HIV prevention. It can be shown that most studies on HIV prevention strategies have focused on the virus, while few studies have focused on the host factors such as inflammation on the genital tract and how interventions used to reduce inflammation on the genital tract affect the commensal bacteria on the genital tract.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Locale

This study was conducted at the Sex Workers Outreach Program (SWOP) clinic in Majengo, Nairobi County, Kenya. Majengo Pumwani Settlement is found in Pumwani Sub-County, Pumwani Location, Pumwani Ward, Kamukunji Sub-County, and Nairobi County. The Project is located within Pumwani area of Nairobi County. Approximate area is 8.2 ha. GPS Coordinates 1°12’30”S and 36°55’10”E.



(*Source*: Kenya Informal Settlements Improvement Project (KISIP))

Figure 3. 1 Pumwani-Majengo Settlement, Nairobi County

(*Source*:Kenya Informal Settlements Improvement Project (KISIP))

Figure 3. 2 Layout-Pumwani-Majengo Settlement, Nairobi County

SWOP is a collaborative research unit housed within the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID). The SWOP clinics have been funded by CDC-PEPFAR since 2005. Currently SWOP-Kenya has ten clinics strategically located within Nairobi County offering services to over 45000 sex workers. The samples were processed at the laboratories based within UNITID.

3.2 Study Cohort

This study was conducted among female sex workers (FSW) at the Sex Workers Outreach Program (SWOP) clinic in Majengo, Nairobi. This program manages a cohort of over 25,000 sex workers including Highly Exposed Seronegative (HESN) individuals, and provides essential HIV prevention and treatment services and other healthcare packages addressing the unique vulnerabilities of sex workers.

3.3 Study Design

This was a longitudinal study nested in a large randomized open label clinical trial currently being done among HIV negative female sex workers. Participants were randomized to 81mg ASA, 325mg ASA and no drug then followed up for 5 months. For the present study, 100 participants (33 taking 81 mg ASA, 33 taking 325 mg ASA and 34 taking no drug) were recruited in the first year and followed up for 5 months. This study lacked a control group due to the absence of a drug group to control those taking ASA and therefore BV status at baseline was used as the individual participant control by comparing their pre-treatment results to those observed during treatment.

Acetylsalicylic acid (ASA) is available in several dosages, including low-dose options like 81 mg (baby aspirin) and 100 mg for cardiovascular protection. Regular doses, such as 325 mg, are commonly used for pain relief and anti-inflammatory effects, while higher doses of 500 mg may be indicated for severe pain or specific clinical situations. One objective of the larger

clinical study was to determine the most effective dosage for reducing inflammation in the genital tract.

3.4 Sample size

A total of 476 participants were mobilized and recruited, with 318 undergoing prescreening. Among those prescreened, 218 were excluded from the study due to testing positive for BV, yeast and classical STIs (syphilis, chlamydia, gonorrhea, trichomonas) and anemia. Overall,100 participants were randomized into three different drug arms (Arm-D, Arm-K and Arm-H) of the study. The drug arms were not disclosed to the laboratory team so as to minimize unconscious bias. Among those enrolled, 38 participants voluntarily discontinued their involvement, including 16 who were lost to follow-up, 11 who self-withdrew, 5 due to pregnancy, 2 who experienced side effects, 1 with recurrent infections, and 1 due to cryotherapy (Figure 3.3). A total of 64 participants completed all visits, Arm-D(no drug)(n=16), Arm-K(81mg) (n=22), Arm-H(325mg) (n=26).

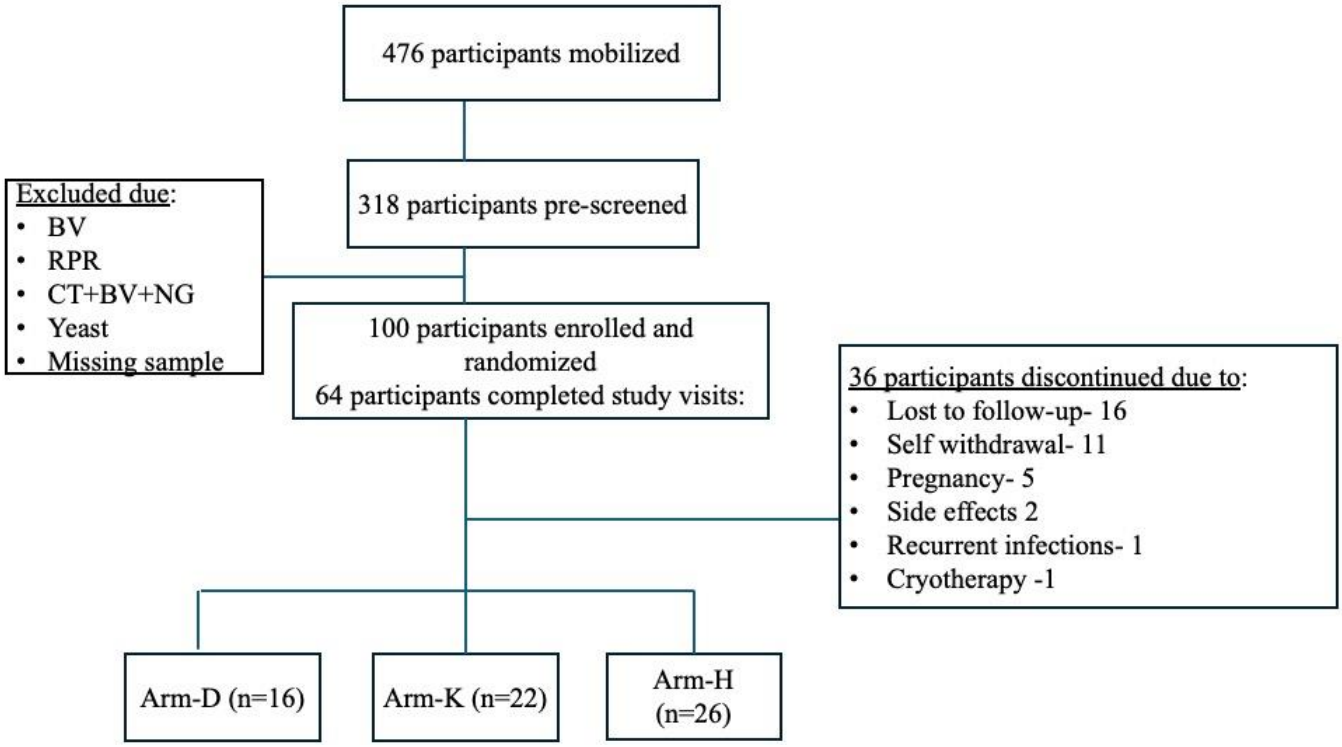


Figure 3. 3 Schematic study profile

3.4.1 Sample Size Calculation

To observe a 30% effect of ASA on BV occurrence, based on error probability of 0.05 and 95% power, it was estimated that 33 participants would be required in each group (Faul et al., 2007). Sample size calculation was done using the formula for comparing proportions as shown below:

$$n = (Z_{\alpha/2}+Z_{\beta})^2 * (p_1(1-p_1)+p_2(1-p_2)) / (p_1-p_2)^2$$

where:

n is the required sample size

$Z_{\alpha/2}$ is the critical value of the Normal distribution at $\alpha/2$ (for instance confidence level of 95%, α is 0.05 and the critical value is 1.96)

Z_{β} is the critical value of the Normal distribution at β (for instance for a power of 80%, β is 0.2 and the critical value is 0.84) p_1 and p_2 are the expected sample proportions of the two groups.

Sample size computation was done using G*power software version 3.1 (Faul et al., 2007).

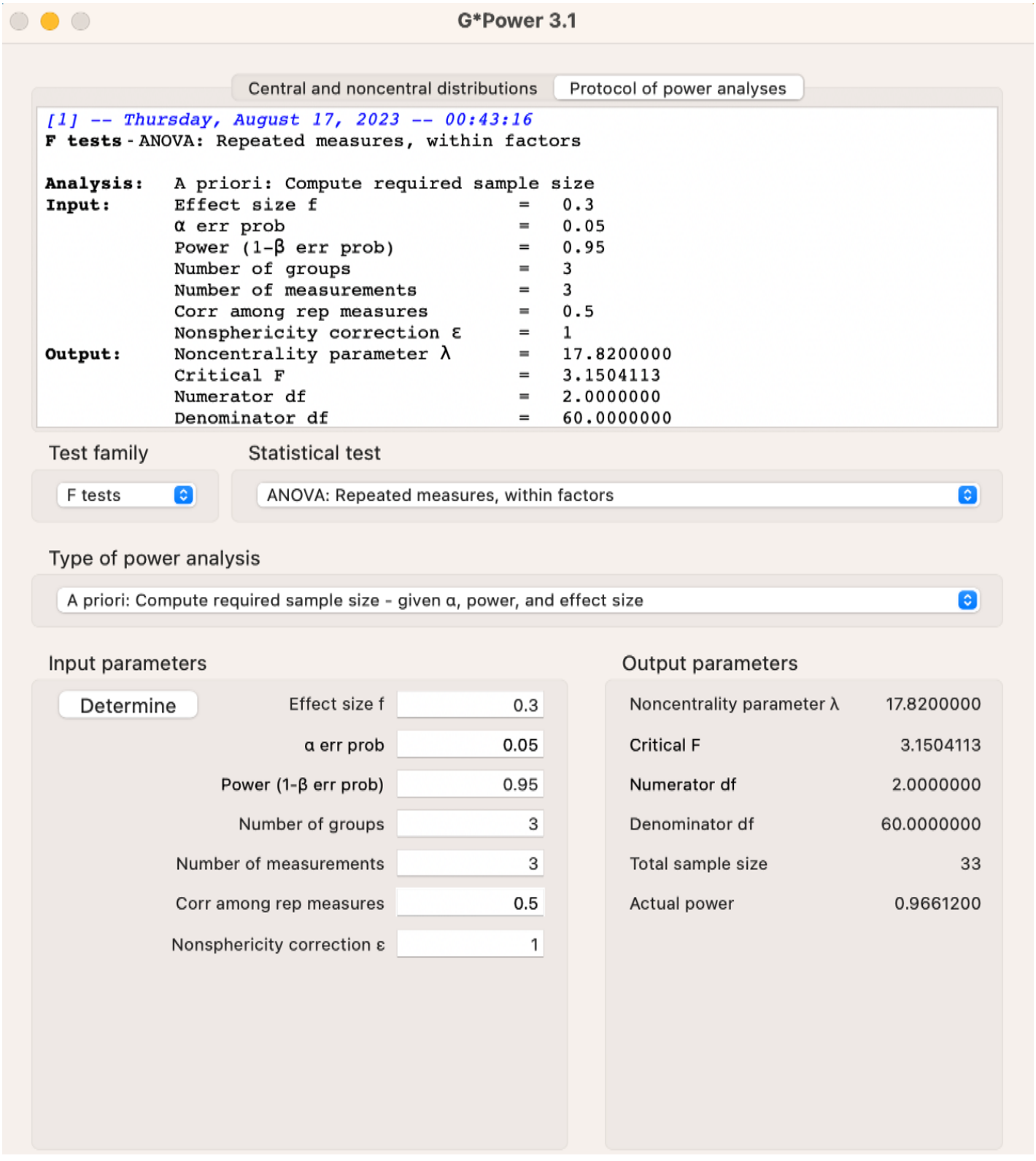


Figure 3. 4 Sample size calculation format using G*power

3.5 Inclusion and Exclusion Criteria

Overall, participants were considered eligible for enrolment in the larger study if they fulfilled all the inclusion criteria and none of the exclusion criteria as stated below. However, the main inclusion criteria for this study was to have participants who had completed the study visits and had all the BV results.

3.5.1 Inclusion Criteria for study participants

Women enrolled met the following criteria:

i. Aged between 18 years and 45 years of age.
ii. HIV negative status
iii. Involved in sex work for a maximum of five years or less
iv. Had uterus and cervix.
v. Consent to take the daily study drug intervention (ASA)
vi. Consent to undergo pelvic exams.
vii. No chronic infection that requires taking any anti-inflammatory drug
viii. No cardiovascular disease

3.5.2 Exclusion criteria

ix. Aged <18 or more than 45 years of age
x. Pregnant in the preceding 12 months
xi. Breast-feeding women
xii. Pregnancy during study
xiii. Tested positive for classical STIs or bacterial vaginosis at enrollment.

xiv. Current participation in a clinical trial

xv. Menopausal

xvi. Active sex work for more than 6 years or no sex work activity

xvii. Having a chronic disease

xviii. Allergy to ASA or an anti-inflammatory drug

xix. Risk of bleeding disorder

xx. History of cardiovascular disease

In reference to sex work activity, the criteria was established to exclude individuals who are Highly Exposed but Seronegative (HESN) from the study. HESN refers to individuals at high risk of HIV exposure, often due to behaviors or circumstances such as having multiple sexual partners or being in a relationship with an HIV-positive individual, yet who remain uninfected despite repeated exposure to the virus (Fowke *et al.*, 1996). These individual were excluded as my group had determined that their vaginal immunology and microbiome are different from other women and therefore would not be representative of the general population of sex workers. Our focus was on including only participants in the New Negative period, defined as those with up to 5 years of self-declared sex work experience as my group has shown their immunology and microbiome is similar to the wider population.

3.6 Sampling design

The sampling design for this study was based on randomization. The research nurse was trained to review inclusion and exclusion criteria for each potential participant for eligibility. Eligible participants were requested to consent and participate in the study. Participants were invited to

randomly pick a numbered piece of paper. Each piece corresponded to one of the drug arms in the study (81 mg, 325 mg and no drug). Those who consented (Consent form in Appendix 5) underwent an external genital examination conducted by the research nurse to check for abnormalities, including irritation, redness, discharge, cysts, genital warts, or other clinical issues affecting the vulva and cervicovaginal mucosa. Those who met the inclusion criteria were enrolled for a 6-month period and had to be willing to take the drug daily for a 5-month period. After the fifth month visit, participants were asked to stop the study drug and to come back two weeks and four later for post-drug follow-up visits.

Consenting participants were screened for BV at baseline, and at subsequent visits that would be at 2 months and at 5 months. The nurse performed an exterior genital examination for abnormalities like any irritation, signs of redness, discharge, cysts, genital warts, or other clinical abnormalities on the vulva and the cervicovaginal mucosa. All participants were enrolled for a 6-month period and had to be willing to take drug and to come back two weeks and four later for post-drug follow-up visits.

3.7 Specimen collection

3.7.1 Vaginal swabs

samples were collected at baseline, 2 months and 5 months. A speculum was gently inserted in the vagina by a nurse. The speculum was used to allow visual examination inside of the vagina and the cervix to check for ulceration, discharge, discoloration, or other clinical abnormalities. A sterile cotton swab was used to collect cervical mucus by rotating the swab at 360º in the

cervical opening. The high vaginal swab was used to make a smear on a microscope slide for Gram staining.

3.7.2 Blood samples

Thirty mls of venous blood were collected and transferred into sodium Heparin tubes then transported to the lab at room temperature.

3.7.3 Cervico-vaginal samples

The cytobrush was gently inserted into the cervical os, rotated 360°, removed, and placed into 50ml falcon tube containing PBS.

3.8 Laboratory procedures

The air-dried slide was Gram stained and the different bacterial morphotypes (*Lactobacillus* spp., *Gardnerella vaginalis and Mobiluncus* spp) visualized using microscopy under magnification x1000 on oil immersion

3.8.1 Gram Staining

Gram staining is widely regarded as the gold standard for diagnosing bacterial vaginosis (BV) due to its ability to provide a direct and reliable assessment of the vaginal microbiota's composition (Money, 2005). Gram stain is preferred for several reasons. First, it allows for the rapid identification of bacterial morphotypes based on their Gram reaction, which is crucial for

diagnosing BV. It differentiates between Gram-positive and Gram-negative bacteria, enabling one to observe shifts in the vaginal microbiome, specifically the reduction of *Lactobacillus* species and the overgrowth of anaerobic bacteria such as *Gardnerella vaginalis* and *Mobiluncus* spp.

Studies have demonstrated that when combined with the Nugent scoring system, Gram staining offers a reproducible and straightforward approach to evaluating these bacterial populations, making it an essential tool in clinical setting. The simplicity of the procedure, along with its cost-effectiveness, ensures that clinicians can obtain results quickly, facilitating timely treatment. In contrast to Gram staining, there are currently no standardized biochemical tests specifically for diagnosing BV (Redelinghuys *et al.*, 2020). Biochemical tests rely on identifying specific metabolic byproducts or enzymatic activities, which can be complex and may not directly correlate with the presence of BV. Furthermore, the absence of a universally accepted biochemical test for BV underscores the importance of Gram staining as the primary diagnostic method (Money, 2005).

First, the smear on the glass slide was air-dried and then stained with crystal violet, which bound to the negatively charged components of the bacterial cell wall. Next, the slide was treated with iodine, which acted as a mordant, forming an insoluble complex with the crystal violet and trapping it within the cell wall. The slide was then decolorized acetone which removed the excess stain, leaving the crystal violet-iodine complex only within the cell walls. Finally, the slide was counterstained with safranin. This counterstaining step provided a visual

distinction between Gram-positive and Gram-negative bacteria. Gram-positive bacteria retained the crystal violet-iodine complex, appearing purple or blue, while Gram-negative bacteria took up the counterstain, appearing pink or red (Moyes *et al*., 2009)..

The Nugent scoring (Figure 3.3) was then used along with microscopic evaluation, to assess the bacterial composition of the smear, and to determine BV diagnosis by quantifying the bacterial morphotypes under microscopy (magnification: 100x with oil immersion). Presence of yeast cells was also determined. The bacterial morphotypes were classified presumptively as:

i. Gram-positive rods (suspected *Lactobacillus* spp.*)* Confirmed by biochemical test, Catalase.

ii. Gram-variable (both Gram positive and negative) rods (suspected *Gardnerella vaginalis*). Confirmed by biochemical tests, Hippurate hydrolysis and starch hydrolysis.

iii. Curved Gram-variable (both Gram positive and negative) rods (suspected *Mobiluncus* spp.). Confirmed by biochemical tests, Oxidase and Catalase.

The Nugent scores were defined as (0-3) for normal, (4-6) for intermediate and (7-10) for BV. In a normal vaginal flora, the Nugent score would be 0-3, with *Lactobacillus* spp. (morphotype I) predominating. This indicates a healthy, balanced vaginal microbiome. In an intermediate state, the Nugent score would be 4-6, with a mixture of *Lactobacillus* spp. and other bacterial morphotypes, indicating a transitional or imbalanced vaginal microbiome. In bacterial vaginosis (BV), the Nugent score would be 7-10, with a significant reduction or absence of *Lactobacillus* spp. and an increase in other bacterial morphotype II (*Gardnerella vaginalis)* and morphotype IV

and curved Gram-variable rods (*Mobiluncus* spp.) indicating an overgrowth of anaerobic bacteria and an imbalanced vaginal microbiome. Only those with BV were treated with Metronidazole. I used the report form on (Appendix 7), to record the results. Most microscopic evaluations and Nugent scoring were performed by myself as the primary investigator to ensure consistency in the assessment.

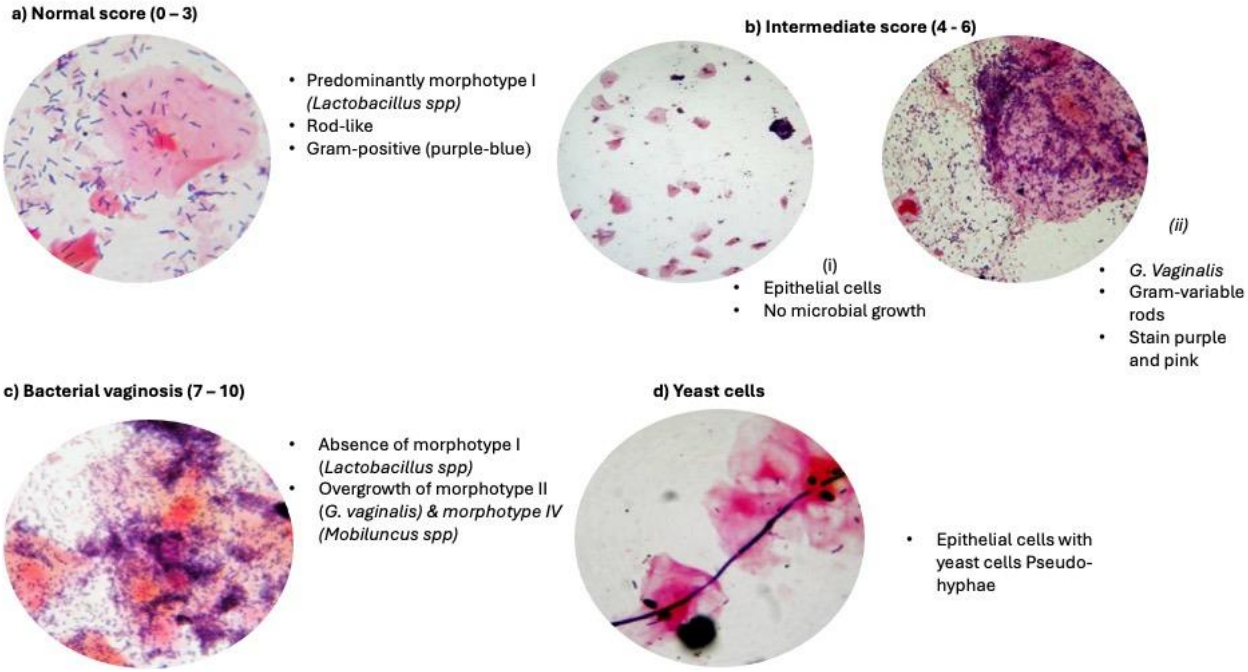
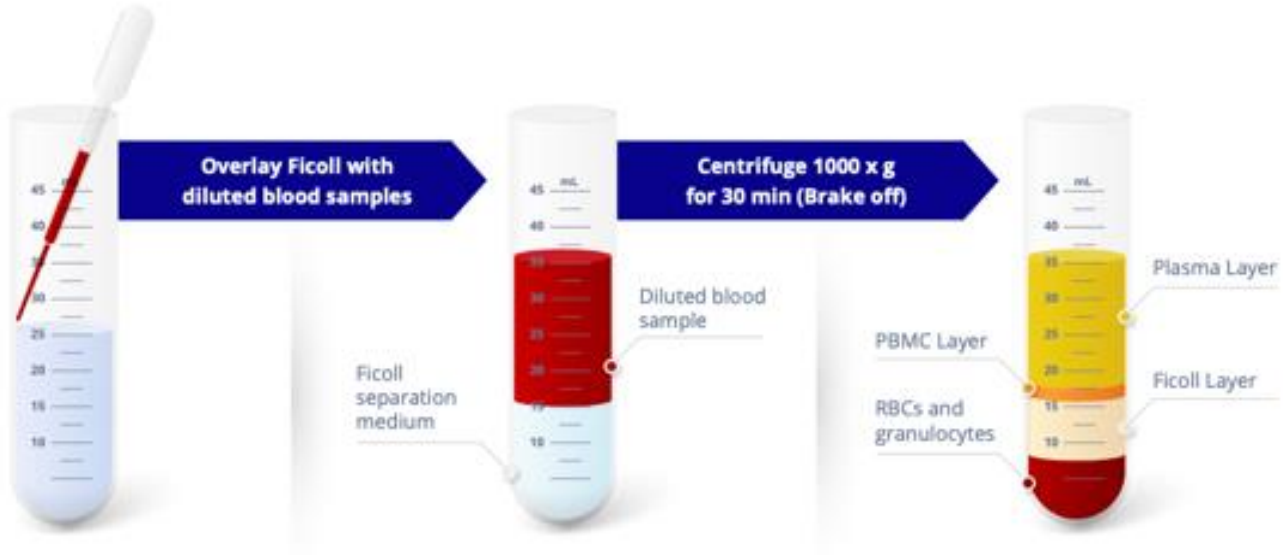


Figure 3. 5 Microscopic field view of a Gram-stained slide.

Key: Microscopic image (Magnification: 100x with oil immersion) showing the composition of the vaginal microbiome under different conditions: a)Normal score (0-3), b)Intermediate score(4-6), c)BV (7-10), d) Presence of yeast cells.

3.8.2 PBMC Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples using Ficoll density gradient. The blood was diluted with an equal volume of phosphate-buffered saline (PBS) to prevent clumping, and the diluted blood was carefully layered over Ficoll-Paque in a 50ml centrifuge tube. The tube was centrifuged at 1600rpm for 20 minutes at room temperature, ensuring that the brake was turned off to maintain the separation layers. After centrifugation, three distinct layers formed, plasma at the top, a buffy coat containing PBMCs in the middle, and red blood cells at the bottom. Using a pipette, the PBMC layer was carefully collected and transferred to a new tube. The PBMCs were washed with PBS by centrifuging at 1600rpm for 10 minutes, discarding the supernatant, and then resuspended the pellet in in RPMI-1640 media containing Penicillin-Streptomycin and fetal bovine serum (FBS). Cell counting was performed using trypan blue and an automated cell counter to assess cell viability and concentration. One million PBMCs were washed by centrifuging at 1600 rpm for 10 minutes, after which the supernatant was discarded. The PBMCs were then processed for staining in preparation for immunophenotyping on the flow cytometer.



Source: Sanguine Biosciences

Figure 3. 6 PBMC Isolation using Ficoll-density gradient technique

3.8.3 CMC Processing

Cervico-mononuclear cells (CMC) were collected using a cytobrush and a plastic spatula, then transferred into a 50 mL conical tube containing 10 mL of phosphate-buffered saline (PBS). To isolate the CMCs from the cell suspension, the tube was vortexed vigorously for 1 minute to dislodge the cells from the cytobrush. In a biosafety cabinet, the mucus on the scraper and cytobrush was squeezed into the fluid suspension. The suspension was then passed through a 100 µm cell strainer into a new conical tube to remove epithelial cells and large mucus particles. The cells were washed by adding 5 mL of RPMI media (supplemented with 10% fetal bovine serum (FBS)) through the filter and centrifuging the filtrate at 1600 rpm for 10 minutes. The supernatant was decanted, and the cell pellet was resuspended by gently flicking the tube, followed by another wash with 5 mL of PBS and centrifugation at 1600 rpm for 10 minutes. The final pellet was resuspended in 100 µL of 2% FBS in 1X PBS (FACS buffer) in preparation for antibody staining.

3.8.4 Flow cytometry

To evaluate changes in immune activation and HIV target cells associated with ASA use, flow cytometric determination of CD4+ T cell expression of CD25 and CD69 was done at each time point on PBMCs (peripheral) and CMCs (mucosal). Following isolation, 1 x 10^6 PBMCs and CMC pellets were immunophenotyped. Both cell types were washed with FACS buffer, while the CMC pellet was resuspended in a blocking solution (0.2 µg/µL mouse IgG, FACS buffer, 10% FBS) for 10 minutes at 4°C, and then washed again with 500 µL of FACS buffer. A mastermix containing fluorochrome-conjugated monoclonal antibodies: Live-Dead-Pe-TxRed, CD3-APC-R700, CD4-APC, CD25-BV786 and CD69-APC-Cy7 was then used to stain the cells for 30 minutes at 4°C. After staining, the cells were washed and fixed in a 1%

paraformaldehyde solution. The stained cells were acquired using a BD FACSCelesta flow cytometer (BD Systems) and analyzed with FlowJo v10.0.8rl software.

3.9 Data Management and Analysis

Nugent score data was entered in a standardized form as attached (Appendix.7)_. All data analyses were done using GraphPad Prism 9.1 (GraphPad Software, La Jolla, CA). Normal distribution of the data was tested by the D'Agostino and Pearson's omnibus normality test to determine if data was normally distributed. If the normality test failed, indicating non-normal data distribution, the non-parametric Kruskal-Wallis test were used to compare changes in BV profiles over time while the Wilcoxon signed-rank test was used to compare BV profiles at different time points within the same individuals. Mann-Whitney U test compared BV profiles between different treatment groups. Fisher's Exact and Chi-square test were used to assess the significance of the association between categorical variables.

The one-way ANOVA test was used to compare the means of the groups to determine if there were any statistically significant differences among them. In addition to the analyses above, comparison of changes in bacterial morphotype scores and T-cell marker expression between time points was done using the Friedman multiple comparison test and Dunn's multiple comparison test as a parametric post-test. Statistical significance was considered a *p value* of ≤ 0.05.

3.10 Ethical Considerations

Ethical approval was obtained from Research ethical committees of University of Nairobi/Kenyatta National Hospital and the University of Manitoba Ethics Review Committee. (Ref.No.KNH/ERC/R/31), (Appendix.3). A research permit from NACOSTI Ref. No.941717 (Appendix.4). was also obtained. Signed consent was obtained from willing participants (Appendix.5)

CHAPTER FOUR

RESULTS

4.1 Sociodemographic and participants' characteristics

The mean age of participants was Arm-D(no drug) (29.7), Arm-K (81mg) (28.9) and Arm-H(325mg) (31.8), (p=0.3487). The majority of the participants were not married (85.9%). Fewer women (43.8%) reported use of intravaginal practices such as douching while (56.3%) reported no douching. Participants across all the study arms did not differ significantly by practicing vaginal douching (p=0.7324), frequency of douching daily (p=0.3237), frequency of douching weekly (p=0.1741), frequency of regular clients (p=0.2147) and the number of times of sex with clients (p=0.0623) (Table 4.1).

Table 4.1 Sociodemographic and behavioral characteristics of participants

Characteristic	D	K	H	*p*-value
	(N=16)	(N=22)	(N=26)	
Age				
Mean ± SD	29.7 ± 7.1	28.9 ± 6.3	31.8 ± 7.9	0.3487
Marital Status				0.5876
Married	2 (12.5%)	2 (9.1%)	0 (0%)	
Not Married	13 (81.3%)	18 (81.8%)	24(92.3%)	
Widow	1 (6.3%)	1 (4.5%)	1 (3.8%)	
Not Married, Living with a Man	0 (0%)	1 (4.5%)	1 (3.8%)	
Practice Vaginal Douching				0.7324
Yes	8 (50.0%)	11 (50.0%)	10 (38.5%)	
No	8 (50.0%)	11 (50.0%)	16 (61.5%)	
Douching Frequency Daily				0.3237
0-1	4 (50.0%)	6(54.5%)	2 (16.7%)	
2-5	4 (50.0%)	5(45.5%)	6 (66.7%)	
5-10	0 (0%)	0 (0%)	1 (8.3%)	
More Than 10	0 (0%)	0 (0%)	1 (8.3%)	
Douching Frequency Weekly				0.1741
2-5	0 (0%)	4 (36.4%)	3 (30.0%)	
5-10	5 (62.5%)	5 (45.5%)	2(20.0%)	
More Than 10	3 (37.5%)	2 (18.2%)	5 (50.0%)	
Number of Regular Clients Last 5 Days				
Median (Range)	1 (0-5)	0.5 (0-7)	0 (0-5)	0.2147
Number of times had Sex with Partner in Last 5 Days				
Median (Range)	0.5 (0-6)	0 (0-3)	0 (0-2)	0.0623

4.2 Impact of ASA on vaginal bacterial profiles and proportions

4.2.1 Comparison of Nugent scores

To assess the impact of ASA on commensal vaginal bacteria, the Nugent score scale was used to categorize the bacterial morphotypes. Within (Arm D-no drug), the median Nugent score was 3 (IQR 0-5), 2 (IQR 2-6) and 3 (IQR 1-4) at visit 1, visit 2 and visit 3 respectively. Within Arm K(81mg) the median Nugent score was 3 (IQR 0-4), 3 (IQR 1-6) and 4 (IQR 1-5) at visit 1, visit 2 and visit 3 respectively and within Arm H (325mg) the median Nugent score was 3 (IQR 1-4), 3 (IQR 1-6), 4 (IQR 2-5) at visit 1, visit 2 and visit 3 respectively. It was observed that there was no significant difference over time intervals in each arm, Arm-D(no drug) (p>0.9999), Arm-K(81mg) (p=0.3403) and Arm-H(325mg) (p=0.2883) respectively **(Figure 4.1)**

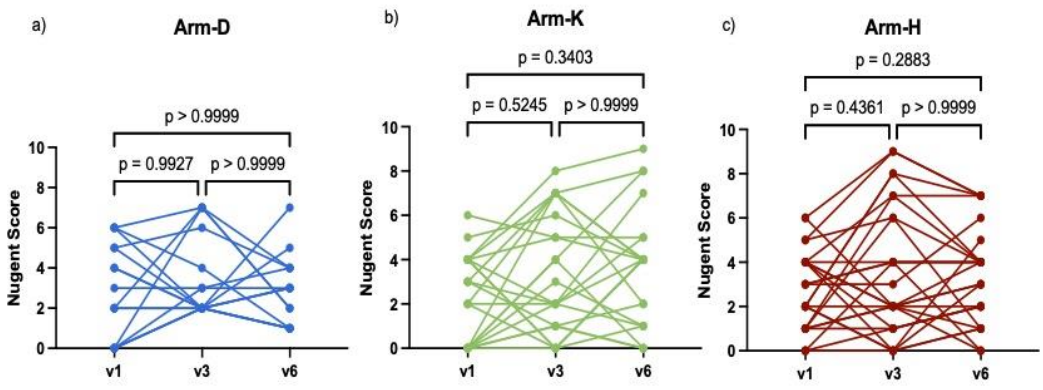


Figure 4. 1 Comparison of Nugent scores

Key: The Nugent scores are plotted on the y-axis, with the time points: V1 – Baseline, V3 - 2

months, V6 - 5 shown on the x-axis in (a) Arm-D, (b) Arm-K, (c) Arm-H

4.2.2 Comparison of BV Diagnosis

The BV diagnosis was categorized into Normal, Intermediate and BV positive.

The morphological characteristics of the vaginal microbiome were assessed at three different visits (v1, v3, and v6) across the three study arms (D, K and H) using microscopy (magnification: 1000x with oil immersion).

At v1, there were no statistically significant differences between the arms, with the majority of samples showing a normal morphology dominated by *Lactobacillus* spp. (p=0.8963). In v3, some differences began to emerge, with more intermediate morphologies (mixed Gram-positive and Gram-negative bacteria) observed in Arm K (6 participants) and Arm H (7 participants) compared to Arm D (2 participants). Additionally, bacterial vaginosis (BV) morphologies characterized by Gram-negative bacteria and clue cells were observed in all three arms (p=0.7437), with Arm D (3 participants), Arm K (5 participants) and Arm H (6 participants) showing these BV-associated morphologies. At v6, the morphological differences became more pronounced, with a statistically significant difference observed between the arms (p=0.04013). Arm D maintained the highest proportion of normal Lactobacillus-dominant morphologies (11 participants), while Arm K and Arm H had more intermediate (10 and 9 participants, respectively) while those with BV-associated morphologies were (1, 4 and 4 participants for Arms D, K and H, respectively).

The comparison of the proportion of women who had BV was assessed within each arm over in each visit. It was observed that there was no significant difference in proportions of women

having BV at each time point within each arm, Arm-D(p=0.1836), Arm- K(p=0.0898) and Arm-H(p=0.1181) respectively **(Figure 4.2).**

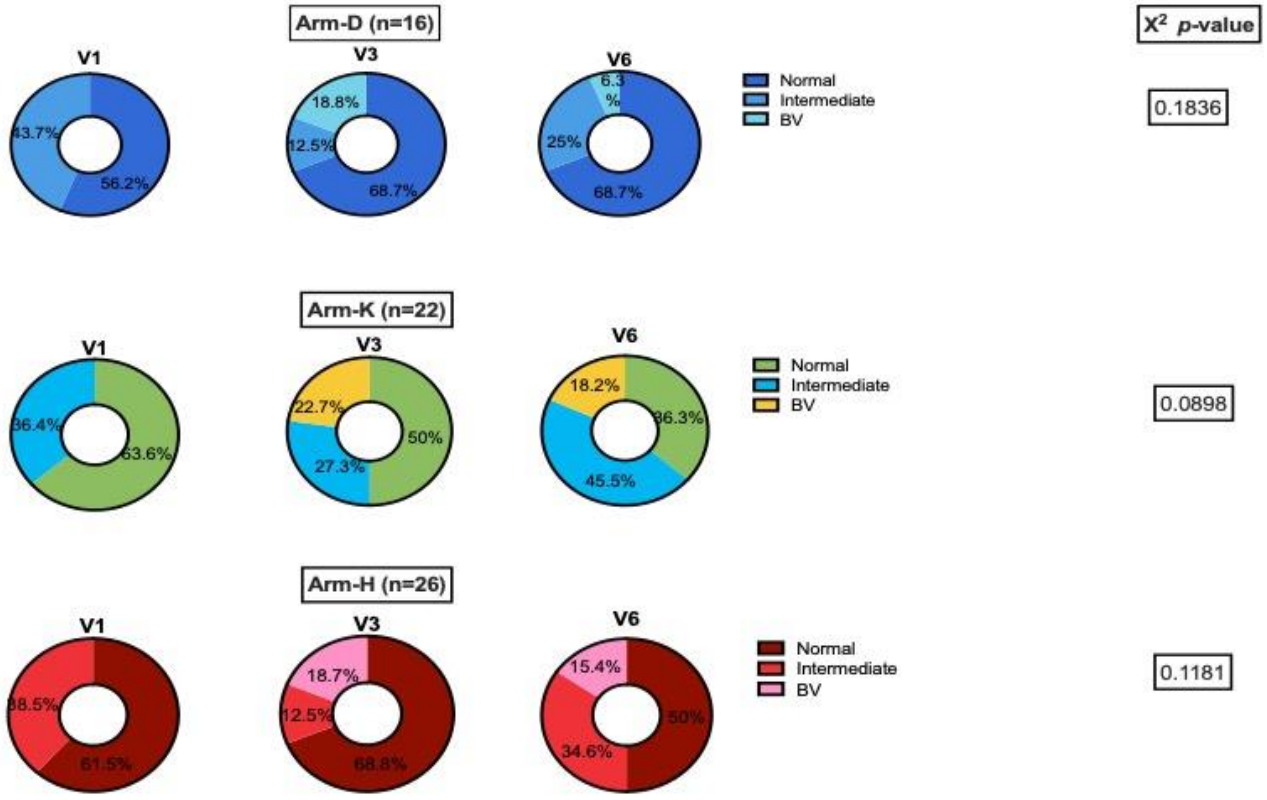


Figure 4. 2 Comparison of Bacterial Vaginosis (BV) diagnosis

Key: Overall BV diagnosis in Arm-D, Arm-K, and Arm-H) every time point (V1 - baseline, V3 - 2 months, and V6 - 5 months).

Furthermore, when combined proportions of women who had normal and intermediate diagnosis versus those who had BV, there was no significant differences in proportion of women having BV between these arms, Arm-D(p=0.5996), Arm-K(p>0.9999) and Arm- H(p=0.07265) respectively **(Figure 4.3).**

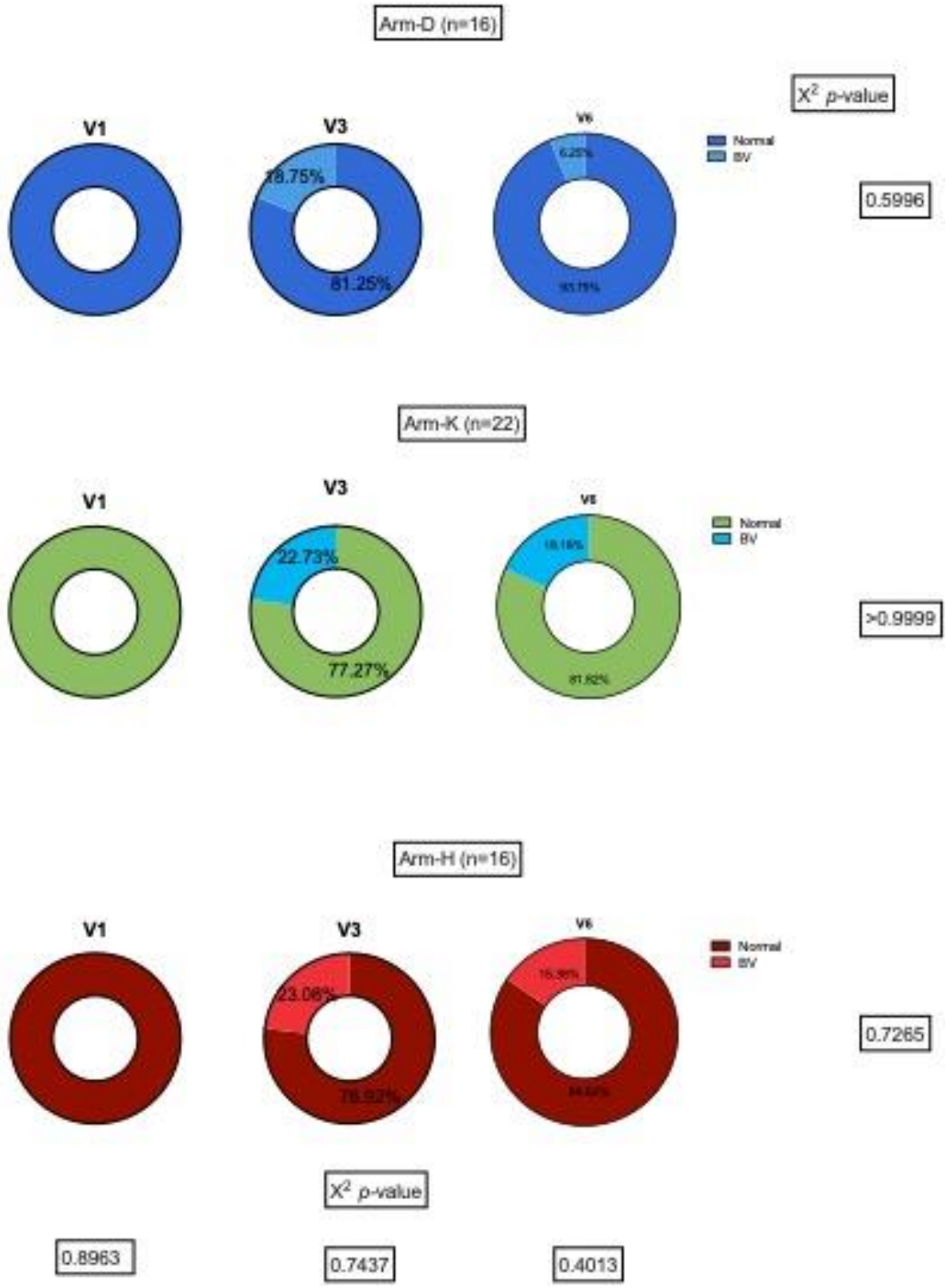


Figure 4. 3 Comparison of proportion of women with combined normal and intermediate versus BV diagnosis

4.2.3 Type I morphotype (*Lactobacillus* spp.) Scores

Changes in proportions of type I morphotype, consistent with *Lactobacillus* spp. were evaluated over time using the Friedmans multiple comparison test **(Figure 4.4).** Within Arm D(no drug), the median *Lactobacillus spp* score was 2 (IQR 0-4), 2 (IQR 0-2) and 1 (IQR 0-3) at visit 1, visit 2 and visit 3 respectively. Within Arm K(81mg) the median the median *Lactobacillus* spp. score was 2 (IQR 0-4), 2 (IQR 0-3) and 0 (IQR 0-3) at visit 1, visit 2 and visit 3 respectively and within Arm H(325mg) the median *Lactobacillus* spp. score was 2 (IQR 0-3), 2 (IQR 0-3), 1 (IQR 0-2) at visit 1, visit 2 and visit 3 respectively. There was no significant difference in the *Lactobacillus* spp. scores at every time point in each arm, Arm-D(p>0.9999), Arm-K(p=0.2918) and Arm- H(p=0.4966) respectively **(Figure 4.4).**

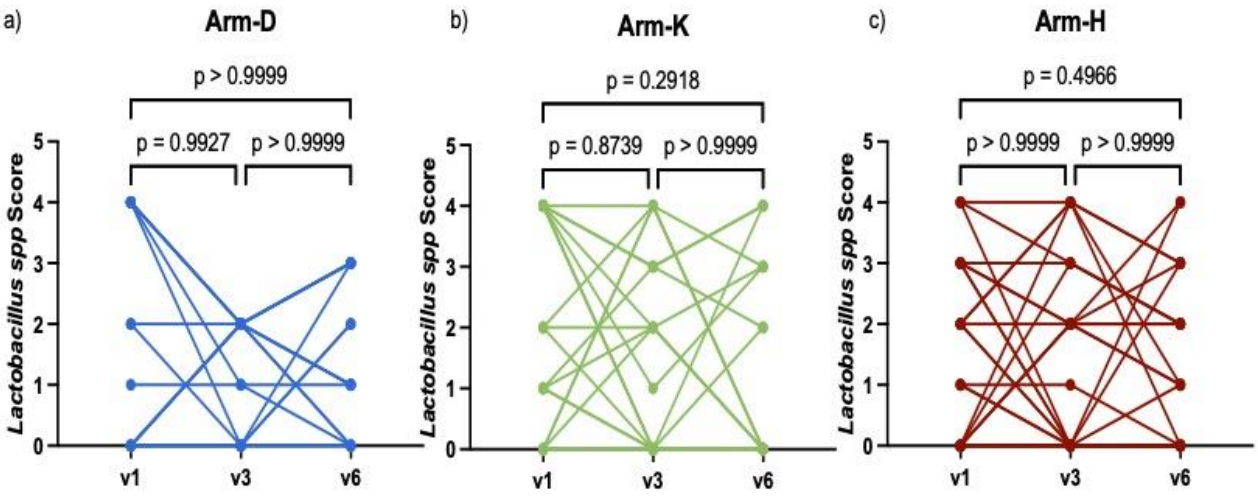


Figure 4. 4 Comparison of *Lactobacillus* spp. scores

Key: *Lactobacillus spp* Scores at different time points (v1, v3, v6) for (a)Arm-D, (b)Arm-K, (c)Arm-H). The *Lactobacillus* spp. Scores are plotted on the y-axis, with the with the timepoints: V1 – Baseline, V3 - 2 months, V6 - 5 shown on the x-axis. Bars represent statistical comparison (ns=not significant).

4.2.4 Type II morphotype (*Gardnerella vaginalis*) Scores

Changes in proportions of type II morphotype, consistent with *Gardnerella vaginalis*, were assessed over time using the Friedmans multiple comparison test **(Figure 4.5)**. Within Arm D(no drug), the median *Gardnerella vaginalis* score was 0 (IQR 0-1), 0 (IQR 0-2) and 0 (IQR 0-0) at visit 1, visit 2 and visit 3 respectively. Within Arm K(81mg) the median the median *Gardnerella vaginalis* score was 0 (IQR 0-0), 0 (IQR 0-2) and 0 (IQR 0-1) at visit 1, visit 2 and visit 3 respectively and within Arm H(325mg) the median *Gardnerella vaginalis* score was 0 (IQR 0-0), 0 (IQR 0-2), 0 (IQR 0-1) at visit 1, visit 2 and visit 3 respectively. It was observed that no significant difference in the *Gardnerella vaginalis* scores at every time point in each arm, Arm- D(p>0.9999), Arm-K(p>0.9999) and Arm-H(p=0.8949) respectively **(Figure 4.5).**

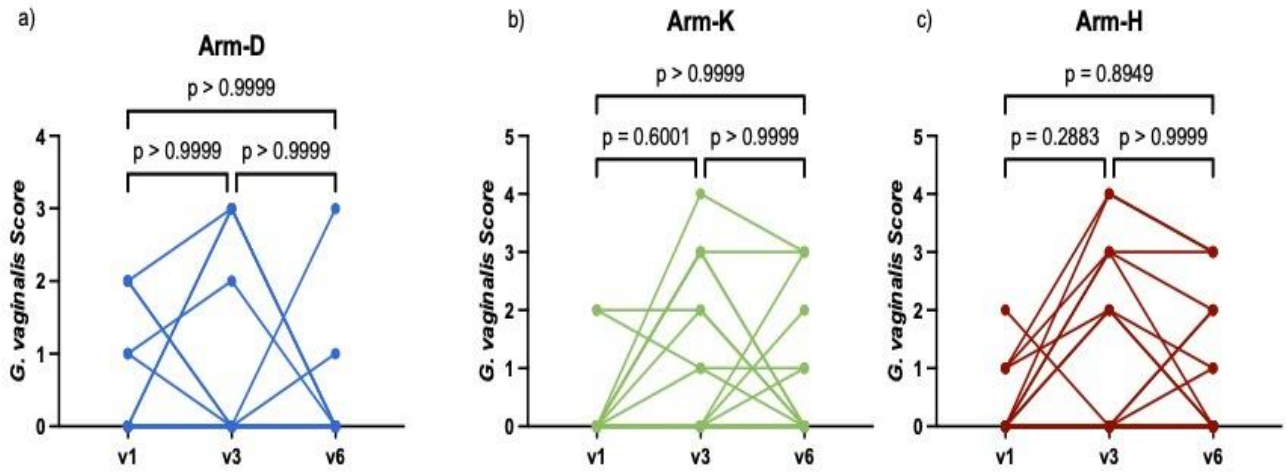


Figure 4. 5 Comparison of *Gardnerella vaginalis* Scores

Key*: Gardnerella vaginalis* Scores at different time points (v1, v3, v6) for (a)Arm-D, (b)Arm K, (c)Arm-H). The *Gardnerella vaginalis* Scores are plotted on the y-a axis, with the time points: V1 – Baseline, V3 - 2 months, V6 - 5 shown on the x-axis. Bars represent statistical comparison (ns=not significant).

4.2.5 Yeast Cells Scores

As yeast is known to cause inflammation, the changes in scores of Yeast cells were assessed **(Figure 4.6)** over time. Despite the fact that few women had yeast infections, no participant in Arm-D (no drug) tested positive for yeast. Instead, those with yeast were found in Arm-K(81mg) (n=3) and Arm-H (325mg) (n=6). The median yeast cells score within arm D, arm K and arm was 0 (IQR 0-0) at visit 1, visit 2 and visit 3 respectively. There was no significant difference in the Yeast cells scores at every time point in each arm, Arm-D(p>0.9999), Arm-K(p>0.9999) and Arm- H(p>0.9999) respectively **(Figure 4.6).**

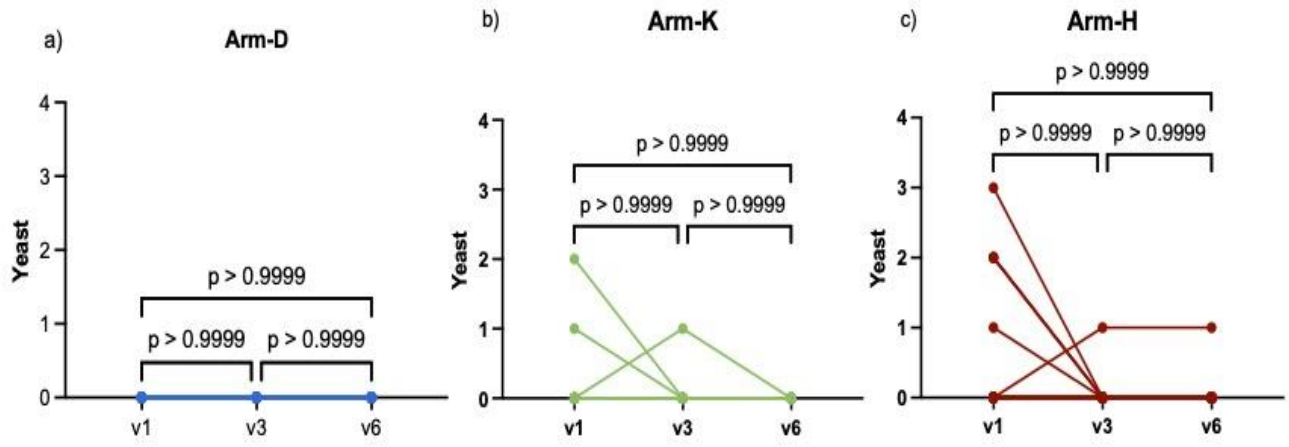


Figure 4. 6 Comparison of Yeast cell scores

Key: Yeast cells Scores at different time points (v1, v3, v6) for (a)Arm-D, (b)Arm-K, (c)Arm-H). The Yeast cells Scores are plotted on the y-axis, with the time points: V1 – Baseline, V3- 2 months, V6 - 5 shown on the x-axis. Bars represent statistical comparison (ns=not significant).

4.2.6 Expression of Immune Activation Markers (CD4+CD25+ and CD4+CD69+)

4.2.6.1 Immune activation markers in PBMCs

The proportions (in percentage) of CD4+CD25+ and CD4+CD69+ T cells were examined across different study visits (v1, v3, v6) for three study arms (Arm-D(no drug), Arm-K,(81mg) Arm-H (325mg)).

A significant difference in the expression of CD4+CD25+ T cells in Arm-D, a significant decrease was observed between v1 and v3 (p=0.0140) and between v1 and v6 (p=0.0080), while there was no significant difference observed between v3 and v6 (p>0.9999) with mean scores 4.36, 6.32, and 6.87 at v1, v3 and v6 respectively.

A similar pattern was observed in the expression of CD4+CD69+ T cells in Arm-D, with significant decreases between v1 and v3 (p=0.0140) and between v1 and v6 (p=0.0400), but no significant change between v3 and v6 (p>0.9999) with mean scores 0.72, 2.13 and 3.54 at v1, v3, and v6 respectively.

In contrast, in the expression of CD4+CD25+ T cells in Arm-K a significant difference was observed between v1 and v6 (p<0.0001), no significant difference between v1 and v3 (p=0.0923) with mean scores 4.50, 6.52 and 11.79 at v1, v3 and v6 respectively, while a significant difference was observed between v3 to v6 (p=0.0261). However, no significant

changes were observed for the CD4+CD69+ T cells in Arm-K across the study visits v1 and v3 (p=0.1922), v3 and v6 (p>0.9999), while v1 and v6 (p=0.1922) with mean scores 1.12, 3.00 and 1.77 at v1, v3 and v6 respectively.

In Arm-H, in the expression of CD4+CD25+ T cells a significant difference was observed between v1 and v3 (p=0.0009), but no significant differences were observed between v1 and v6 (p=0.2143) or v3 and v6 (p=0.2143) with means scores 4.32, 7.01and 5.33 at v1, v3 and v6 respectively. No significant differences was observed in the expression of CD4+CD69+ T cells in Arm-H, v1 and v3 (p=0.0552), v3 and v6 (p>0.9999), v1 and v6 (p=0.2143) with means scores 1.13, 4.28 and 2.78) at v1,v3 and v6 respectively.

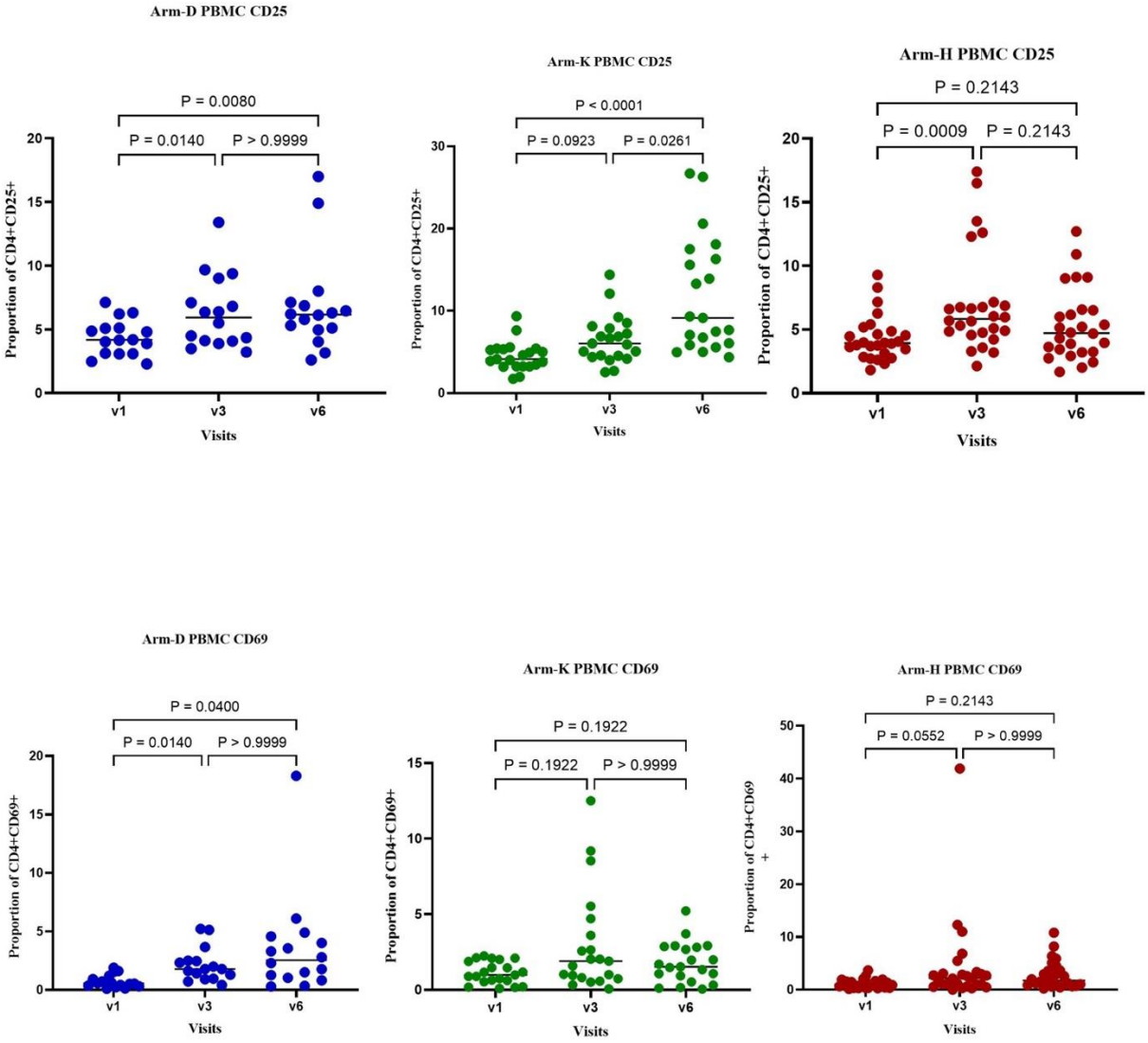


Figure 4. 7 Expression of immune activation markers in PBMCs

Key: Immune activation markers CD25 and CD69 at different time points (v1, v3, v6) for (a) Arm-D, (b) Arm-K, (c) Arm-H). The Activation markers are plotted on the y-axis, with the time points: V1 – Baseline, V3- 2 months, V6- 5months shown on the x-axis. Bars represent statistical comparison (ns=not significant).

4.2.6.2 Immune activation markers in CMCs

The proportions of CD4+CD25+ and CD4+CD69+ T cells were examined in CMCs (cervical mononuclear cells) across different study visits (v1, v3, v6) for three study arms (Arm-D(no drug), Arm-K (81 mg), Arm-H (325 mg)).

In Arm-D, a significant difference was observed in the expression of CD4+CD25+ T cells between v1 and v3 (p=0.0240) and between v1 and v6 (p=0.0240), but there was no significant difference observed between v3 and v6 (p>0.9999) with mean scores 4.50, 7.00 and 6.87) for v1, v3 and v6 respectively. While in the expression of CD4+CD69+ T cells in Arm-D, there was a significant difference between v1 and v3 (p=0.0140), but no significant differences between v1 and v6 (p=0.1555) or between v3 and v6 (p>0.9999) with means scores 3.00, 5.83 and 3.54 at v1, v3 and v6 respectively.

In Arm-K, a significant difference was observed in the expression of CD4+CD25+ T cells between v1 and v3 (p=0.0449) and between v1 to v6 (p=0.0020), but there was no significant difference between v3 and v6 (p=0.9912) with means scores 6.92, 10.62 and 12.64 at v1, v3 and v6 respectively. However, no significant differences were observed for the CD4+CD69+ T cells in Arm-K across the study visits, v1 and v3 (p=0.9912), v3 and v6 (p=0.4329), v1 and v6 (p>0.999) with mean scores 44.72, 41.21 and 44.41) at v1,v3,and v6 respectively.

In Arm-H, in the expression of CD4+CD25+ T cells, no significant differences were observed between v1 and v3 (p=0.4467), v1 and v6 (p=0.1229) or v3 and v6 (p>0.9999) with mean scores 6.884, 10.84 and 11.36 at v1, v3 and v6 respectively. While no significant differences was observed in the expression of CD4+CD69+ T cells in Arm-H, v1 and v3 (p>0.9999), v3 and v6 (p>0.9999), v1 and v6 (p>0.9999) with mean scores 33.38, 43.91 and 45.1) at v1, v3 and v6 respectively.

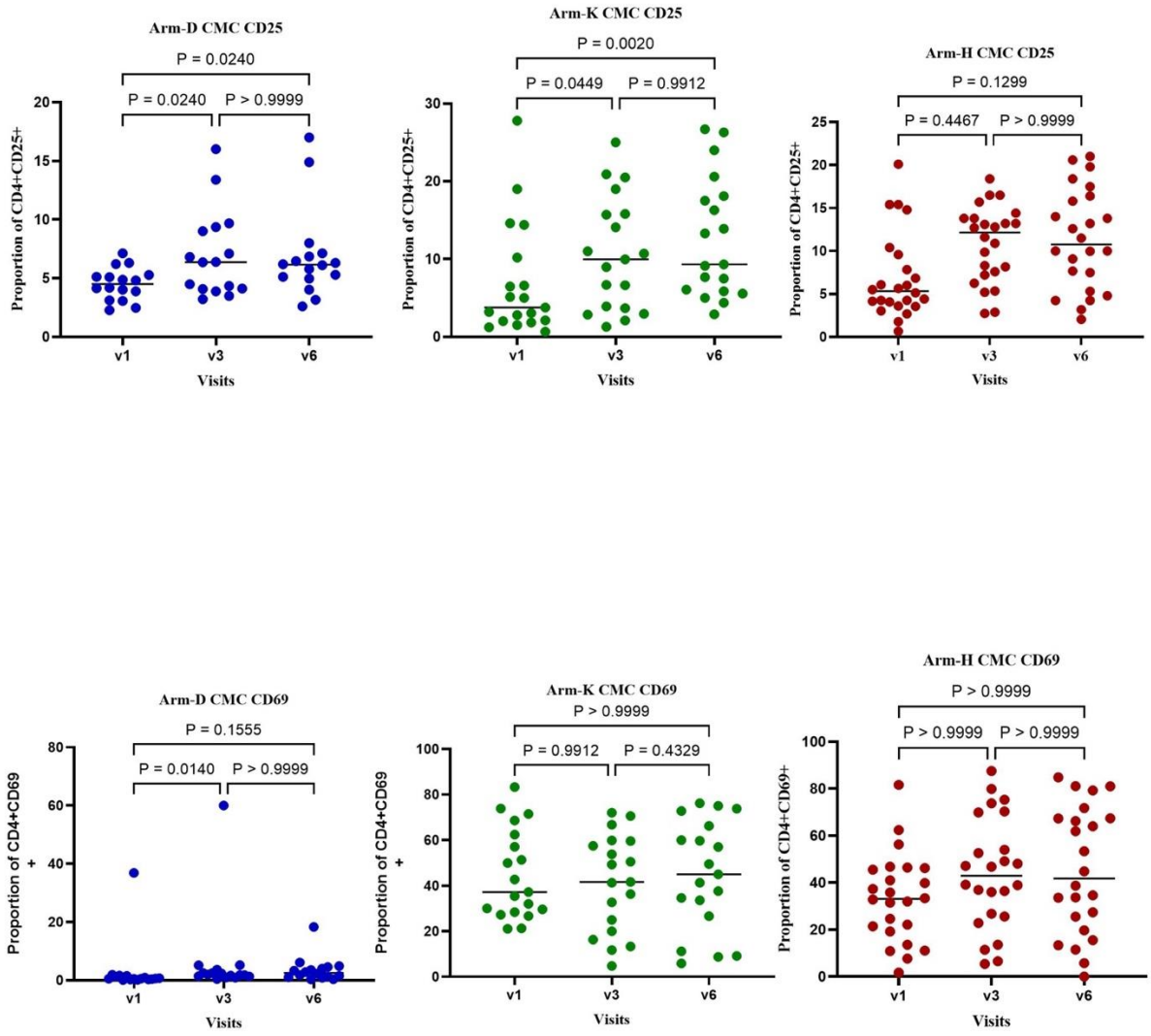


Figure 4. 8 Expression of activation markers CD25 and CD69 in CMCs

Key: Immune activation markers CD25 and CD69 at different time points (v1, v3, v6) for (a) Arm-D, (b) Arm-K, (c) Arm-H). The Activation markers are plotted on the y-axis, with the time points: V1 – Baseline, V3- 2 months, V6- 5months shown on the x-axis. Bars represent statistical comparison (ns=not significant).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

In this study it was hypothesized that daily intake of ASA will not alter the commensal vaginal bacteria and yeast. Therefore, the impact of daily ASA intake on commensal vaginal bacteria was evaluated by population changes over time.

The mean ages of participants in Arms D (29.7 years), K (28.9 years), and H (31.8 years) groups were similar, with no statistically significant differences (p=0.3487). Age has been shown to influence vaginal microbiome composition and susceptibility to infections. Younger women often have a higher prevalence of Lactobacillus-dominant microbiomes, which are associated with lower infection rates. Conversely, older women may experience shifts toward more diverse and potentially pathogenic communities due to hormonal changes and alterations in immune system (Ravel *et al.*, 2011).

The data indicates that unmarried participants had the greatest frequency across all groups, with 81.3% , 81.8% and 92.3% in arms D, K and H respectively with no statistically significant differences (p=0.5876). Most female sex workers are usually unmarried and in unstable relationships given their kind of work. This is consistent with the findings of the current study. However, previous research also suggests that marital status can impact sexual behavior and health practices where married women tend to have different sexual health outcomes compared

to unmarried women, often experiencing lower rates of sexually transmitted infections (STIs) due to more stable sexual partnerships.

Marital status has a complex and sometimes contradictory association with bacterial Vaginosis (BV), with some studies showing higher prevalence in married or single women, while others show no significant link. This may be because marital status is not as strong a risk factor as other factors like the number of sex partners and unprotected sex, which are more likely to be behaviorally linked to both marital status and BV. Some studies show married women, or those with similar partnership structures to married individuals, may have higher rates of BV, potentially due to factors like sex without condoms. Other studies found no statistically significant association between marital status and BV, suggesting marital status itself is not a direct cause.

Research shows that vaginal douching disrupts the natural vaginal microbiome, which is crucial for maintaining vaginal health. The introduction of foreign substances through douching can lead to a decrease in beneficial *Lactobacillus* species, which play a vital role in preventing infections. This disruption can create an environment conducive to the overgrowth of harmful bacteria, subsequently increasing the risk of bacterial vaginosis (BV) and sexually transmitted infections (STIs) (Martino *et al*, 2002). In previous research done, it has been found that a significant association between douching and the prevalence of BV, suggesting that the practice may compromise the natural defenses of the vagina (Brotman *et al.*, 2008).

In the present study, the practice of vaginal douching was prevalent among participants, Arm D (50%), Arm K (50%) and Arm H (38.5%) across the groups aligning with previous research that highlights the prevalence of this practice among women of certain demographics such as female sex workers (Fonck *et al.*, 2001) This consistency in findings highlights the deep-rooted nature of douching within various demographics, reflecting a broader trend observed in the literature. Despite the variations in douching frequency, with some participants engaging in daily or weekly practices, statistical analysis revealed no significant differences among the groups (Table 4.1). This finding suggests that while douching is a common practice, it does not necessarily correlate with improved vaginal health.

The relationship between sexual activity and vaginal health is well-documented; previous research has shown that individuals engaging in high-risk sexual practices, such as having multiple sexual partners, unprotected sex, and douching exhibit greater diversity in their vaginal microbiota compared to those practicing lower-risk behaviors. Furthermore, a significant decrease in the abundance of *Lactobacillus* spp. which are critical for maintaining vaginal health, has been observed in these populations. This reduction in *Lactobacillus spp* is associated with an increased susceptibility to infections, including bacterial vaginosis and sexually transmitted infections (STIs) (Wessels *et al*, 2017). In my study, the median number of regular clients reported in the last five days was 1 for arm D, 0.5 for arm K, and 0 for arm H, with no significant differences observed (p=0.2147). Notably, participants in arm D reported a median of 0.5 sexual encounters with partners during this period, indicating some level of sexual activity.

These findings align with another study that explored the impact of sexual activity on activation and memory phenotypes of peripheral T cells among female sex workers.. Specifically, the study indicated that interruptions in sex work led to significant changes in blood-derived T cell populations, including alterations in T cell activation and differentiation markers. This suggests a potential reduction in the risk of HIV transmission and related infections during periods of sex-work interruption (Omollo *et al.*, 2016). The vaginal microbiome is affected by the local immune environment. Changes in T cell activation and differentiation may modify the host's immune response to microbial communities, impacting the balance between beneficial and harmful bacteria. A more balanced immune response can help maintain a healthier vaginal microbiome. Secondly, the interruption of high-risk sexual behaviors may result in reduced inflammation within the vaginal environment. A less inflammatory setting can promote the growth of Lactobacillus species, which are essential for maintaining vaginal health and preventing infections.

While my study focused primarily on Gram staining and microscopy to assess the presence of *Lactobacillus* spp., *Gardnerella vaginalis* and other bacterial populations, the isolation and characterization of bacterial vaginosis (BV) pathogens represent a critical aspect of understanding the microbiological landscape associated with BV. The Gram stain method remains the gold standard for diagnosing bacterial vaginosis (BV). While alternative diagnostic methods have been proposed, none have proven to be superior to the standardized Gram stain technique. Alternatives such as gas-liquid chromatography, vaginal cultures, and liquid-based Papanicolaou smears offer practical advantages in sampling and laboratory transport; however, these methods have primarily been explored in research settings and would necessitate

significant changes in smear interpretation protocols. Reports indicate a general lack of sensitivity for these alternatives, although they exhibit reasonable specificity. Currently, nucleic acid techniques have not demonstrated utility for the clinical diagnosis of the complex microbial imbalances associated with BV, though they may hold promise for future applications. Researchers are actively investigating genetic approaches, including RNA-based methods, to evaluate the vaginal microbiome. Despite their potential, these advanced techniques remain impractical for clinical use due to their complexity and cost. Therefore, the Gram stain method continues to be the most effective diagnostic tool available for BV at this time.

Isolation techniques involve culturing bacteria from clinical samples on selective media, allowing for the identification and characterization of specific pathogens that contribute to BV. This process typically includes the use of anaerobic culture conditions, which requires an anaerobic chamber, as many BV-associated bacteria are anaerobes or microaerophiles.

Previous studies have highlighted the significance of isolating and characterizing BV pathogens to establish a clearer understanding of their roles in the pathogenesis of BV. Some studies utilized culture-independent methods such as 16S rRNA gene sequencing, which allowed for a more comprehensive identification of the complex microbial communities present in the vaginal microbiome (Macklaim *et al*, 2013). These studies identified not only *Gardnerella vaginalis* but also other key bacteria such as *Atopobium vaginae, Prevotella* spp. *and Mobiluncus* spp. which are often overlooked in traditional culture methods.

In contrast, my approach, which relied on Gram staining and microscopy, provided a rapid assessment of the microbial population but lacked the specificity and depth offered by isolation and characterization techniques. Gram staining and assessment by Nugent score is widely used at the clinic level and therefore was used in this clinical study as it represents the technique primarily used for clinical assessments. Gram staining is a valuable preliminary method for identifying Gram-positive and Gram-negative bacteria, yet it does not allow for the detailed identification of individual species. This limitation is significant, as the pathogenic potential of different bacterial species can vary widely, for example, while *Gardnerella vaginalis* is commonly associated with BV, it is not always the sole or primary pathogen involved, other bacteria may play synergistic roles in the dysbiotic state.

The findings from studies employing isolation and characterization techniques have revealed that the microbial diversity in BV is often greater than what can be assessed through microscopy alone. For example, the application of culture techniques could lead to the identification of previously unrecognized pathogens and provide insights into their metabolic capabilities (Hummelen *et al.*, 2010). This information is crucial for understanding how these pathogens interact with the host and contribute to inflammation and disease progression.

At the baseline visit-v1, all three study arms showed a predominance of normal vaginal morphology characterized by Lactobacillus-dominant bacteria, since participants with BV were excluded from the study. The similar distributions across the arms at this initial visit

suggest that the randomization was effective, and the study groups were well-balanced at the start.

However, the differences started to emerge by the v3 visit, where some participants in Arm K and Arm H began to develop intermediate morphologies, indicating a shift towards a more diverse vaginal microbiome with a mix of Gram-positive and Gram-negative bacteria. Notably, BV-associated morphologies were also observed in a few participants in these two arms at v3, despite the exclusion of BV at baseline.

At v6, the differences between the arms became statistically significant (p=0.04013). Arm D maintained the highest proportion of normal *Lactobacillus*-dominant morphologies, while Arm K and Arm H had more participants with intermediate and BV-associated morphologies. These findings underscores the importance of considering the dynamic nature of the vaginal microbiome and the potential for targeted interventions to maintain a healthy, *Lactobacillus*-dominant state, even in the face of hormonal and menstrual cycle fluctuations.

Bacterial morphogroups characterization was done into *Lactobacillus spp, Gardnerella vaginalis* and yeast cells populations in the female genital tract before and after use of 81mg, 325mg of ASA and no drug for a period of 5 months. The findings of this study demonstrated that the daily use of ASA, whether at 81 mg, 325 mg had no effect on the composition of these vaginal microbiota. Notably, there were no significant changes observed in the populations of

morphogroups characterized by *Lactobacillus* spp. Arm-D (p>0.9999), Arm-K (p=0.2918) and Arm-H (p=0.4966), *Gardnerella vaginalis*,Arm- D (p>0.9999), Arm-K (p>0.9999) and Arm-H (p=0.8949) or yeast cells, Arm-D (p>0.9999), Arm-K (p>0.9999) and Arm-H (p>0.9999) before and after ASA use.

5.1.1 Changes in the *Lactobacillus spp* populations in the female Genital Tract before and after use of 81mg, 325mg of ASA and No drug

In evaluating changes in morphotype I (*Lactobacillus* spp.) populations in the genital tract before and after use of 81mg, 325mg and no drug, our findings showed that ASA had no impact on morphotype I *Lactobacillus* spp. Arm-D(no drug)(p>0.9999), Arm-K(81mg)(p=0.2918) and Arm-H(325mg)(p=0.4966) and no changes were observed in their population. Therefore, maintaining a healthy vaginal microbiome dominated by *Lactobacillus* spp. could help in preventing the overgrowth of other pathogens and the subsequent inflammatory response. Vaginal microbiota is predominated by morphotype I (*Lactobacillus* spp.) known as the biomarkers for an optimal microbiota, metabolize certain drugs, potentially reducing their protective levels against HIV. *Lactobacillus* spp. produce lactic acid, hydrogen peroxide, and bacteriocins, which contribute to a lower pH, enhance *Lactobacillus* spp. adhesion to epithelial cells, and hinder pathogenic bacteria.

Additionally, the production of lactic acid and bacteriocins that directly kill or inhibit bacterial and viral pathogens, as well as the stimulation of host defense mechanisms against pathogens, are also important anti-pathogenic mechanisms employed by *Lactobacillus* spp. in the female

genital tract. Some *in vitro* studies have demonstrated that lactic acid has the ability to inactivate pathogens on the genital tract and prevent colonization of host cells by pathogenic microbes (Petrova *et al*, 2015).

The pilot study conducted a genital microbiome analysis on the ASA arm, identifying two major microbial communities among the participants, *Lactobacillus*-dominant (LD) communities, which were less common (n = 22, 33%), and non-*Lactobacillus*-dominant (non-LD) communities, which were more prevalent (n = 45, 67%). The non-LD taxa exhibited high proportions of diverse microbial communities primarily composed of *Gardnerella vaginalis*, *Prevotella*, and *Atopobium vaginae*. Notably, the majority of women (n = 24, 77.4%) in the ASA arm maintained unchanged microbial diversity over time. In contrast, a small proportion shifted from a non-LD state to an LD state (12.9%), while three women (9.7%) transitioned from *Lactobacillus*-dominant to non-Lactobacillus-dominant post-treatment. Importantly, there were no significant changes in microbial diversity over time with ASA use (Lajoie, Julie *et al*, 2018). The findings of my study align closely with those of the pilot study (Lajoie, Julie *et al*, 2018). I observed no changes in the *Lactobacillus* spp. population before and after the use of 81mg, 325mg of ASA and no. My results showed no alterations in Nugent scores, indicating that ASA did not impact these scores. This stability suggests that ASA use did not lead to increased inflammation, supporting Lajoie *et al*.,'s conclusions about the protective role of *Lactobacillus* species in maintaining vaginal health.

Furthermore, the analysis of bacterial vaginosis (BV) diagnosis within each study arm and comparisons between the arms at various time points (Figure 4.2) yielded consistent results that ASA did not impact BV diagnosis. Since having a positive BV diagnosis at pre-screening was an exclusion criterion, none of the women in the study had BV at baseline (V1). However, during the subsequent time points (V3 - 2 months, and V6 - 5 months), some participants had developed BV. They were treated accordingly and allowed to continue in the study.

5.1.2 Changes in *Gardnerella vaginalis* populations before and after use of 81mg, 325mg of ASA and No drug

My findings also showed that ASA had no impact on *Gardnerella vaginalis*, Arm-D (no drug) (p>0.9999), Arm- K (81mg) (p>0.9999) and Arm-H (325mg) (p=0.8949) and no changes were observed in their population at each visit in every arm. This also suggested that a LD vaginal microbiome can effectively inhibit the overgrowth of *Gardnerella vaginalis*, thereby maintaining an optimal vaginal microbiome. Disruption of this LD vaginal microbiome can lead to the proliferation of *Gardnerella vaginalis* and other BV-associated bacteria in the genital tract which can have negative consequences for women's reproductive health. Therefore, preserving and promoting a LD vaginal microbiome is crucial for preventing the overgrowth of *Gardnerella vaginalis* and maintaining overall vaginal health.

While my study focuses on ASA's impact on *Lactobacillus* spp. and *Gardnerella vaginalis*, it is important to note that other factors, such as a diverse vaginal microbiome, can influence the efficacy of drugs in HIV prevention strategies. Some studies done at the Centre for AIDS

Program of Research in South Africa (CAPRISA) 004 Trial, evaluating topical vaginal PrEP with 1% Tenofovir found that BV-associated bacteria in the vaginal microbiome metabolized the drug and reduced its efficacy therefore lowering its protection levels in women at risk of HIV acquisition (Cheu *et al.*, 2020). A more diverse microbiome is linked to inflammation, due to higher concentrations of inflammatory cytokines, hence a compromised genital epithelial barrier, and an increased risk of sexually transmitted infections. This is consistent with previous research that has shown that BV-associated bacteria can metabolize certain drugs, potentially leading to reduced efficacy and increased inflammation. The inflammatory responses triggered by these bacteria can compromise treatment outcomes, highlighting the importance of maintaining a balanced vaginal microbiome (McKinnon *et al.*, 2018).

The findings of my study provide valuable insights into the relationship between ASA use and the vaginal microbiome, particularly regarding *Gardnerella vaginalis*, a key bacterium often associated with bacterial vaginosis (BV). My results indicate that ASA does not significantly alter the levels of *Gardnerella vaginalis*, suggesting that its use does not disrupt the existing microbial balance within the vaginal environment.

In a previous research done among women in South Africa which emphasize the potential negative impact of BV-associated bacteria on drug efficacy (McKinnon *et al*, 2018), while my study suggests that ASA may actually help preserve the effectiveness of therapeutic interventions. The unchanged levels of *Gardnerella vaginalis* during ASA use imply that this medication does not induce an inflammatory environment that could diminish drug efficacy.

This is a significant finding, as it supports the idea that ASA may contribute to a healthier vaginal microbiome, thereby preventing the complications associated with dysbiosis and inflammation.

Moreover, the stability of the vaginal microbiome observed in my study aligns with the notion that a balanced microbial environment is essential for optimal vaginal health (McKinnon *et al*, 2018). The researchers highlighted that the presence of BV-associated bacteria can lead to dysbiosis, which alters the microbial landscape and may hinder treatment outcomes (McKinnon *et al*, 2018). In our study, the lack of significant changes in *Gardnerella vaginalis* levels indicates that ASA use does not disrupt this balance, thereby reinforcing the importance of maintaining microbial stability for effective treatment.

Therefore, maintaining a balanced and less diverse vaginal microbiome is generally considered more optimal for maintaining vaginal health and reducing the risk of infections and inflammation.

5.1.3 Changes in Yeast Cell populations in the female Genital Tract before and after use of 81 mg, 325 mg of ASA and No drug

In the context of yeast infection, in the vaginal tract, yeast-induced inflammation occurs when yeast overgrowth triggers a robust inflammatory response, resulting in high concentrations of pro-inflammatory cytokines in the genital tract (Fidel Jr, 2002). This inflammatory state caused

by the *Candida* spp. overgrowth disrupts the integrity of the vaginal epithelial barrier. The compromised vaginal epithelium creates an environment that is more permissive for the entry and replication of HIV. The inflammatory mediators and disruption of the mucosal barrier facilitate HIV's ability to penetrate and infect the underlying tissue and target immune cells (Nazli *et al.*, 2010). By mitigating the inflammatory response and restoring the integrity of the genital mucosal defenses, interventions targeting yeast overgrowth could potentially lower the risk of HIV acquisition (Donders *et al.*, 2017).

One study observing the prevalence of yeast infections in women with BV and found that co-infection with Candida spp. was common in their population. The study reported that women with BV were more likely to experience yeast infections, leading to increased inflammation and symptomatic discomfort (Sobel, Jack D. *et al.*, 2024). In contrast, our findings indicate a low prevalence of yeast infections among participants, particularly in Arm-D, which suggests that the use of specific interventions or treatments may have contributed to a reduced incidence of yeast infections. This discrepancy may highlight the effectiveness of our approach in managing yeast-related symptoms compared to the findings of (Sobel, Jack D. *et al*, 2024).

A previous study examining the relationship between yeast infections and inflammatory markers in the vaginal environment reported that the presence of yeast was associated with elevated levels of inflammatory cytokines, suggesting that yeast infections can exacerbate inflammatory responses in the vagina (Cheng *et al.*, 2024). In our study, the absence of significant yeast cell scores and the lack of inflammation-related symptoms in Arm-D provide

additional evidence for the importance of managing vaginal microbiome can mitigate the risk of inflammation. This contrasts with Cheng et al.'s findings, where yeast infections were linked to heightened inflammation, suggesting that our intervention may have effectively reduced the inflammatory potential of the vaginal environment.

A study in Mombasa found that fluctuations in yeast populations were often associated with changes in bacterial communities, particularly during episodes of BV (Masese *et al.*, 2015). In my study, the lack of significant yeast presence across all arms, especially in Arm-D, suggests that the interventions applied may have stabilized the vaginal microbiome, preventing the dysbiosis often seen in other studies. This stability contrasts with the findings of a study that had shown that shifts in microbial populations led to increased yeast colonization, indicating that our approach may differ in its effectiveness at maintaining a balanced vaginal microbiome (Masese *et al*, 2015).

Findings from this study show that there was no significant changes, Arm-D (p>0.9999), Arm-K (p>0.9999) and Arm-H (p>0.9999) in the population of yeast cells with ASA use. Though a few women tested positive *Candida* yeast throughout the study, the scores did not exhibit any substantial variations. This findings concurs with previous studies that suggest a complex interplay between *Lactobacillus* spp. dominance, BV, and yeast presence. In the U.S, some *in vitro* studies have indicated that *Lactobacillus* spp. dominance can impede the growth of *Candida albicans* through various mechanisms such as competition for nutrients and adhesion

sites, or by secreting fungicidal substances, which occurred when yeast was inversely correlated with *Lactobacillus* spp. dominance (Smritee *et al*, 2021).

5.1.4 Activation markers CD25+ and CD69+ on CD4+T cells

The analysis of activation markers CD4+CD25+ and CD4+CD69+ in peripheral blood mononuclear cells (PBMCs) and cervical mucosal cells (CMCs) across the different study arms (D, K, and H) provides critical insights into the immune response following use of acetylsalicylic acid. In Arm D, significant fluctuations in CD4+CD25+ expression were observed in both PBMCs (Figure 4.7) and CMCs (Figure 4.8) respectively.

In Arm K, the results were mixed for PBMC CD25, with one significant measurement ($p < 0.0001$) and others showing no significance. However, CMC CD4+CD25+ consistently demonstrated significant activation across all time points, with *p*-values ranging from 0.002 to 0.0449. This consistency indicates a robust response in the genital tract, highlighting the importance of local immune activation in the context of treatment. Conversely, CD4+CD69+ expression in both PBMCs and CMCs did not show significant fluctuations, suggesting that while CD4+CD25+ may be a more sensitive marker of immune response, CD4+CD69+ might not reflect the same level of activation under these conditions.

Arm H presented a different picture, with only one significant result for PBMC, CD4+CD25+ ($p = 0.0009$) and no significant changes observed in CMC CD4+CD25+ or CD4+CD69+ across

all time points. This lack of consistent immune response may suggest that patient characteristics or baseline immune status could influence the effectiveness of acetylsalicylic acid use.

The significant expressions of CD4+CD25+ and CD4+CD69+ in PBMCs and CMCs in Arms D and K suggest that acetylsalicylic acid may effectively modulate inflammatory responses, potentially providing therapeutic benefits in conditions characterized by immune dysregulation.

My study had a high attrition rate resulting from many women not completing their scheduled visits. This incomplete data collection may have impacted the findings and limited the overall results. The absence of whole genome sequencing in this analysis restricted my ability to accurately identify and differentiate between microbial species, which is crucial for understanding the complexities of the microbiome. While the main clinical study was assessing all of the inflammation and cellular activation parameters, this thesis specifically focused on the microbiome only, potentially overlooking important interactions between microbial communities and immune responses. To address these limitations, future analyses will aim to integrate both microbiome and immune parameters together.

5.2 Conclusions

The study concluded the following:

i) There was no significant change Arm-D(p>0.9999), Arm-K(p=0.2918) and Arm- H(p=0.4966) in the population of *Lactobacillus spp* and therefore ASA had no impact on *Lactobacillus spp* population.

ii) There was no significant change Arm-D(p>0.9999), Arm-K(p>0.9999) and Arm- H(p=0.8949) in the population of *Gardnerella vaginalis* and therefore ASA had no impact on *Gardnerella vaginalis.*

iii) There was no significant change Arm-D(p>0.9999), Arm-K(p>0.9999) and Arm- H(p>0.9999) in the population of yeast cell and therefore ASA had no impact on yeast cell populations

Therefore, these findings suggested that the daily intake of ASA did not alter commensal vaginal bacteria and yeast in the genital tract. Hence controlling genital inflammation using ASA may be a safe strategy that preserves the vaginal.

5.3 Recommendations

Acetylsalicylic acid (ASA) is a known anti-inflammatory drug. However, there is lack of information on its role on disrupting essential processes or bacterial structures as its antibacterial function is still unknown. Therefore, future longitudinal studies should:

(i) Evaluate the impact ASA and other anti-inflammatory drugs on the diverse profile of the vaginal microbiota.

(ii) Investigate the interactions between the impact on vaginal microbiota and drug pharmacokinetics.

(iii) It is also crucial for researchers using prevention approaches to understand the mechanism and processes the genital microbiota use to influence genital inflammation.

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APPENDICES

Appendix 1: Research Approval for the study

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Internal Memo

FROM: Dean, Graduate School **DATE:** 5th October, 2022

TO: Ms. Onyango Anne Wendy Adhiambo **REF:** 156/27017/2014
Department of Biochemistry,
Microbiology & Biotechnology

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

We acknowledge receipt of your Research Proposal after fulfilling recommendations raised by the Graduate School Board of 28th June, 2022.

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking and Progress Report Forms per semester. The Forms are available at the University's Website under Graduate School webpage downloads.

Also, please ensure that you publish article(s) from your thesis before submitting it to Graduate School for examination as per the Commission for University Education and Kenyatta University guidelines.

Thank you.

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

CC. Chairman, Department of Biochemistry, Microbiology & Biotechnology

Supervisors:
1. Dr. Anthony Kebira
   C/o Biochemistry, Microbiology & Biotechnology Dept.
   Kenyatta University

2. Prof. Keith Fowke
   Department of Medical Microbiology & Infectious Diseases
   University of Manitoba, Canada
   C/o Biochemistry, Microbiology & Biotechnology Dept.
   Kenyatta University

jc/ovw

Appendix 2: Authorization Letter

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Our Ref: I56/27017/2014

DATE: 5th October, 2022

Director General,
National Commission for Science, Technology
and Innovation
P.O. Box 30623-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR MS. ONYANGO ANNE WENDY ADHIAMBO REG. NO. I56/27017/2014

I write to introduce Ms. Onyango Anne Wendy Adhiambo who is a Postgraduate Student of this University. She is registered for M.Sc. degree programme in the **Department of Biochemistry, Microbiology & Biotechnology.**

Ms. Onyango intends to conduct research for a M.Sc. thesis Proposal entitled, **"Impact of Daily Aspirin Intake on Commensal Vaginal Bacteria."**

Any assistance given will be highly appreciated.

Yours faithfully,

PROF. ELISHIBA KIMANI
DEAN, GRADUATE SCHOOL

JG/eww

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Ref. No.KNH/ERC/R/31

7th March, 2022

Dr. Joshua Kimani
Co- Investigator
Institute of Tropical and Infectious Diseases (UNITID)
Faculty of Health Sciences
University of Nairobi

Dear Dr. Kimani,

Re: Approval of Annual Renewal – Preventing HIV by Targeting the Immune System instead of the Virus (P638/09/2018)

Your communication dated 24th February 2022 refers.

This is to acknowledge receipt of the study progress report and hereby grant annual extension of approval for ethics research protocol P638/09/2018.

The approval dates are 26th February 2022- 25th February 2023.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc.) are submitted for review and approval by KNH- UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH- UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) 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*).

Appendix 3: Ethics Approval

Appendix 4: NACOSTI Research License

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **941717**

Date of Issue: **14/February/2023**

RESEARCH LICENSE

This is to Certify that Ms.. Anne Wendy Adhiambo of Kenyatta University, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Nairobi on the topic: Impact of Daily Aspirin Intake on Commensal Vaginal Bacteria for the period ending : 14/February/2024.

License No: **NACOSTI/P/23/23211**

941717

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions

Appendix 5: Informed Consent

Informed consent form:

Preventing HIV infection by targeting the immune system instead of the virus - (P638/08/2018)

Participants Information and Consent form

This information will be communicated orally in English, Swahili or other Kenyan dialect of participant's preference. The form is also available written in Swahili.

Investigators:

Dr Keith Fowke, University of Manitoba, 745 Bannatyne, room 539, Winnipeg, MB, Canada 1-204-789-3818

Dr. Joshua Kimani, Medical Microbiology, University of Nairobi, tel. 714851, P.O Box 19676, Nairobi, Kenya

Dr Julie Lajoie, University of Manitoba, 745 Bannatyne, room 539, Winnipeg, MB, Canada, 1-294-789-3296 (study coordinator)

You are being asked to participate in a human research study. Please take your time to review this consent form and discuss any questions you may have with the research nurse or the clinician. You may also discuss your participation with your regular doctor, family, and friends before making your decision. If you don't understand words or information you may read in this form, please do not hesitate to ask the research nurse or clinician to explain anything that you do not clearly understand.

Financial support of this study: To conduct this study the investigators are receiving financial support from the Canadian Institute of Health Research (CIHR).

Informed consent form:

Preventing HIV infection by targeting the immune system instead of the virus - (P638/08/2018)

Background Information

The University of Nairobi and University of Manitoba joint basic science research program is conducting studies to determine the relationship between the immune system and susceptibility to sexually transmitted infections (STI) with the goal of developing vaccines or treatments for STIs and HIV.

Purpose of study:

Currently, there is no vaccine against HIV. However, Pre-exposure prophylaxis-PrEP is available in Kenya has been proven to prevent HIV infection. However, condom use is, the only method widely available to protect against HIV infection during sexual intercourse. This study is being conducted to analyse if acetylsalicylic acid (also called ASA or aspirin) can be used to decrease inflammation and the number of HIV target cell (cells that HIV infects) in your genital tract (female part) and blood. Inflammation has been associated with an increased risk of HIV infection. This study does not intend to determine if the drugs can prevent HIV infection. Aspirin is not approved for the purpose of preventing HIV infection, rather, the study will try to determine if it can decrease inflammation and HIV target cell numbers at the genital tract. The drugs being tested in this study is approved as anti-inflammatory drugs. We are trying to assess if its effect can be extended to the genital tract and can decreased the number of HIV target cells. It is important to understand that taking aspirin does **NOT** protect you from getting HIV or other sexual transmitted infections and you should continue to regular condom use and PrEP if you are currently using it. If you are not currently taking PrEP to prevent HIV infection and wish to start, please let the clinical staff know and they will be helping you with this. Taking PrEP won't affect your participation in this study.

You are being asked to take part in this study because you are HIV negative and do not have any chronic disease.

Appendix 6:Questionnaire

Preventing HIV by targeting the immune system instead of the virus
Visit 1 Questionnaire

ID **Clinic No:**

Date

Randomization arm: A, B, C, D

Number of pills given:

1.	**Study number**		
2.	**Signed consent form?** ***If No, stop completion of the questionnaire immediately***	0. No 1. Yes	
3.	**Date of visit**	DD/MM/YY	
4.	**Identification of the person completing the questionnaire (initials)**		
5.	**Age** **Date of birth (DD/MM/YY)**		____ years ____/____/_____
6.	**Country of origin**		
7.	**Marital status**	1. Not married, not living with a man 2. Not married, living with a man 3. Married, not living with a man 4. Married, living with a man 5. Widowed, not living with a man 6. Widowed, living with a man	
8.a	**How many years of school have you completed?**		____ years
8.b	**What is your level of education?**		
9.	**Are you taking hormonal contraceptive?**	0.No If no go to question 10 1.Yes	
9b	**What type of contraceptive do you take?**	0. Oral (please select Combined or single hormone) 1. Injection (DMPA) 2. Implant 3. Intra uterine device 4. Other, specify_____	
9c	**Where do you get your hormonal contraception?**	1. SWOP clinics 1. Other clinics/pharmacist If 1, please indicates where	
9d	**If you are using DMPA as contraceptive method, when did you get your last injection**		Day/Month/Year ____/____/_____
9e	**If you are using DMPA, when is your next injection scheduled?**		Day/Month/Year ____/____/_____
9f	**If you are using Oral contraception what is type that you are using?**		

9g	**When did you start using your current method of contraception?**		____ years _____ months
10a	**How many times have you been pregnant?**		
10b	**When is the last time you were pregnant?**		
10c	**How many children did you have?**		
10d	**How many children alive do you have?**		
11	**Do you drink alcohol?**	1.Yes 2.No if No go to Q.12	
11b	**How much and how often do you drink alcohol?** **(Consumption per week)** **1 consumption = 1 beer or 1 glass of wine**	1. Occasionally 2. 0-5 3. 5-10 4. 10 and more	
12.	**Do you take non-prescribed or prescribed drugs?**	0.no 1.yes	 1.prescribed 2. non prescribed
12b	**If yes, where did you get them?**	1. Physician 2. Pharmacist 3. Traditional healer	
12c	**If yes, which one and for which reason**	Drugs: Reason:	
13	**Do you practice oral sex?**	0. No (go to 14) 1. Yes	
13a	**Did you practice oral sex during the last 7 days?**		
13b	**How often did you practice oral sex during the last 7 days?**		
14.	**Do you practice anal sex?**	0. No 1. Yes	
14b	**Did you practice anal sex during the last 7 days?**		
14c	**How often did you practice anal sex during the last 7 days?**		
15.	**Did you have oral sex (penis in the mouth) and/or anal sex (penis in the anus) in the last 7 days?**	0=No 1=Yes (anal) 2=Yes (oral) 3=Yes (both: oral and anal)	
16a.	**Have you ever received a blood transfusion?**	0. No → Question 17 1. Yes	
16b.	**If yes, how old were you when you had a blood transfusion for the first time?**	____ years	I
17a.	**Except for vaccinations or DMPA injection, did you recently receive medicinal injections to treat a health problem?**	0. No → Go to part II or III 1. Yes	
17b.	**Where did you receive this injection?**	1. Public clinic 2. Private clinic 3. Traditional healer	

Appendix 7:Bacterial Vaginosis Report Form

Preventing HIV infection by using low dose anti-inflammatory: IIQ.2

Bacterial Vaginosis Diagnosis Report Form.

Epithelial cells at 100x all others at 1000x
<1 per field=1+
1-5 per field=2+
5-30 per filed=3+
>30 per field=4+

Morphotype	point	morphotype	point
4+ Lacto	0	1+ G.vag	1
3+ Lacto	1	2+ G vag	2
2+Lacto	2	3+ G. vag	3
1+ Lacto	3	4+ G. vag	4
0+ Lacto	4	1-2+ G. vag	1
		3+-4 G. vag	2

Patient number and visit							
Date collected:	Day/Month/Year						
Sample condition	Good or Unacceptable (indicate nature)						
Date stained	Day/Month/Year						
Date read	Day/Month/Year						
WBC	1 to 4+						
Clue cells	1 to 4+						
Lactobacilli	Lg pos rods						
(LB)	Inter pos rods						
G.vaginalis/ acteroides	Sm gram pos/neg rods						
Mobiluncus	Curved						
Fusobacterium							
Others							
BV score (0 to 10)							
Yeast	1 to 4+						
Diagnosis	BV, Normal, Intermediate						

Read by (initials):_____ Reviewed by (initals):_____