Longitudinal impact of PrEP use and BV treatment on vaginal microbiota in adolescent girls and young women at high risk of acquiring HIV in KwaZulu-Natal

Presented by

Noluthando Mazibuko-Munonde (209502316)

Submitted in partial fulfillment of the requirements for the degree

of **Doctor of Philosophy (Medical Microbiology)**

Principal Supervisor: Dr Sinaye Ngcapu

School of Laboratory Medicine and Medical Sciences; Department of Medical Microbiology; Nelson R. Mandela School of Medicine, College of Health Sciences; University of KwaZulu-Natal; Durban, South Africa

2025

PREFACE

I, Noluthando Mazibuko-Munonde, declare that the experimental work in this dissertation is conducted solely by myself unless otherwise detailed by reference or acknowledged. The experimental work detailed in this thesis was conducted at the Centre for the AIDS Program of Research in South Africa Laboratory, Doris Duke Medical Research Institute Building, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, South Africa, under the supervision of Dr Sinaye Ngcapu.

This study represents the original work by the author and has not been otherwise submitted in any form to any other University. Where use of the work by others has been made, it is duly acknowledged in the text.

Noluthando Mazibuko-Munonde. Date: 25 August 2025

Candidate

Dr Sinaye Ngcapu Date:

Supervisor

DECLARATION 1-Plagiarism

I, Noluthando Mazibuko-Munonde declare as follows:

The research described in this dissertation, except otherwise specified, is my original work.

This dissertation has not been presented for degree purposes or examination by any other academic institution.

This dissertation does not comprise other data, pictures, graphs, or information acquired from other researchers unless purposely acknowledged as being obtained from other researchers.

This dissertation does not comprise writing that is obtained from other researchers unless specifically acknowledged as being obtained from other researchers. Where other written sources have been quoted, then:

Their words have been amended, but the overall information accredited to them has been referenced. In cases where there are exact words were used, their writing has been located inside quotation marks and referenced.

Where I have repeated a publication whereby, I am an author, co-author, or editor I specified in detail which portion of the journal was written by me only and had completely referenced the aforementioned publication.

This thesis does not contain texts, tables, or pictures copied and pasted from the internet unless exclusively acknowledged, and the information is detailed in the thesis and the reference section.

DECLARATION 2 - Publications

The journals presented in this dissertation have originated from the CAPRISA 082, CAPRISA 084 and CAPRISA 090 studies, and the research was carried out as part of the DSI-NRF Centre of Excellence in HIV Prevention, which is sponsored by the Department of Science and Innovation, and the National Research Foundation. This thesis constitutes work carried out by myself and fulfils the requirements for the degree of Doctor of Philosophy by research manuscripts.

The following contributions were made to the publications of the following manuscripts

1. **Noluthando Mazibuko-Motau[1,2‡],** Parveen Sobia[1,2‡], Jiawu Xu[3], Joseph, Ahmed Elsherbini[3], James E San[4], Lara Lewis[1], Andile Mtshali[1,2], Gugulethu Mzobe[1,2], Lungelo Ntuli[1,2], Salim S. Abdool Karim[1,5], Leila E. Mansoor[1], Quarraisha Abdool Karim[1,5], Douglas S Kwon[3,6,7†], Derseree Archary[1,2†], Sinaye Ngcapu[1,2†*]. **Vaginal microbial shifts are unaffected by oral pre-exposure prophylaxis in South African women.** Scientific reports, Published. **Contribution:** Together with Dr Sinaye Ngcapu, we conducted all laboratory experiments, analysed and wrote the manuscript which has been published in the Scientific reports. Dr Parveen Sobia contributed as a co-author and reviewed the draft manuscript.

2. Mazibuko-Munonde N[1,2]*, Kama OA [1]*, Letsoalo M[1]*, Ngcobo S[1], Radebe P[3], Gumbi P[1,3] Mehou-Loko C[4], Bunjun R, Mtshali A[1,2], Mzobe GF[1,2], Magini SN[1,2], Ntuli L[1,2], Maphumulo N[1], Mntambo N[1], Radzey N[4], Abrahams AG[4], Sibeko S[5], Harryparsad R[4], Manhanzva MT[4], Meyer B[4], Ahmed N[6], Potloane D,[1] Horsnell W[4], Liebenberg LJP[1,2], Archary D[1,2], Sivro A[1,2,7,8], Abdool Karim Q[1], Bekker LG[4,6], Passmore JS[1,3,9], Humphries H[10,11], Masson L[1,4,13,14], Jaspan HB[4,10#], Ngcapu S[1,2#]. **Impact of Vaginal Insertion Products, Sexual Activity, and Vaginal Microbiome Dynamics on Genital Inflammation Markers Among South African Women.** Submitted, Microbiome; Under review. **Contribution**: Together with Dr Sinaye Ngcapu, we conceptualised the study. Samukelisiwe Ngcobo and I conducted the assessment of T cells. Marothi Letsoalo, Olona Kama, Sinaye Ngcapu and myself contributed to the analysis and wrote the manuscript.

ETHICAL APPROVAL

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (REF: BE603/18).

PRESENTATIONS EMANATING FROM THIS THESIS

1.1 Oral presentations

1. **Noluthando Mazibuko-Motau[1,2‡],** Parveen Sobia[1,2‡], Jiawu Xu[3], Joseph, Ahmed Elsherbini[3], James E San[4], Lara Lewis[1], Andile Mtshali[1,2], Gugulethu Mzobe[1,2], Lungelo Ntuli[1,2], Salim S. Abdool Karim[1,5], Leila E. Mansoor[1], Quarraisha Abdool Karim[1,5], Douglas S Kwon[3,6,7†], Derseree Archary[1,2†], Sinaye Ngcapu[1,2†*]. ***The transient and persistent shifts in vaginal microbial communities not influenced by PrEP***.

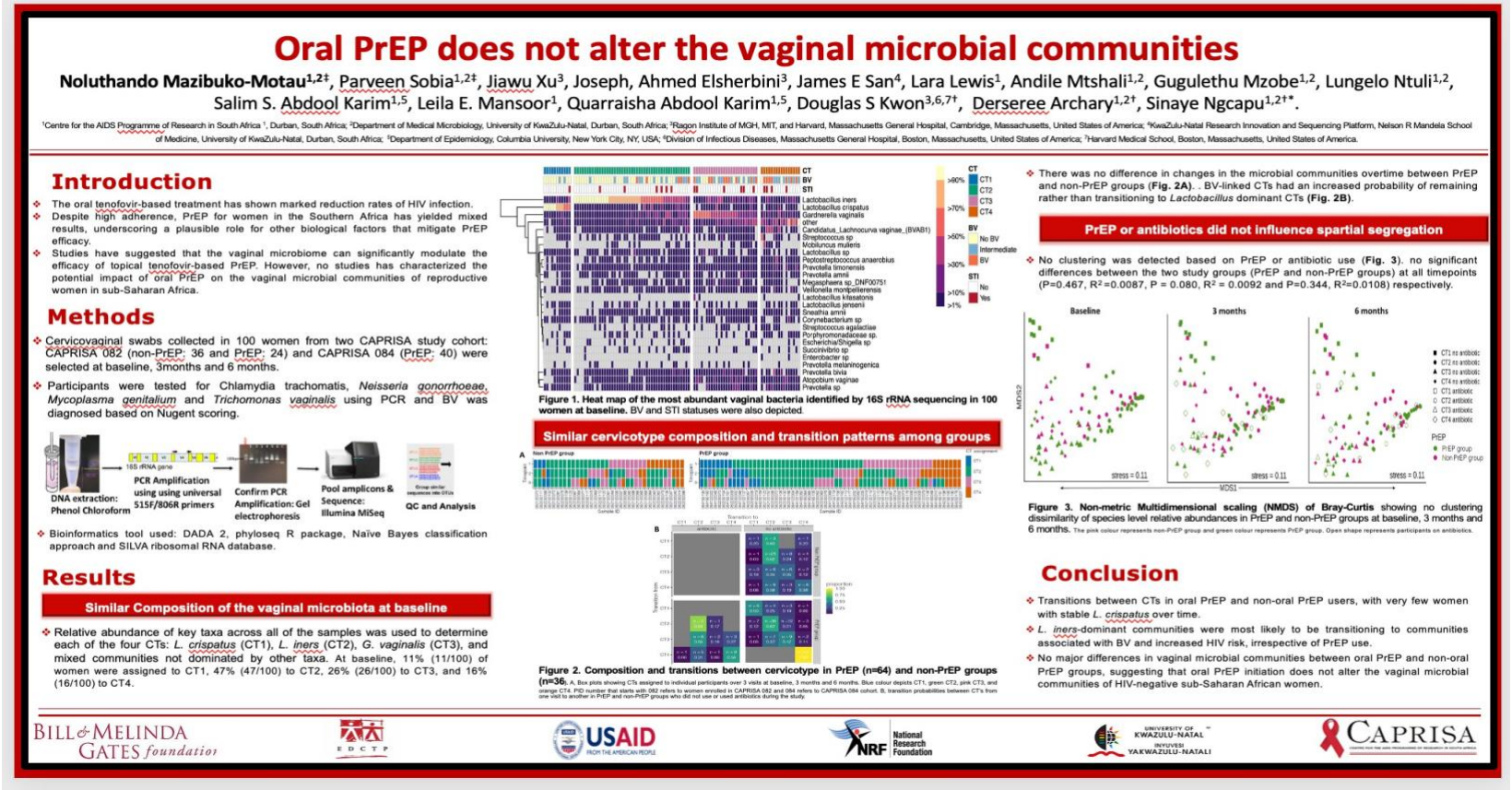
   School of Laboratory Medicine and Medical Science Research Symposium; oral category; 26 November 2022; University of KwaZulu-Natal, South Africa

2. **Noluthando Mazibuko-Motau[1,2‡],** Parveen Sobia[1,2‡], Jiawu Xu[3], Joseph, Ahmed Elsherbini[3], James E San[4], Lara Lewis[1], Andile Mtshali[1,2], Gugulethu Mzobe[1,2], Lungelo Ntuli[1,2], Salim S. Abdool Karim[1,5], Leila E. Mansoor[1], Quarraisha Abdool Karim[1,5], Douglas S Kwon[3,6,7†], Derseree Archary[1,2†], Sinaye Ngcapu[1,2†*]. ***The transient and persistent states of cervicovaginal microbiota not influenced by PrEP.***

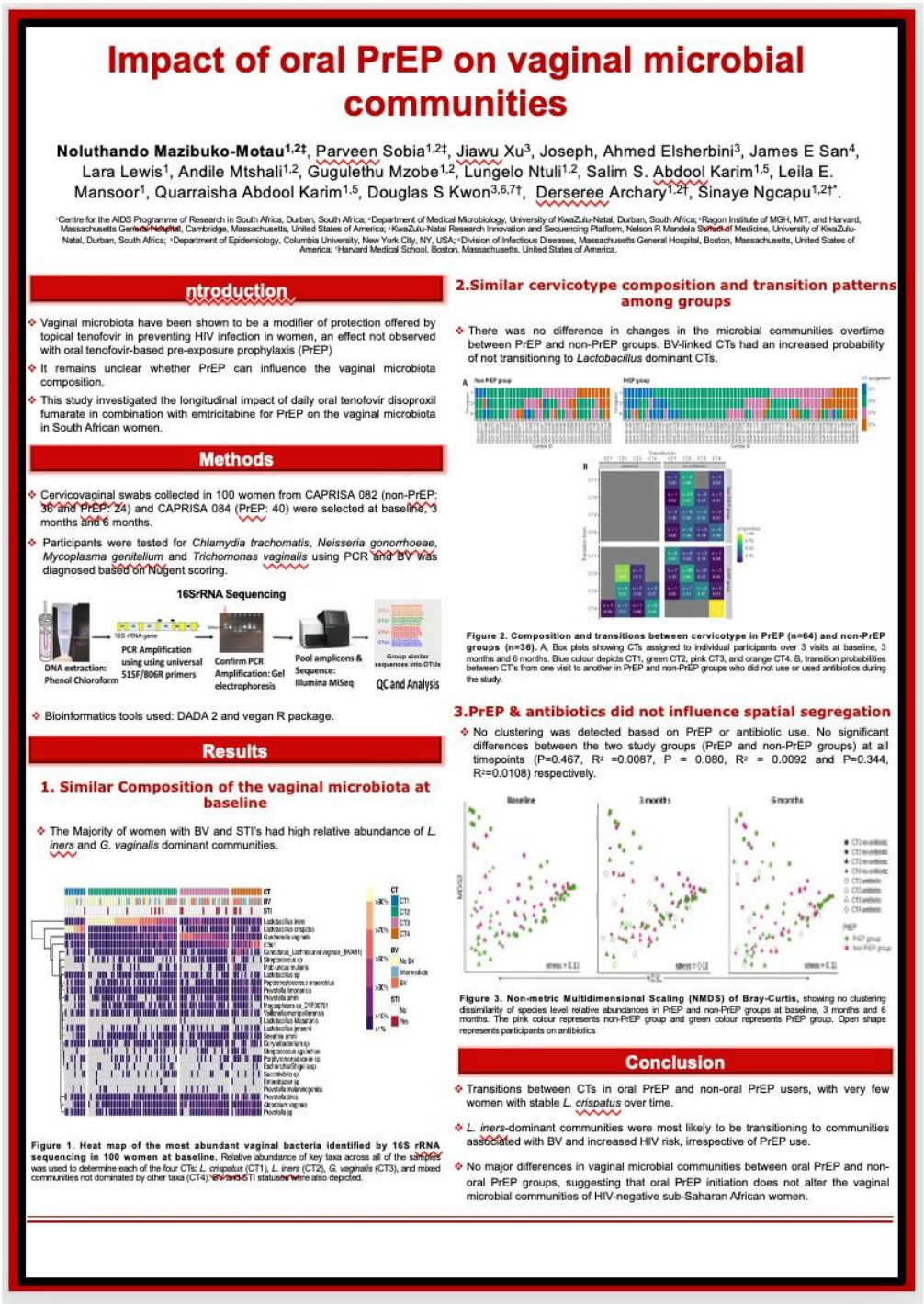
   The 2nd African Microbiome Symposium in Stellenbosch; Oral category; 7-9 December 2022; University of Stellenbosch, South Africa.

1.2 Poster presentations

Noluthando Mazibuko-Motau[1,2‡], Parveen Sobia[1,2‡], Jiawu Xu[3], Joseph, Ahmed Elsherbini[3], James E San[4], Lara Lewis[1], Andile Mtshali[1,2], Gugulethu Mzobe[1,2], Lungelo Ntuli[1,2], Salim S. Abdool Karim[1,5], Leila E. Mansoor[1], Quarraisha Abdool Karim[1,5], Douglas S Kwon[3,6,7†], Derseree Archary[1,2†], Sinaye Ngcapu[1,2†*]. ***Oral PrEP does not alter the vaginal microbial communities.*** The 9th Federation of Infectious Diseases Societies of South Africa (FIDSSA) Congress; best e-poster category; 3-5 November 2022; at the Elangeni & Maharani Hotel, Durban, South Africa.



Noluthando Mazibuko-Motau[1,2‡], Parveen Sobia[1,2‡], Jiawu Xu[3], Joseph, Ahmed Elsherbini[3], James E San[4], Lara Lewis[1], Andile Mtshali[1,2], Gugulethu Mzobe[1,2], Lungelo Ntuli[1,2], Salim S. Abdool Karim[1,5], Leila E. Mansoor[1], Quarraisha Abdool Karim[1,5], Douglas S Kwon[3,6,7†], Derseree Archary[1,2†], Sinaye Ngcapu[1,2†*]. ***Impact of oral PrEP vaginal microbial communities*. 18th International Congress of Immunology (IUIS 2023);** poster category; 27 November - 2 December 2023, Cape Town, South Africa.



DEDICATIONS

I dedicate this thesis to my daughters, Arabile Zesuliwe Munonde and Rethabile Simelokuhle Munonde. You are the light of my life and the source of my greatest purpose. Your presence inspires me to dream bigger and strive harder every day. I also extend this dedication to my other daughters, Sisanda Andile Mdluli, Sitota Ziphezinhle Atiso, and Thingolwenkosazane Mbongwe, this work is also for you girls. I hope that one day, as you read these words, you will feel encouraged to aim for the stars, knowing that with determination and resilience, anything is possible. May this serve as a testament to the power of perseverance and a reminder to always reach for your dreams.

ACKNOWLEDGMENTS

The work detailed in this thesis would not have been possible without the incredible contributions of several individuals whom I would like to acknowledge:

My supervisor, Dr Sinaye Ngcapu, for his patience, guidance and mentoring throughout the course of my PhD studentships in his lab. His time and expertise were key to the success of this work, as well as shaping me into the scientist I am today. The financial support he provided when the funding ran out.

A special thank you goes to all the members of the inflamed group and CAPRISA fellows, for the amazing moments we have shared, the laughs, support and encouragement.

I would like to give a special mention to Miss Olona Kama, Mr Marothi Letsoalo and Dr Emmanuel San James for their expertise and massive support during data Analysis.

I would also like to acknowledge our collaborators, Kwon Lab (Harvard University), for their input into this research and expertise with microbiome sequencing and Jaspan Lab for their support with microbiome sequencing. A special thanks to Dr A. Mtshali, Dr G Mzobe and Mr L Ntuli for lending a helping hand.

Thank you to all the women who participated in the CAPRISA 008, CAPRISA 084, CAPRISA 082 and CAPRISA 090 study cohorts, without their willingness to participate, this study would not have been possible.

I would like to extend my appreciation to the University of KwaZulu-Natal: Talent Excellence Scholarship and Poliomyelitis Research Foundation [PRF; Grant no: 21/59)] for financial support. This research was funded by University-Southern African Fogarty AITRP Programme (grant#D43TW00231), National Research Fund Thuthuka Research Grant (grant# TTK160510164586), and Poliomyelitis Research Foundation Research Grant (grant#16/17)

I would also like to acknowledge members of my family. My parents and sisters for their support. My husband, Karabo Munonde, for all the support and encouragement he gave me to complete this degree. Thank you for all your sacrifices Munonde, unwavering support and for loving me through this PhD journey of my studies.

TABLE OF CONTENTS

List of Figures

List of Tables

LIST OF ABBREVIATIONS

AGYW	Adolescent girls and young women
ARV	Antiretroviral
ASV	Amplicon sequence variants
BV	Bacterial vaginosis
BV	Bacterial Vaginosis
Cl	Confidence Interval
CST	Community state type
CT	Community type
CV	Cervicovaginal
DADA	Divisive Amplicon Denoising Algorithm
FACTS-001	Follow on African Consortium for Tenofovir Studies
FDA	Food and Drug Administration
FGT	female genital tract
FTC	emtricitabine
H_2O_2	hydrogen peroxide
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSRC	Human Sciences Research Council
HSV-2	Herpes simplex virus-2
IDUs	injecting drug users
Ig	Immunoglobulin
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
iPrEX	Pre-exposure Prophylaxis Initiative trial
LMICs	Low and low middle incime countries
MCP-1	Monocyte chemoattractant protein1
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
NF-κB	Nuclear factor kappa B
NMDS	Non-metric dimensional scaling
PCR	Polymerase chain reaction
pMTCT	pre-exposure prophylaxis for the prevention of mother-to-child transmission
PrEP	Pre-exposure prophylaxis
SCFAa	short-chain fatty acids
STI	Sexually transmitted infection
TDF-FTC	tenofovir disoproxil fumarate + emtricitabine
TFF	Tumor Necrosis Factor
TFV	Tenofovir
TLR	Toll like-receptor

TNF-α	Tumor necrosis factor alpha
UNAIDS	Joint United Nations Programme on HIV and AIDS
VIPs	Vaginal insertion products
VM	Vaginal microbiome
VMT	Vaginal microbiome transplantation
Voice	Vaginal and Oral Interventions to Control the Epidemic

THESIS CONTEXT

Chapter 1 provides an overview of the study design, including its background, rationale, aims, objectives, and methodologies. It also contains a comprehensive literature review exploring the relationship between the vaginal microbiome, PrEP, and vaginal insertion products (VIPs). The chapter highlights factors influencing the vaginal microbiome, the implications of dysbiosis, and various strategies for managing or treating dysbiotic vaginal microbiota.

Chapter 2 investigates the interaction between daily oral tenofovir-based pre-exposure prophylaxis (PrEP) and vaginal microbiota in a cohort of 100 South African women, of whom 64 were taking PrEP and 34 were not. This chapter examines whether bacterial vaginosis (BV) modifies the efficacy of oral PrEP. The findings show similar vaginal microbial profiles between women taking oral PrEP and those not using it. While some shifts in vaginal microbiota were observed, these were not attributable to PrEP use. The results support the continued provision of oral PrEP to women regardless of BV status.

Chapter 3 evaluates the influence of the vaginal microbiome on the efficacy of 1% tenofovir gel, a topical antiretroviral used as PrEP in women. The study concludes that tenofovir concentrations are not affected by the composition of the vaginal microbiota, reinforcing the gel's consistent efficacy across diverse microbial profiles.

Chapter 4 Characterises the vaginal microbiome of South African adolescent girls and adult women using VIPs and assesses the effects of sexual activity and VIP use on microbial dynamics and markers of genital inflammation. The findings demonstrate that vaginal microbiota diversity and the timing of sexual activity significantly influence immune responses. However, VIP use did not affect microbiota composition or immune modulation. These results underscore the importance of microbial diversity and the timing of sexual activity in maintaining vaginal health and immune function.

Chapter 5 summarises the key findings of the thesis, integrating insights from each chapter and discussing their broader implications for understanding the interplay between vaginal microbiota, PrEP, VIPs, and genital health.

ABSTRACT

Background: The HIV epidemic in South Africa, particularly in high-burden regions such as KwaZulu-Natal, remains a significant public health challenge, with persistently high prevalence and incidence rates, especially among young women. The vaginal microbiome plays a crucial role in modulating HIV susceptibility and influencing the efficacy of pre-exposure prophylaxis (PrEP). However, little is known about the longitudinal impact of oral tenofovir disoproxil fumarate and emtricitabine (TDF-FTC) PrEP on the vaginal microbiome, nor the mechanisms by which specific bacterial taxa may modulate the pharmacokinetics and effectiveness of topical tenofovir gel. Additionally, the effects of vaginal insertion products (VIPs) and recent sexual activity on microbial composition, genital inflammation, and HIV susceptibility remain poorly understood.

Methods: Chapter 2 examined the longitudinal effects of daily oral PrEP on the vaginal microbiota in 100 women enrolled in the CAPRISA 082 and 084 trials, with swabs collected at baseline, 3 months, and 6 months for 16S rRNA sequencing. Chapter 3 evaluated the relationship between cervicovaginal tenofovir (TFV) concentrations and microbial community state types (CSTs) in 69 women from the CAPRISA 008 trial, where TFV levels were measured by LC-MS/MS and microbiota composition was analyzed using 16S sequencing. In Chapter 4, the vaginal microbiomes of 219 adolescent girls and 107 adult women from the MIST cohort study were examined, with cytokine levels and immune activation assessed via multiplex bead array and T-cell analysis. All data were analyzed using R and SAS, linking microbiome composition, immune responses, and HIV prevention strategies.

Results: In **Chapter 2**, we observe that only 8% (3/36) of women in the non-PrEP group and 13% (8/64) in the PrEP group had *L. crispatus*-dominated microbial communities. We investigate the dynamics of microbial community transitions over time, finding no significant difference in the rate or pattern of these transitions between the PrEP and non-PrEP groups. While *Lactobacillus iners*-dominated communities were less likely to transition, those that did tended to shift towards communities associated with bacterial vaginosis (BV) and increased HIV risk, independent of PrEP or antibiotic use. These findings suggest that microbial community stability and shifts towards BV-associated states are significant in the context of

HIV risk, irrespective of PrEP use. In **Chapter 3**, we extend this exploration by examining the influence of vaginal microbiota on tenofovir (TFV) concentrations. We found that bacterial taxa distributions and community state types (CSTs) were similarly represented in both high and low TFV concentration groups. Additionally, there were no significant differences in the median log TFV concentrations between women with CST IV or *Lactobacillus*-dominated CSTs ($p>0.005$). This suggests that while vaginal microbiota composition influences several aspects of vaginal health, it does not significantly impact TFV absorption or concentration in this cohort. **Chapter 4** shifts focus to the vaginal microbiome of South African adolescent girls and adult women who use vaginal insertion products (VIPs). The study reveals regional differences in hygiene practices, with women from the Western Cape predominantly using soap and water, while a substantial proportion of women in KwaZulu-Natal utilised VIPs for both hygiene and sexual enhancement. The vaginal microbiome of adolescent girls was predominantly dominated by *L. iners* and BV-associated communities, a profile also observed in adult women. Non-users of VIPs displayed higher levels of pro-inflammatory cytokines (MIP-1β, MIP-1α, IFN-α, IL-6, G-CSF), while VIP users demonstrated elevated levels of IFN-α and G-CSF, which could indicate an immune response to alterations in the vaginal microbiome.

Conclusion:

This dissertation demonstrates that the initiation of PrEP does not significantly alter vaginal microbiota or influence tenofovir concentrations, underscoring the critical role of adherence and timely application in achieving effective HIV prevention. The study concluded that vaginal microbiota diversity and sexual activity timing significantly influenced immune responses. These findings highlight the complex interplay between microbiota composition, sexual behaviour, and immune modulation in HIV susceptibility. Further investigation is warranted to identify the underlying biological mechanisms mediating these effects, such as hormonal fluctuations, mucosal repair processes following intercourse, and functional changes in the vaginal microbiome.

CHAPTER 1

1. INTRODUCTION

1.1 The Human Immunodeficiency Syndrome Virus (HIV) Epidemic in South Africa

In 2023, UNAIDS estimated that 39.9 million [36.1 million–44.6 million] people were living with HIV, and 1.3 million [1 million–1.7 million] had new infections that occurred the same year (1). South Africa, home to just 1% of the global population, bears a disproportionate 18% of the global HIV burden, with approximately 1,000 new infections recorded daily (2). The province of KwaZulu-Natal is at the epicentre of the epidemic, where four out of 11 districts report HIV prevalence rates among pregnant women exceeding 40%, and the remaining districts range between 30% and 40% (3). In one of KwaZulu-Natal's most heavily affected rural districts, HIV prevalence among women utilizing antenatal services escalates from one in ten by age 16 to one in three by age 20, and to one in two by age 24 (4). The community's HIV incidence rates are alarmingly high; in the placebo arm of the CAPRISA 004 trial, which assessed the safety and efficacy of 1% tenofovir gel among urban and rural women in KwaZulu-Natal, an incidence rate of 9.1% per 100 woman-years of follow-up was observed, despite monthly HIV risk-reduction counselling and testing (5). Similarly, high HIV incidence rates have been reported among postpartum women in peri-urban communities within the province (6, 7). Addressing these challenges requires innovative, context-specific interventions and sustained commitment to reducing the burden of HIV, particularly in high-prevalence regions like KwaZulu-Natal.

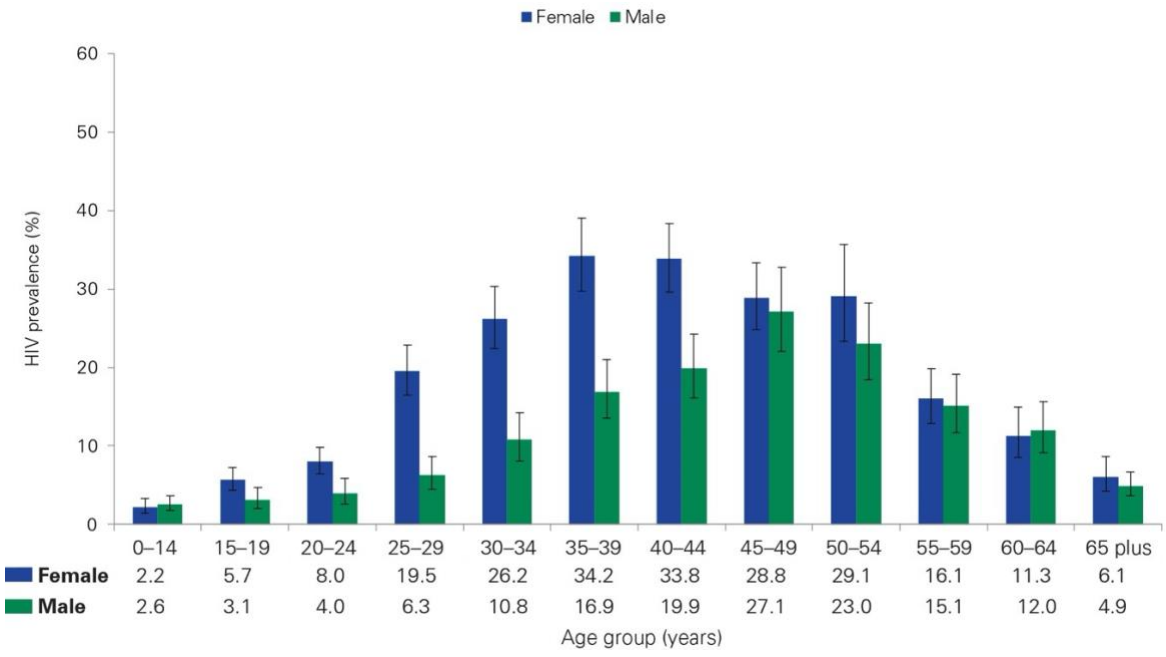


Figure 1.1: HIV prevalence by sex and age in South Africa, 2022. Adapted from Human Sciences Research Council (HSRC, https://hsrc.ac.za, accessed November 2024).

1.2 Factors influencing HIV risk in women

The heightened vulnerability of young women to HIV infection arises from a combination of behavioral, biological, and social factors. While the specific behavioral, physiological, and immunological mechanisms rendering younger women more susceptible to HIV than older women remain incompletely understood, certain key drivers have been identified. Behavioural risks include engagement in new and potentially higher-risk sexual activities, often involving multiple partners (8-16). Moreover, young women frequently experience domestic abuse and sexual violence, with rape posing the greatest risk for those under 18 years old (17, 18). Alarmingly, nearly one-third of adolescent girls in South Africa report forced sexual initiation, highlighting the widespread nature of sexual violence in this region (17, 18). Intimate partner violence (OR 1.48, 95% CI 1.15–1.89) and male-dominated relationship dynamics (OR 1.52, 95% CI 1.13–2.04) further exacerbate HIV risk (19). Practices like dry sex, aimed at sexual enhancement and hygiene, also contribute to risk, as women use substances such as powders, creams, herbs, or detergents to dry and tighten the vagina (20-23). Additionally, sexual debut exposes young women to sexually transmitted pathogens, such as herpes simplex virus type-2 (HSV-2) and human papillomavirus (HPV), which are associated with a 2- to 3-fold increased risk of HIV acquisition (24, 25).

Beyond behavioural factors, biological vulnerabilities significantly elevate HIV risk among adolescent girls and young women in sub-Saharan Africa. Women are, on average, twice as likely as men to contract HIV from a single sexual encounter due to several biological mechanisms (26). The extensive mucosal surface of the vagina provides a pathway for viral entry, while the female genital tract's high immune cell activation and elevated expression of HIV co-receptors in cervical cells further enhance susceptibility compared to men (27, 28). Genital trauma, often resulting from forced or unwanted sexual intercourse, also facilitates HIV transmission (29). Young women's developing reproductive systems must navigate a delicate balance between tolerating semi-allogeneic male semen and mounting immune responses against pathogens. However, genital inflammation, characterised by elevated pro-

inflammatory cytokines, has been linked to a 3-fold increased risk of HIV acquisition, as demonstrated in a recent microbicide trial of tenofovir gel (30). A key driver of such inflammation is bacterial vaginosis (BV), a common microbial imbalance among reproductive-age women. Non-optimal cervicovaginal microbiota, marked by low *Lactobacillus* abundance and high bacterial diversity, contribute to increased genital inflammation, thereby heightening HIV risk and leading to adverse reproductive outcomes (31, 32).

1.3 ARV-based pre-exposure prophylaxis (PrEP): Efficacy, Adherence, and the Role of Vaginal Microbiota

Since 2010, the results of several clinical trials have demonstrated the preventative benefits of antiretroviral (ARV) drugs, offering renewed hope in reducing sexually acquired HIV infections. Pre-exposure prophylaxis (PrEP) trials using tenofovir alone or in combination with emtricitabine (Truvada®) have shown both safety and efficacy in preventing HIV acquisition among diverse groups, including men who have sex with men, transgender women, injecting drug users, serodiscordant couples, and heterosexual individuals (33-38). Importantly, no major safety concerns have been identified. A consistent finding across these trials is that PrEP efficacy is highly dependent on adherence, emphasizing the importance of consistent use. While PrEP trials in men report consistent protective benefits ranging from 44% to 96% with both daily and intermittent dosing strategies (33-38), trials evaluating oral and topical PrEP among women in southern Africa have produced mixed results (5, 39-41). While the CAPRISA 004 trial reported a 39% reduction in HIV incidence with topical tenofovir gel, subsequent trials such as VOICE and FACTS 001 showed little to no protective effect, largely attributed to poor adherence. Similarly, oral PrEP trials like FEM-PrEP and Partners PrEP showed divergent outcomes, with efficacy highly dependent on adherence and possibly influenced by biological factors such as vaginal microbiota composition and mucosal immunity. This variability in trial outcomes underscores the need to investigate host, microbial, and behavioural factors that may modulate PrEP efficacy, which forms a central premise of this thesis.

Recent research has proposed vaginal microbiota as a key factor in the reduced effectiveness of topical tenofovir (TFV) in protecting women from HIV infection (42, 43), a limitation not

observed with oral TFV-based PrEP (44). Proteomics analyses from the CAPRISA 004 trial revealed that 1% tenofovir gel significantly lowered the risk of HIV acquisition in women with *Lactobacillus*-dominant vaginal microbiota compared to those with a non-*Lactobacillus*-dominant microbiota (42). In contrast, reduced TFV levels were observed in vaginal specimens from women with a *Gardnerella vaginalis*-dominant microbial community, suggesting rapid drug depletion in the presence of high levels of *G. vaginalis* (42). Similar findings using Gram stain techniques supported this observation (43). Cervical tissue tenofovir concentrations were higher in women with Nugent scores ≤3 (indicative of *Lactobacillus* dominance) but were lower in women diagnosed with bacterial vaginosis, which is associated with elevated Gardnerella vaginalis levels (43). While both studies demonstrated a negative correlation between TFV levels and BV-associated bacteria, they also highlighted several limitations that warrant further investigation.

1.4 Rationale with Identified Research Gaps and Aims

The HIV epidemic in South Africa, particularly in high-burden regions like KwaZulu-Natal, remains a significant public health challenge despite the availability of interventions such as pre-exposure prophylaxis (PrEP) and risk-reduction counselling. Persistent high HIV prevalence and incidence rates, especially among young women, underscore the need to address unresolved vulnerabilities. Existing gaps in research highlight the limited understanding of how oral tenofovir disoproxil fumarate and emtricitabine (TDF-FTC) PrEP impacts the vaginal microbiome over time and the mechanisms by which vaginal microbiota influence the efficacy of topical tenofovir gel. Additionally, the role of specific bacterial taxa in modulating tenofovir levels and the impact of vaginal practices, such as the use of vaginal insertion products (VIP), on microbial dynamics and genital inflammation remain poorly understood. To address these gaps, longitudinal studies utilising 16S rRNA metagenomic sequencing are essential to elucidate changes in vaginal microbiota associated with PrEP use and to identify microbial contributors to drug efficacy. Furthermore, investigating the correlation between microbial alterations, inflammatory markers, and HIV susceptibility will provide critical insights to optimize PrEP strategies and design culturally relevant interventions that account for host-microbiome interactions. In this dissertation, the following Aims and objectives will be addressed:

Aim 1: To investigate the impact of longitudinal oral tenofovir disoproxil fumarate + emtricitabine (TDF-FTC) PrEP on the vaginal microbiome of healthy South African women.

Hypothesis: Use of oral TDF-FTC PrEP alters the composition and diversity of the vaginal microbiome in healthy South African women, with potential implications for microbial stability, vaginal health, and PrEP efficacy.

Aim 2: To longitudinally evaluate the impact of the vaginal microbiota on concentrations of genital tract tenofovir *in vivo*.

Hypothesis: BV-associated bacterial taxa, characterized by reduced *Lactobacillus* dominance and increased diversity of anaerobic bacteria, are associated with lower concentrations of topical tenofovir in the genital tract among women using tenofovir-based pre-exposure prophylaxis (PrEP).

Aim 3: To assess whether intravaginal practices among these populations lead to alterations in the vaginal microbiome and examined the correlation between these microbial changes and cellular and cytokine markers of genital inflammation.

Hypothesis: Intravaginal practice use leads to alterations in the vaginal microbiome, and these microbial changes influence concentrations of cellular and cytokine markers of genital inflammation.

1.2. LITERATURE REVIEW

1.2.1. PREP AND HIV PREVENTION

Despite extensive prevention campaigns and scale-up of antiretroviral therapy (ART), South Africa remains an epicentre of the HIV pandemic (1, 45). The province of KwaZulu-Natal is the epicentre of the HIV-1 epidemic in South Africa (1, 46). In this region, 56% of all people living with HIV are indeed women underscoring the gender-biased disparity of the HIV infection dynamic. HIV occurs predominantly through unprotected sexual intercourse. Among young women aged between 15-24 years (47), HIV infections were 44% higher than infections in men of the same age. Young women remain vulnerable and acquire HIV about 5-7 years earlier than men, emphasizing the need to find urgent and novel prevention technologies to prevent and reduce incident infections in this key population.

Biological and behavioural factors make women particularly vulnerable to HIV acquisition. Biological risk factors include the large surface area of the female genital tract (FGT) which provides increased opportunities for target CD4+T cell infections (48-50), pre-existing genital inflammation that can impact barrier function (51, 52), vaginal microbial dysbiosis (53), and sexually transmitted infections (54). Behavioural factors can significantly alter HIV risk as well. These include age-disparate sexual coupling (55, 56), age at sexual debut (57-59), multiple concurrent sexual partners (60), low marriage rates (61), disempowerment of women, the inability to negotiate safe sex practices leading to low or no condom use (62), gender-based violence (63) and the use of vaginal insertion products (64) that further increases HIV risk especially in women.

ARVs were first shown to reduce sexual HIV acquisition in the CAPRISA 004 Tenofovir Gel Trial. The trial was conducted in 889 rural and urban South African women, and the results showed that tenofovir gel used before and after sex reduced the acquisition of HIV infection in women by 39% (95% Confidence Interval (CI): 6;60) overall, and by 54% in women who used the gel consistently(5). Thereafter, the Pre-exposure Prophylaxis Initiative trial (iPrEX) showed that daily oral tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) combination (Truvada) reduced HIV incidence by 44% (95% CI 15;63) among 2,499 men or transgender women who have sex with men (35) (Figure 1). Further, the effectiveness of daily

oral PrEP in heterosexual men and women was shown in the Partners PrEP trial (33) and the Botswana TDF2 trial (38). The Partners PrEP trial, which included 4,758 HIV discordant couples from Kenya and Uganda showed that daily oral TDF and TDF/FTC reduced HIV incidence by 67% (95% CI 44; 81) and 75% (95% CI 55; 87) respectively, while the Botswana TDF2 trial, conducted among 1,200 heterosexual men and women from the general population, found that daily oral TDF-FTC reduced HIV incidence by 62% (95% CI 22; 83) (Figure 1.2). The use of ARVs for treatment of HIV-infected patients has also recently been shown in a randomized clinical trial to prevent onward transmission of HIV to their uninfected partners (TasP). The HIV Prevention Trials Network 052 trial (HPTN), conducted among 1,763 HIV-discordant couples from 9 countries, showed that HIV transmission was reduced by 96% (95% CI, 73;99.5) when antiretroviral therapy (ART) was initiated in patients with CD4 counts between 350 and 550 cells/mm (65). This series of breakthroughs in HIV prevention, combined with the recent approval of the first ARV drug (Truvada®) for reducing the risk of sexually acquired HIV infection by the U.S. Food and Drug Administration (FDA) (66), has made the use of ARV drugs, as part of a comprehensive HIV prevention package, a reality.

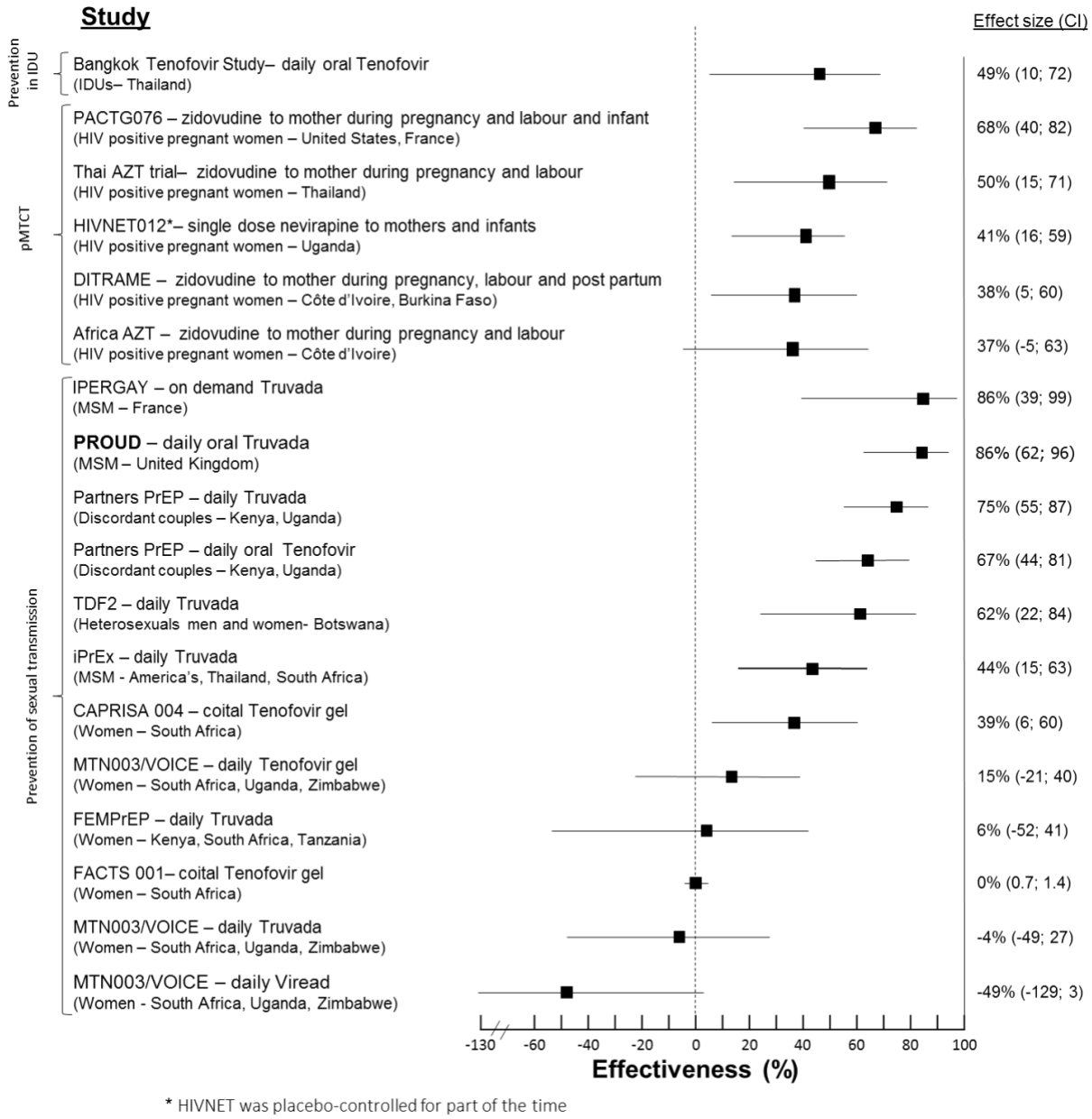


Figure 1.2: Results of placebo-controlled randomized control trials assessing the effectiveness of ARV pre-exposure prophylaxis for the prevention of mother-to-child transmission (pMTCT), as well as transmission through heterosexual and homosexual intercourse and in injecting drug users (IDUs): (adapted from (67-72).

Although daily oral TDF-FTC has been demonstrated to be effective in MSM and transgender women globally and heterosexual men and women in Africa, some clinical trials have failed to show efficacy due to poor adherence to daily dosing. The lack of protective effect observed in

the FEM-PrEP (40) and the Vaginal and Oral Interventions to Control the Epidemic (VOICE) trials (41) can partially be explained by suboptimal adherence. In the FEM-PREP trial (40), only 24% of the women allocated to the daily oral TDF/FTC group had detectable drug levels. Similarly, in the VOICE trial, only 25%, 29%, and 30% of women allocated to the daily tenofovir gel, daily oral TDF/FTC and daily oral TDF groups respectively, had detectable drug levels (41). The challenges and complexities of adherence to daily oral PrEP and coitally dependent gel use are evident from the data emanating from the VOICE (41) and Follow on African Consortium for Tenofovir Studies (FACTS 00) (73), as well as those from the open-label extension studies.

Novel PrEP agents and innovative delivery systems such as slow release formulations of ARVs monthly vaginal rings, LA parenteral agents and lenacapavir, investigated by the current protocol, are needed to support adherence and offer independent and discreet use by vulnerable, HIV uninfected young women, the key population under study. The Phase 3 PURPOSE 1 (74) and 2 (75) trials assessed the efficacy and safety of lenacapavir, a long-acting HIV-1 capsid inhibitor, for pre-exposure prophylaxis (PrEP) in high-risk populations. PURPOSE 1 included adolescent girls and young women in South Africa and Uganda, while PURPOSE 2 targeted cisgender men, transgender women, and nonbinary individuals who have sex with men across multiple regions. In PURPOSE 1, no HIV infections occurred among 2134 participants receiving twice-yearly subcutaneous lenacapavir (0 per 100 person-years; 95% CI, 0.00 to 0.19), whereas 39 infections were observed among 2136 participants in the daily oral emtricitabine-tenofovir alafenamide (F/TAF) group (2.02 per 100 person-years; 95% CI, 1.44 to 2.76) and 16 infections among 1068 participants in the F/TDF group (1.69 per 100 person-years; 95% CI, 0.96 to 2.74) (74) PURPOSE 2 reported similar efficacy, with lenacapavir significantly reducing HIV incidence compared to oral PrEP options (75). Across both trials, adherence to daily oral PrEP was low, but no significant safety concerns were identified. Injection-site reactions were more common in the lenacapavir group, occurring in 68.8% of participants compared to 34.9% in the placebo injection group, with only 0.2% discontinuing due to these reactions. These results establish lenacapavir as a highly effective long-acting PrEP option to address adherence challenges and reduce HIV incidence in diverse at-risk populations (74, 75).

Despite its proven efficacy in clinical trials, the real-world implementation of PrEP faces significant challenges, particularly among women in high-burden regions like sub-Saharan Africa. Adherence remains a critical barrier, with many studies reporting low levels of consistent usage, undermining its protective potential (68, 71, 76). This challenge is compounded by sociocultural factors, stigma, and structural inequalities that limit women's agency in negotiating safer sexual practices (77, 78) or accessing healthcare services. Moreover, biological complexities, such as variations in genital tract drug concentrations influenced by vaginal microbiota (53) and inflammation (79), add layers of complexity to PrEP efficacy. Long-acting formulations, such as injectable lenacapavir (74), offer a promising solution to adherence challenges, simplifying the regimen and reducing reliance on daily dosing. However, the successful rollout of such interventions requires addressing systemic barriers, enhancing community engagement, and ensuring equitable access. PrEP remains a cornerstone of HIV prevention, but its true potential will be realized only through integrated strategies that combine biomedical innovation with a focus on social determinants of health.

1.2.2. VAGINAL MICROBIOME IN HEALTHY WOMEN

A genital environment not dominated by *Lactobacillus* species may facilitate transmission of sexually transmitted infections (STIs), HIV, as well as increase the risk of urogenital disease, miscarriages, preterm births and sepsis in pregnant women (80-82). *Lactobacillus* species (*L. crispatus, L. gasseri* and *L. jensenii*), which are well established as healthy vaginal commensal organisms, play a role in inhibiting the colonization and survival of reproductive tract pathogens, such as Herpes simplex virus-2 (HSV-2), *Neisseria gonorrhoeae,* and uropathogenic *Escherichia coli* as they produce lactic acid, H_2O_2 and bacteriocins (80, 83). This may not be perfectly applicable to women of African descent (84). The predominance of *Lactobacillus* spp is associated with healthy pregnancy outcomes, lack of abnormal vaginal symptoms, urogenital disease and reduced risk for several STIs, including HIV (85). *Lactobacillus* spp. are thought to provide protection by lowering the environmental pH of the lower genital tract through production of lactic acid, by producing various bacteriostatic and bacteriocidal compounds such as hydrogen peroxide (H_2O_2), and/or through competitive exclusion of other organisms (86). A number of different *Lactobacillus* species have been associated with genital health, with the most frequent and abundant being *L. crispatus, L. gasseri,* and *L. jensenii* (86).

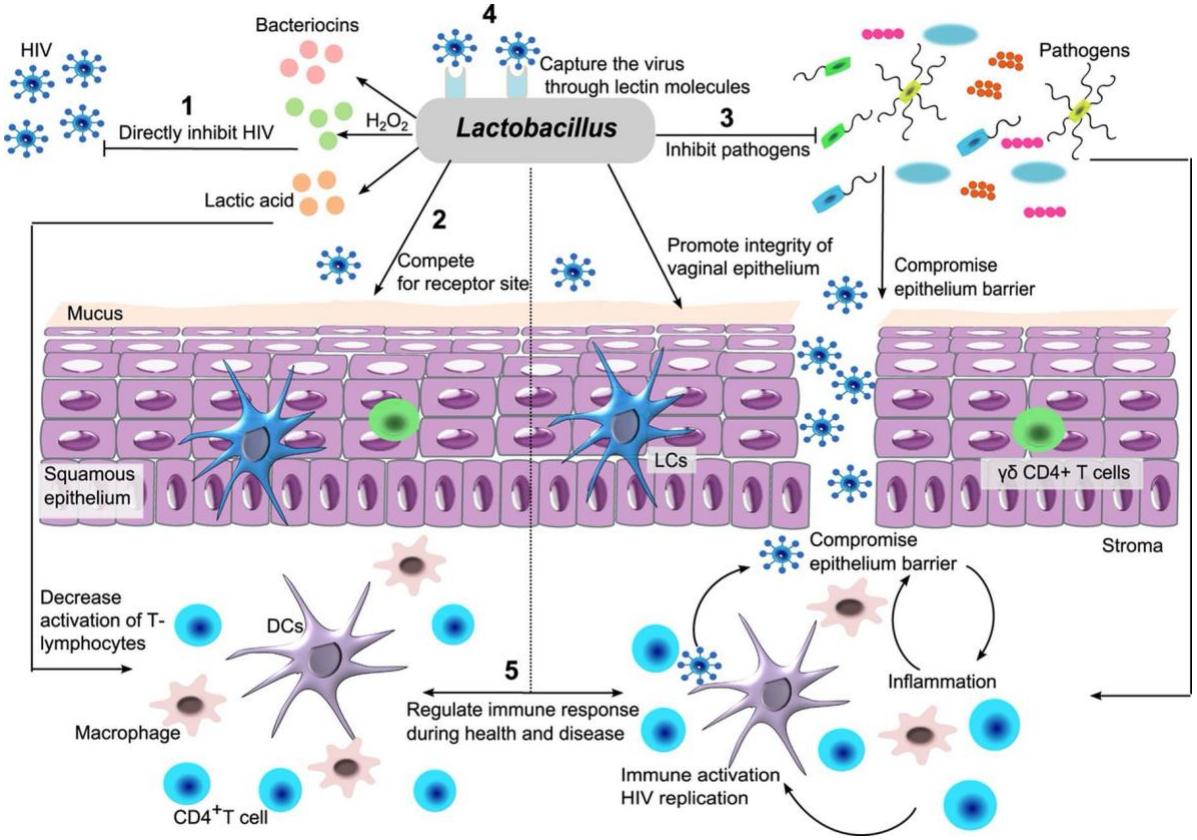


Figure 1.3: Normal vaginal microbiota marked by *Lactobacillus* dominance and *Lactobacillus* mechanism of action against pathogens [adapted from Petrova *et al*, 2013 (85)].

1.2.3. NON OPTIMAL VAGINAL MICROBIOME: VAGINAL DYSBIOSIS

BV is the most common cause of vaginal discharge in women of reproductive age, although its prevalence varies considerably between ethnic groups. In KwaZulu-Natal, the prevalence of BV was reported to be >50% in Black pregnant women living in peri-urban communities (87, 88). The aetiology of BV is not known, although it has been associated with sexual activity (89). BV is a microbial dysbiosis typified by the replacement of commensal *Lactobacillus* spp. with commensal anaerobes, often resulting in vaginitis and discharge (88, 90). This depletion is generally accompanied by the intense overgrowth of commensal vaginal anaerobic bacteria, which can reach densities 100-1000 times higher than found in healthy vaginas and display significantly increased bacterial diversity (90). Culture of organisms from the genital tract of

women with BV typically yields a spectrum of facultative anaerobic commensals, including *Gardnerella vaginalis, Atopobium vaginae, Eggerthella* spp., *Prevotella* spp., BVAB2 (Clostridia-like bacteria), *Megasphaera* type 1, *Mobiluncus* spp, *Ureaplasma urealyticum* and *Mycoplasma hominis (91)*. Absence of *Lactobacillus* species combined with the presence of *G. vaginalis, A. vaginae,* and/or *Prevotella* spp. (91) have been suggested to be highly accurate indicators of BV (92, 93). The diverse microbiota are reminiscent of bacterial communities found associated with the condition known as bacterial vaginosis (BV), which is also characterized by a lack of *Lactobacillus* species, can present clinically with vaginal discharge, a fish-like odor, vaginal discomfort and urinary symptoms and colonization with a diverse spectrum of primarily anaerobic bacteria (91), elevated genital inflammation and thus increased HIV risk (6, 31, 84).

The presence and persistence of BV-associated bacteria, including *Atopobium*, *Mobiluncus*, *Bacteroides*, *Prevotella*, *Clostridiales*, and *Sneathia* have been associated with recurrent BV (94-97). A recent study found that higher concentrations of Megasphaera phylotype 2 and BV-associated bacteria at initial diagnosis, as well as higher concentrations of *Gardnerella vaginalis* posttreatment, were associated with recurrent BV (98). *Atopobium vaginae* is frequently detected with *G. vaginalis* in patients with recurrent BV (99). The role of G. vaginalis in BV is not without controversy, however. While actual causes of poor treatment response rates are still being evaluated, the current model of the etiology of BV focuses on the importance of *Gardnerella* species. It postulates that virulent strains of *Gardnerella* form an adherent biofilm on the vaginal epithelium in which other species can proliferate, resulting in a polymicrobial biofilm (100-104). This adherent biofilm is only weakly affected by the innate immune response (105, 106), and the interplay with the host immune system is only partially understood (107). BV development may be triggered by sexual transmission of a mix of bacteria including *Gardnerella*, which can adhere to epithelial cells in the presence of lactobacilli. It has been suggested that, unlike strictly anaerobic bacteria associated with BV, *Gardnerella*, a facultative anaerobe, can tolerate the high redox potential created by the *Lactobacillus*-dominated healthy vaginal microbiome (102, 105, 108). The metabolism of *Gardnerella spp.*, in turn, results in a local increase in pH and decrease of the redox potential, favouring growth of iron-dependent anaerobes (109) and suppression of lactobacilli (110). Recently, the species previously known as *Gardnerella vaginalis* was shown to comprise at

least 13 different species of which four were named (111). Clinical studies indicated that various *Gardnerella* species may contribute differently to the pathogenesis of BV. A higher abundance of *G. vaginalis* and *G. swidsinskii* was found to be related with vaginal symptoms of abnormal odor and discharge and the relative abundances of *G. vaginalis*, *G. swidsinskii*, and *G. piotii* but not *G. leopoldii* were strongly associated with BV microbiome (112, 113).

1.2.3.1. Human vaginal community state types (CSTs) identification

The vaginal microbiota consists of a diverse community of microorganisms, including bacteria, fungi, viruses, and protozoa. However, our knowledge of the relative abundance of non-bacterial communities is still incomplete. One of the initial studies exploring the vaginal microbiota analysed a cohort of 396 reproductive-aged women from North America, representing four different ethnic backgrounds. The study categorized microbial communities within the vagina into five distinct Community State Types (CSTs). CST-I is primarily dominated by *Lactobacillus crispatus*. CST-II is characterised by a prevalence of *L. gasseri*. CST-III is marked by *L. iners* as the dominant species. CST-V is primarily represented by *L. jensenii*. While CST-IV, has a higher diversity and evenness comprises a diverse community of facultative and anaerobic bacteria (114). Additionally, CST IV was further subdivided into three subtypes: CST IV-A, characterized by high to moderate relative abundance of BVAB1 and *Gardnerella vaginalis*; CST IV-B, marked by the moderate to high relative abundance of *G. vaginalis* and *Atopobium vaginae*; and CST IV-C which is characterised by the low relative abundances of *G. vaginalis*, BVAB1, and *Lactobacillus* species. CST IV-C samples observed either had relatively even distributions of *Prevotella species* or the dominance of *Streptococcus agalactiae*. This classification has provided a valuable framework for understanding the variations in vaginal microbiota composition among individuals.

1.2.3.2 The adverse effects of BV

The cervicovaginal microbiota plays a crucial role in maintaining gynecological health and influencing disease outcomes. This complex ecosystem, dominated primarily by *Lactobacillus* species, provides a protective barrier against pathogenic microorganisms by maintaining an acidic environment and producing antimicrobial substances. Disruptions to this microbiota, often referred to as dysbiosis, have been associated with adverse gynecologic outcomes,

including bacterial vaginosis, increased susceptibility to STIs, and a higher risk of preterm birth in pregnancy. Additionally, imbalances in the cervicovaginal microbiota are linked to conditions such as pelvic inflammatory disease and cervical cancer progression. Understanding the importance of this microbial community offers opportunities for targeted therapeutic interventions, such as probiotics, prebiotics, and microbiota restoration therapies, to improve women's reproductive health and reduce disease burden.

1.2.3.3. Diverse vaginal microbiota causing inflammation and risk for HIV

The diverse microbial communities have been closely associated with elevated genital inflammation and increased HIV risk, likely by increasing mucosal HIV target cell frequency and T cell activation (6, 84, 115). Specific bacteria linked to bacterial vaginosis (BV), such as *Prevotella*, *G. vaginalis*, *Sneathia*, *Atopobium vaginae*, and *Mobiluncus*-have been significantly associated with genital inflammation and heightened HIV susceptibility in women (84, 116, 117). Genital inflammation, defined by elevated levels of five or more pro-inflammatory cytokines (e.g., MIP-1α, MIP-1β, IP-10, IL-8, MCP-1, IL-1α, IL-1β, IL-6, TNF-α) above the 75th percentile, is critical for mounting an immune response but can also compromise the genital epithelial barrier, increasing access to HIV target cells (51). Masson et al., (2015) in a sub-analysis of the CAPRISA 004 study, demonstrated that elevated pro-inflammatory cytokines in genital inflammation were strongly associated with HIV infection risk among South African women. Specifically, chemokines such as MIP-1α, MIP-1β, and IP-10, which were independently linked to HIV seroconversion, were also elevated in women with BV (51, 52, 118). These chemokines bind to the CCR5 HIV co-receptor, facilitating the recruitment of HIV target cells to the female genital tract (FGT) (6, 119). Similarly, Gosmann et al., (2017) found that low-diversity *Lactobacillus*-dominated microbial communities were linked to lower levels of genital inflammation, while diverse *Lactobacillus*-deficient communities correlated with increased pro-inflammatory cytokine and chemokine levels, thereby raising HIV infection risk (6, 84).

In vitro studies have confirmed the role of dysbiotic bacteria in increasing genital inflammation. Co-cultures of *G. vaginalis* and *L. iners* with cervical and vaginal epithelial cells led to elevated pro-inflammatory cytokine production (120) and weakened epithelial barrier

integrity (121). Recognition of these bacteria by antigen-presenting cells through Toll like-receptor 4 (TLR4) activation triggers the NF-κB pathway, promoting pro-inflammatory cytokine release and potentially increasing HIV susceptibility (84). Additionally, mucosal inflammation diminishes the efficacy of HIV prevention strategies, such as tenofovir gel, as demonstrated by McKinnon et al., (2018) in the CAPRISA 004 study (79). High levels of protection were observed in women without genital inflammation, whereas no protection was evident in those with inflammation. Furthermore, Selhorst et al., (2017) showed that women with genital inflammation were infected by less-fit HIV variants (122). Other factors influencing genital cytokine levels include seminal fluid exposure (123) and genital infections such as STIs (124). Collectively, these findings emphasize the importance of addressing genital inflammation and vaginal dysbiosis to reduce HIV risk and enhance the efficacy of PrEP.

1.2.4. ADAPTIVE IMMUNE RESPONSES AGAINST VAGINAL BACTERIA

Adaptive immune responses against bacteria in the FGT remain largely unexplored. However, in the gut there is significant evidence that these responses play an important role in mediating disease pathogenesis and regulating microbial community composition. It is well established that IgA is abundant in the human gut and functions to coat and regulate enteric commensal bacteria (125-129). Few studies, however, have characterized B cell response against bacteria in the FGT. In a recent study of White Dutch women with a high frequency of *L. crispatus* dominant communities, a higher percentage of FGT bacteria were coated with IgA than women with diverse anaerobe dominant communities (130). The study suggested IgA responses against *L. crispatus* may help to maintain this taxon and suppress inflammation in the cervicovaginal mucosa. Such studies have not been performed using samples from women in Low and Low Middle Income Countries (LMICs). There is a large body of recent work that has identified antigen-specific T cell responses directed toward multiple bacteria taxa from the enteric microbiome in both mice and humans (129, 131-138). In mice it has been demonstrated that specific enteric bacteria not only stimulate antigen-specific T cell responses, but also polarize helper T cell differentiation (133, 136, 139, 140). Further, multiple studies show that enteric bacteria-specific T cells play critical roles in driving or preventing disease in models of intestinal inflammation and autoimmune disease, and the balance between inflammation and protection is dependent on the phenotype of the relevant gut bacteria-specific T cell responses induced (131, 132, 141, 142). Some of these T cell responses exacerbate mucosal inflammation

while others have been shown to promote antigen-specific regulatory T cells (143-145). In a study involving Kenyan sex workers, effective treatment of vaginal dysbiosis was linked to a reduction in the numbers of CD4+CCR5+ and CD4+CD69+ T cells (146). Additionally, in a cohort of healthy Belgian women, the presence of *Lactobacillus crispatus* and *Lactobacillus jensenii* was associated with fewer CD3+HLA-DR+ and CD3+CD4+CCR5+ T cells (147). These studies further supported a causative role for a BV-type vaginal microbiota in eliciting genital inflammation and increasing the risk of HIV. Overall, characterization of antigen-specific bacteria-directed T and B cell responses in the FGT provides an opportunity to better understand how to effectively modulate vaginal microbiota composition and host genital inflammation to devise strategies to mitigate adverse health outcomes for women globally.

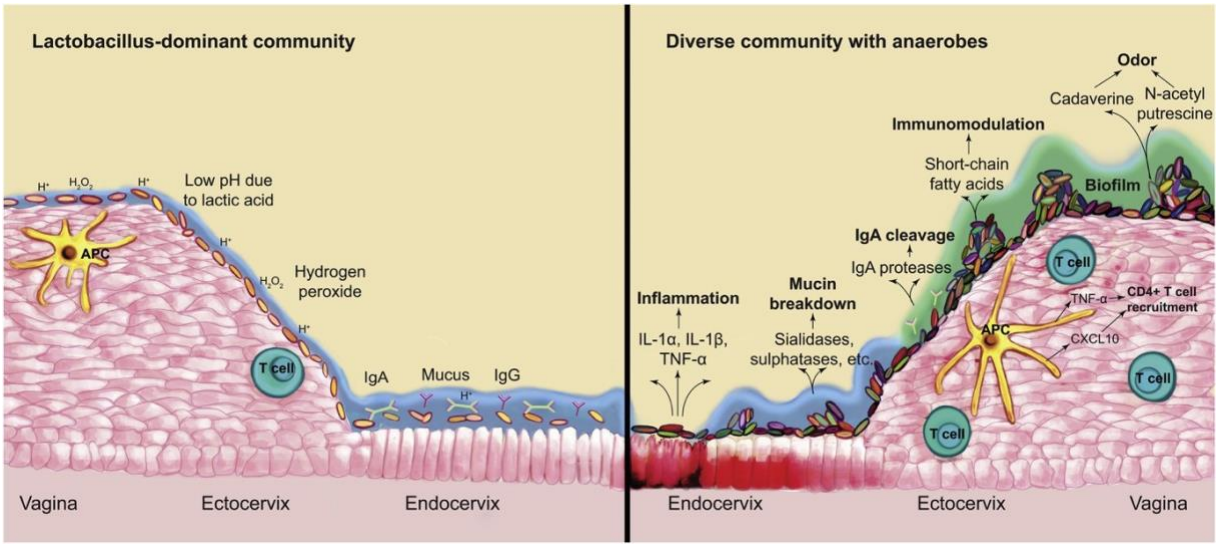


Figure 1.4: *Lactobacillus*-Dominated Vaginal Microbiome versus Diverse community dominated by anaerobes. In a vaginal microbiome dominated by *Lactobacillus* species, such as *L. crispatus*, these bacteria produce lactic acid, bacteriocins, and hydrogen peroxide (H_2O_2). These substances help protect against bacterial vaginosis (BV)-related bacteria and other infections. (**b)** In a BV-associated microbiome, bacteria like *Gardnerella* induce inflammation in the vaginal tract and form a biofilm on vaginal epithelial cells. This biofilm can increase antibiotic resistance and make probiotic treatments less effective. BV-related bacteria also produce short-chain fatty acids (SCFAs) that raise vaginal pH. Additionally, the breakdown of amino acids and mucosal proteins by these bacteria results in the production of amines and a thinner mucosal layer.

1.2.3.4 Impact of Vaginal Dysbiosis on STIs

Vaginal dysbiosis is strongly linked to an elevated risk of sexually transmitted infections (STIs), including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, herpes simplex virus type 2 (HSV-2), and human papillomavirus (HPV) (148, 149). While minimal genital inflammation is crucial for clearing infections, excessive inflammation can disrupt the epithelial barrier, facilitating the entry and establishment of HIV and STI-associated pathogens. For instance, HSV increases the risk of HIV acquisition by causing mucosal barrier disruption through lesions, which subsequently induces chemokine production and the recruitment of CCR5-expressing immune cells (118). Research has shown a significant association between increased HIV acquisition and pre-existing HSV-2 infection in high-risk Kenyan women (150). HPV, the most prevalent viral STI in the female genital tract, is linked to various genotypes associated with cervical cancer and genital warts (151). Co-infections of HIV with other STIs, such as gonorrhoea and chlamydia, exacerbate infectiousness and transmission risks, complicating HIV treatment efforts (152). Furthermore, South African women infected with *C trachomatis*, *N gonorrhoeae*, and *T. vaginalis* have been observed to exhibit elevated levels of genital tract pro-inflammatory cytokines, including IL-12, IL-10, TNF-α, and IFN-γ (124). High STI prevalence has been documented among high-risk African women in clinical studies such as the Partners PrEP trial (33), as well as in the prematurely terminated VOICE (41) and FEM-PrEP (40) trials. These findings underscore the severe consequences of STIs, particularly their role in undermining HIV prevention efforts by compromising mucosal barriers in adolescent girls and young women.

1.2.5 MANAGEMENT OF VAGINAL DYSBIOSIS

BV, though not classified as an infection per se, is typically managed with either oral or topical metronidazole (MTZ) or clindamycin as the standard of care. Emerging experimental alternatives include antiseptic treatments, pro- and prebiotics, and vaginal microbiome transplantation (153-157). BV treatment decreases the abundance of BV-associated microbes; however, re-colonisation by *Lactobacillus* species (excluding *L. iners*) is often slow or unlikely. Consequently, recurrence rates remain high, reaching up to 60% within six months of treatment (156, 158, 159). Several studies report a modest reduction in BV-associated bacterial abundances post-treatment, inversely correlated with increased Lactobacillus levels

(160-162). While antibiotics effectively and rapidly alleviate symptoms, even in non-recurrent cases, they are associated with significant recurrence rates (163). One factor contributing to this recurrence is the presence of antibiotic-tolerant biofilms, which harbour reservoirs of persister cells (156, 163). Antibiotic treatments often fail to eradicate these biofilms and can lead to further vaginal dysbiosis and complications such as candidiasis (164). Additionally, clindamycin use may trigger *Clostridium difficile* infections, antibiotic-associated diarrhoea, and gastrointestinal side effects (165, 166). Resistance to metronidazole among *Gardnerella* strains has also been documented (167, 168).

Probiotic and antibiotic combinations have shown promise in improving BV cure rates without adverse events (169, 170). However, trial heterogeneity—differences in probiotic strains, dosages, durations, delivery routes, and study populations—complicates interpretations. The first clinical trial (2005) tested 100 mg clindamycin ovules for three days, followed by tampons containing 10^8 CFU of *L. gasseri*, *L. casei rhamnosus*, and *L. fermentum*, compared to a placebo. No significant difference was observed in cure rates (56% vs. 62%, placebo) (171). BV cure was assessed by Amsel criteria and did not show a significant difference between the two groups (56% probiotic versus 62% control group). In a second study 400mg of metronidazole orally for seven days followed by vaginal pessary containing *L.acidophilus* (10^7 CFU) for twelve days also did not affect BV cure (72% vs. 73% in controls) (172). A seven day course of two percent vaginal clindamycin cream directly followed by vaginal capsules containing *L.gasseri* and *L.rhamnosus* (each 10^8- 10^9 CFU) for ten days with the probiotic treatment repeated after each menstruation for ten days over three menstrual cycles resulted in slightly greater BV cure rates in the probiotic group compared to the controls (65% versus 46%) (173). Similar results were seen in studies that administrated 500mg metronidazole orally for seven days followed by oral administration of *L. rhamnosus* and *L. reuteri* (each 10^9 CFU) for 30 days (88% versus 40% in the control group) (174) or when administering 300mg oral clindamycin for seven days followed by vaginal capsules containing 10^9 CFU of *L. casei rhamnosus* for seven days (83% vs. 35% in controls) (175). The latest clinical trial testing combination treatment showed that administration of one probiotic capsule containing *L. plantarum*, *L. fermentum* and *L. gasseri* (10^8 CFU in total) together with metronidazole twice daily for 10 days followed by one probiotic capsule daily for 10 days lengthened the relapse of BV symptoms up to 51% compared to placebo (176).

Antiseptic treatments for BV have been less well studied than antibiotics but they are similar with regard to their effectiveness and are also associated with high recurrence rates and deterioration of the healthy vaginal microbiome (104, 177). Studies of probiotics and prebiotics are generally inconclusive: they appear not to result in lasting benefits (156). Few probiotic trials have been conducted on *L. crispatus* with promising results. Bohbot et al., (2018) observed that the time to first recurrence was shorter, with a higher recurrence rate in the placebo group compared to the treatment group (178). Additionally, recent double-blinded clinical trials involving *L. crispatus* CTV-05 (LACTIN-V) have been shown to significantly reduce recurrence rates of bacterial vaginosis (BV). Clinical trials found that women who received LACTIN-V after antibiotic treatment experienced longer BV-free intervals compared to placebo groups. Specifically, LACTIN-V improved the establishment of *L. crispatus* in the vaginal microbiome, a key factor in preventing recurrence (179, 180). Another approach that has been considered is vaginal microbiome transplantation after a course of antibiotic treatment had a promising effect in one study of five patients, but relied on successful antimicrobial treatment prior to the transplantation (181). An alternative or additional treatment of interest is the investigational drug candidate, PM-477 (currently known as BNT331), an endolysin which has previously shown its efficacy and specificity against *G. vaginalis* strains in a series of *in vitro* and *ex vivo* experiments (182, 183). In addition, PM477 leads to efficient killing of several other BV-associated species in vitro, such as *L. iners*, *A. vaginae* and *P. bivia*, but not the beneficial lactobacillus strains (*L. crispatus*, *L. jensenii* or *L. gasseri*). However, the argument is that PM477 cannot work alone but in combination with other antibiotics or probiotics/ live biotherapeutic products to effectively eliminate BV-associated microbiomes and prevent a recurrence. Therefore, there is a need for a combination treatment that can eliminate or disrupt biofilm, treat BVAB, and probiotics that can colonise FGT and establish *L. crispatus*-dominated communities to improve women's genital health.

1.2.6. VAGINAL MICROBIOTA and AGE

Women undergo many physiological challenges, which are largely driven by hormonal fluctuations. In every stage of life, from birth, including puberty, reproductive age, and menopause, the composition of Vaginal microbiota is mainly impacted (184). In the early stages

of life, the mother's vaginal microbiota predominantly colonises the exterior and interior surfaces of a child delivered vaginally (185). At birth, infants experience increased oestrogen levels, influenced by their mother's circulating hormones. However, these levels decline in the first weeks of life, and they generally remain low until puberty begins. Fluctuations in oestrogen play a key role in shaping the composition of vaginal microbiota. During periods of low oestrogen, such as before puberty, the vaginal epithelium may be thinner, and glycogen production, which is an energy source for beneficial bacteria like lactobacilli, may be diminished. As oestrogen levels increase with the start of puberty, the vaginal environment becomes more conducive to the growth of lactobacilli, aiding in the development of a balanced and protective vaginal microbiota (186). During the reproductive years between 20–30 and 31–43 years of age, the VMB of women is deemed to be stable. However, when Menopause occurs, due to the decline in estrogen levels. For women aged between 55-75 years, it is believed that *Lactobacilli* are either absent or present in low concentrations. Although several studies suggested a stable bacterial community during puberty and reproductive age, it should be noted that the studies were not performed in Africa. Therefore, there is a need to use 16S rRNA metagenomic sequencing to determine longitudinal changes in vaginal bacterial communities to determine normal or healthy vaginal microbiota in African populations.

1.2.7. THE ROLE OF VAGINAL MICROBIOTA IN MODULATING PREP EFFICACY

Tenofovir (TFV), a widely used antiretroviral drug in pre-exposure prophylaxis (PrEP) strategies, has been extensively studied for its efficacy. Emerging evidence suggests that the vaginal microbiota plays a pivotal role in modulating the protective efficacy of vaginally administered TFV. Initial in vitro studies by Pyles et al., (2014) demonstrated that non-*L. iners* species enhanced the protective effect of topical TFV against HIV in vaginal epithelial co-cultures, whereas *L. iners* and highly diverse microbiota diminished its efficacy (187). Klatt et al., (2017), in a retrospective analysis of the CAPRISA 004 trial, further corroborated this, showing reduced TFV levels in women with *G. vaginalis*-dominated microbial communities. They found that TFV was metabolized rapidly in the presence of *G. vaginalis*, while *Lactobacillus* species did not metabolize the drug. Women with *Lactobacillus*-dominant microbiota achieved 61% HIV prevention efficacy, compared to only 18% in those with diverse vaginal microbiota (53). These findings were supported by studies showing higher cervical tissue TFV levels in women with a Nugent score ≤3 (indicating *Lactobacillus* dominance)

compared to those with bacterial vaginosis (BV) dominated by *G. vaginalis* (43). Further investigations by Cheu et al., (2020) revealed that BV-associated bacteria (BVAB) could extensively metabolize TFV, dapivirine (DPV), and tenofovir alafenamide (TAF), whereas *Lactobacillus*-dominant samples did not metabolize these drugs (188). These findings emphasize the importance of understanding vaginal microbiota's drug metabolism propensity before offering PrEP. However, contrasting evidence from the ASPIRE study indicated no significant difference in DPV efficacy between women with and without BV, suggesting that DPV efficacy might be microbiome-independent (189).

In contrast to vaginally applied TFV, oral PrEP (TDF/FTC) efficacy appears unaffected by the vaginal microbiome. Heffron et al., (2017), in a secondary analysis of the Partners PrEP study, demonstrated that oral PrEP efficacy remained consistent across women with normal microbiota, intermediate microbiota, and BV, as assessed by Nugent scoring (44, 190). Additionally, we recently conducted a longitudinal study in black South African women that showed that oral PrEP did not impact temporal shifts in vaginal microbiota composition (190). These findings suggest that the route of PrEP administration (oral versus topical) may influence drug metabolism and efficacy (42). Overall, these studies highlight the need for further research to optimize PrEP delivery methods and evaluate the impact of diverse vaginal microbiota on PrEP efficacy, particularly as new PrEP drugs are developed for market introduction. Longitudinal studies are critical to determining whether distinct vaginal microbial communities influence the performance of current and emerging PrEP formulations.

1.2.8. THE IMPACT OF INTRAVAGINAL PRACTICES ON VAGINAL MICROBIOME

Various vaginal practices have been reported in Southern African countries, including the insertion of herbal or non-herbal preparations, antiseptic cleansing, and the use of drying agents, all of which can disrupt the vaginal microbiome (191). One notable practice, commonly referred to as "dry sex," involves inserting drying agents such as herbs or dry cloth into the vagina to create tightness, aiming to enhance male sexual pleasure. However, for women, this practice can have adverse effects, including disruption of the vaginal microbiome, an increased risk of bacterial vaginosis (BV), and a heightened susceptibility to HIV. Additionally, the friction caused by these practices can lead to tearing of the delicate vaginal mucosa, increasing

HIV acquisition risk. The use of harmful chemicals further exacerbates the problem by causing inflammation, lesions, and alterations to the natural vaginal pH, all of which compromise vaginal health and increase vulnerability to infections (12, 191, 192).

A meta-analysis pooling data from Indonesia, Thailand, Mozambique, and South Africa found that women engaging in intravaginal practices had higher rates of BV and HIV acquisition compared to those who only cleansed with water (64). Furthermore, Alcaide et al. demonstrated that douching is linked to increased mucosal inflammation, potentially facilitating HIV acquisition and transmission (193). High prevalence rates of these practices have been self-reported in various African communities; for example, a Kenyan study among sex workers reported 86% to 100% engagement in vaginal practices (194). In South Africa, a household survey in KwaZulu-Natal Province found 90% of women reported using some form of vaginal practice, whereas in Cape Town, Western Cape Province, significantly lower prevalence rates (26%–29%) were observed (195-197). Beyond intravaginal practices, many women use a range of feminine hygiene products to cleanse the genital area, aiming to remove sweat, odor, or discharge. Crann et al., (2018) found that women using such products had three times the odds of experiencing adverse health conditions such as BV, urinary tract infections (UTIs), or sexually transmitted infections (STIs) (198, 199).

Practices such as douching, using soaps or antiseptics, and inserting herbal or chemical products can alter the vaginal pH, damage the epithelial barrier, and increase the likelihood of inflammation. Studies have linked vaginal washing with a higher likelihood of detecting BV-associated bacteria, including *BVAB1*, *A. vaginae*, *G. vaginalis*, and *Megasphaera* spp. (200). A meta-analysis of 13 cohort studies involving 14,874 women found that intravaginal practices, such as using cloth, drying products, and soap, were associated with increased HIV acquisition and the development of bacterial vaginosis. Intermediate vaginal flora and BV were also linked to a higher risk of HIV acquisition (64). Six studies involving 6,503 women examined the relationship between intravaginal practices and the development of bacterial vaginosis (BV) or disturbance of vaginal flora (201-206). Unadjusted analyses from three studies suggested a significant association between intravaginal douching or washing and BV in women with normal vaginal flora at baseline (summary effect: 1.20, 95% CI 1.03–1.40, I^2 0%) (204-206).

However, this association was attenuated in adjusted analyses that included different studies (summary effect: 1.12, 95% CI 0.82–1.54, I^2 49.2%) (202-204, 206). In Zimbabwe and Uganda, the insertion of substances to dry or tighten the vagina before intercourse was linked to incident BV in univariable analyses, but this association was not significant in multivariable analyses (201). Only two studies (201, 202) investigated the impact of intravaginal practices on trichomoniasis and candidiasis. Hawes et al. found no significant association between douching and trichomoniasis or candidiasis (201), while the insertion of substances to dry or tighten the vagina was associated with vaginal candidiasis in univariable analyses (202). While the specific impact of these practices on the vaginal microbiome is not fully understood, these findings highlight the need for longitudinal studies to explore their temporal associations and causality, as well as their potential influence on PrEP efficacy and HIV acquisition. Given the vagina's natural self-cleansing properties, education and awareness about safe practices are essential to safeguard women's reproductive health.

Reference List

1. (UNAIDS) JUNPoHA. 2023. *Global HIV & AIDS statistics — Fact sheet*
2. UNAIDS. 2016. *Global AIDS update - 2016*, Joint United Nations Programme on HIV/AIDS (UNAIDS), Geneva, Switzerland
3. Department of Health. 2015. *The 2013 National Antenatal Sentinel HIV Prevalence Survey South Africa*, Department of Health, Pretoria
4. Kharsany AB, Frohlich JA, Yende-Zuma N, Mahlase G, Samsunder N, Dellar RC, Zuma-Mkhonza M, Abdool Karim SS, Karim QA. 2015. Trends in HIV prevalence in pregnant women in rural South Africa. *J Acquir Immune Defic Syndrome*
5. Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany ABM, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mlotshwa M, Morris L, Taylor D, on behalf of the CAPRISA 004 Trial Group. 2010. Effectiveness and Safety of Tenofovir Gel, an Antiretroviral Microbicide, for the Prevention of HIV Infection in Women. *Science* 329: 1168-74
6. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, Padavattan N, Desai C, Droit L, Moodley A, Dong M, Chen Y, Ismail N, Ndung'u T, Ghebremichael MS, Wesemann DR, Mitchell C, Dong KL, Huttenhower C, Walker BD, Virgin HW, Kwon DS. 2017. Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. *Immunity* 46: 29-37
7. Masson L, Passmore JA, Liebenberg LJ, Werner L, Baxter C, Arnold KB, Williamson C, Little F, Mansoor LE, Naranbhai V, Lauffenburger DA, Ronacher K, Walzl G, Garrett NJ, Williams BL, Couto-Rodriguez M, Hornig M, Lipkin WI, Grobler A, ..., Abdool Karim SS. 2015. Genital Inflammation and the Risk of HIV Acquisition in Women. *Clin Infect Dis*
8. Jewkes RK, Dunkle K, Nduna M, Shai N. 2010. Intimate partner violence, relationship power inequity, and incidence of HIV infection in young women in South Africa: a cohort study. *The Lancet* 376: 41-8
9. Masson L, Mlisana K, Little F, Werner L, Mkhize NN, Ronacher K, Gamieldien H, Williamson C, Mckinnon LR, Walzl G. 2014. Defining genital tract cytokine signatures of sexually transmitted infections and bacterial vaginosis in women at high risk of HIV infection: a cross-sectional study. *Sexually transmitted infections* 90: 580-7

10. Dunkle KL, Jewkes RK, Brown HC, Gray GE, McIntryre JA, Harlow SD. 2004. Gender-based violence, relationship power, and risk of HIV infection in women attending antenatal clinics in South Africa. *Lancet* 363: 1415-21
11. Stirling M, Rees H, Kasedde S, Hankins C. 2008. Introduction: Addressing the vulnerability of young women and girls to stop the HIV epidemic in southern Africa. *AIDS* 22 Suppl 4: S1-3
12. Chersich MF, Rees HV. 2008. Vulnerability of women in southern Africa to infection with HIV: biological determinants and priority health sector interventions. *AIDS* 22 Suppl 4: S27-40
13. Abdool Karim Q, Kharsany AB, Leask K, Ntombela F, Humphries H, Frohlich JA, Samsunder N, Grobler A, Dellar R, Abdool Karim SS. 2014. Prevalence of HIV, HSV-2 and pregnancy among high school students in rural KwaZulu-Natal, South Africa: a bio-behavioural cross-sectional survey. *Sex Transm Infect* 90: 620-6
14. Boily M-C, Baggaley RF, Wang L, Masse B, White RG, Hayes RJ, Alary M. 2009. Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies. *The Lancet infectious diseases* 9: 118-29
15. Freeman EE, Weiss HA, Glynn JR, Cross PL, Whitworth JA, Hayes RJ. 2006. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *Aids* 20: 73-83
16. Critchlow CW, Wölner-Hanssen P, Eschenbach DA, Kiviat NB, Koutsky LA, Stevens CE, Holmes KK. 1995. Determinants of cervical ectopia and of cervicitis: age, oral contraception, specific cervical infection, smoking, and douching. *American journal of obstetrics and gynecology* 173: 534-43
17. UNICEF. UNAIDS.(1999). *Children orphaned by AIDS. Frontline responses from eastern and southern Africa*
18. Quinn TC, Overbaugh J. 2005. HIV/AIDS in women: an expanding epidemic. *Science* 308: 1582-3
19. Jewkes R, Dunkle K, Nduna M, Levin J, Jama N, Khuzwayo N, Koss M, Puren A, Duvvury N. 2006. Factors associated with HIV sero-status in young rural South African women: connections between intimate partner violence and HIV. *International journal of epidemiology* 35: 1461-8

20. Hilber AM, Chersich MF, van de Wijgert JHHM, Rees H, Temmerman M. 2007. Vaginal practices, microbicides and HIV: what do we need to know? *Sexually Transmitted Infections* 83: 505-8
21. Alcaide ML, Rodriguez VJ, Fischl MA, Jones DL, Weiss SM. 2017. Addressing intravaginal practices in women with HIV and at-risk for HIV infection, a mixed methods pilot study. *Int J Womens Health* 9: 123-32
22. van De Wijgert JH, Mason PR, Gwanzura L, Mbizvo MT, Chirenje ZM, Iliff V, Shiboski S, Padian NS. 2000. Intravaginal practices, vaginal flora disturbances, and acquisition of sexually transmitted diseases in Zimbabwean women. *J Infect Dis* 181: 587-94
23. Sandala L, Lurie P, Sunkutu MR, Chani EM, Hudes ES, Hearst N. 1995. 'Dry sex' and HIV infection among women attending a sexually transmitted diseases clinic in Lusaka, Zambia. *Aids* 9 Suppl 1: S61-8
24. Glynn JR, Biraro S, Weiss HA. 2009. Herpes simplex virus type 2: a key role in HIV incidence. *AIDS* 23: 1595-8
25. Houlihan CF, Larke NL, Watson-Jones D, Smith-McCune KK, Shiboski S, Gravitt PE, Smith JS, Kuhn L, Wang C, Hayes R. 2012. Human papillomavirus infection and increased risk of HIV acquisition. A systematic review and meta-analysis. *AIDS* 26: 2211-22
26. Centers for Diseases Control and Prevention. 2013. *HIV Transmission Risk. Available from: http://www.cdc.gov/hiv/policies/law/risk.html. [Accessed: 29 January 2016]*
27. Fish EN. 2008. The X-files in immunity: sex-based differences predispose immune responses. *Nat Rev Immunol* 8: 737-44
28. Prodger JL, Gray R, Kigozi G, Nalugoda F, Galiwango R, Hirbod T, Wawer M, Hofer SO, Sewankambo N, Serwadda D, Kaul R. 2012. Foreskin T-cell subsets differ substantially from blood with respect to HIV co-receptor expression, inflammatory profile, and memory status. *Mucosal Immunol* 5: 121-8
29. Klot JF, Auerbach JD, Berry MR. 2013. Sexual violence and HIV transmission: summary proceedings of a scientific research planning meeting. *Am J Reprod Immunol* 69 Suppl 1: 5-19
30. Masson L, Passmore JA, Liebenberg LJ, Werner L, Baxter C, Arnold KB, Williamson C, Little F, Mansoor LE, Naranbhai V, Lauffenburger DA, Ronacher K, Walzl G, Garrett

NJ, Williams BL, Couto-Rodriguez M, Hornig M, Lipkin WI, Grobler A, ..., Abdool Karim SS. 2015. Genital inflammation and the risk of HIV acquisition in women. *Clin Infect Dis* 61: 260-9

31. Lennard K, Dabee S, Barnabas SL, Havyarimana E, Blakney A, Jaumdally SZ, Botha G, Mkhize NN, Bekker LG, Lewis DA, Gray G, Mulder N, Passmore JS, Jaspan HB. 2018. Microbial Composition Predicts Genital Tract Inflammation and Persistent Bacterial Vaginosis in South African Adolescent Females. *Infect Immun* 86
32. Masson L, Passmore J-AS, Liebenberg LJ, Werner L, Baxter C, Arnold KB, Williamson C, Little F, Mansoor LE, Naranbhai V. 2015. Genital inflammation and the risk of HIV acquisition in women. *Clinical Infectious Diseases*: civ298
33. Baeten JM, Donnell D, Ndase P, Mugo NR, Campbell JD, Wangisi J, Tappero JW, Bukusi EA, Cohen CR, Katabira E, Ronald A, Tumwesigye E, Were E, Fife KH, Kiarie J, Farquhar C, John-Stewart G, Kakia A, Odoyo J, Mucunguzi A, Nakku-Joloba E, Twesigye R, Ngure K, Apaka C, Tamooh H, Gabona F, Mujugira A, Panteleeff D, Thomas KK, Kidoguchi L, Krows M, Revall J, Morrison S, Haugen H, Emmanuel-Ogier M, Ondrejcek L, Coombs RW, Frenkel L, Hendrix C, Bumpus NN, Bangsberg D, Haberer JE, Stevens WS, Lingappa JR, Celum C. 2012. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med* 367: 399-410
34. Choopanya K, Martin M, Suntharasamai P, Sangkum U, Mock PA, Leethochawalit M, Chiamwongpaet S, Kitisin P, Natrujirote P, Kittimunkong S, Chuachoowong R, Gvetadze RJ, McNicholl JM, Paxton LA, Curlin ME, Hendrix CW, Vanichseni S. 2013. Antiretroviral prophylaxis for HIV infection in injecting drug users in Bangkok, Thailand (the Bangkok Tenofovir Study): a randomised, double-blind, placebo-controlled phase 3 trial. *The Lancet* 381: 2083-90
35. Grant RM, Lama JR, Anderson PL, McMahan V, Liu AY, Vargas L, Goicochea P, Casapia M, Guanira-Carranza JV, Ramirez-Cardich ME, Montoya-Herrera O, Fernandez T, Veloso VG, Buchbinder SP, Chariyalertsak S, Schechter M, Bekker LG, Mayer KH, Kallas EG, Amico KR, Mulligan K, Bushman LR, Hance RJ, Ganoza C, Defechereux P, Postle B, Wang F, McConnell JJ, Zheng JH, Lee J, Rooney JF, Jaffe HS, Martinez AI, Burns DN, Glidden DV. 2010. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N Engl J Med* 363: 2587-99

36. McCormack S, Dunn D, On behalf of the PROUD Study Group. 2015. Pragmatic Open-Label Randomised Trial of Preexposure Prophylaxis: The PROUD Study. [Abstract 22LB]. In *Conference on Retroviruses and Opportunistic Infections (CROI), 23-26 February*. Seattle, Washington

37. Molina J-M, Capitant C, Spire B, Pialoux G, Chidiac C, Charreau I, Tremblay C, Meyer L, Delfraissy J-F, On behalf of the ANRS Ipergay Study Group. 23-26 February 2015. On Demand PrEP With Oral TDF-FTC in MSM: Results of the ANRS Ipergay Trial. [Abstract 23LB]. In *Conference on Retroviruses and Opportunistic Infections (CROI)*. Seattle, Washington

38. Thigpen MC, Kebaabetswe PM, Paxton LA, Smith DK, Rose CE, Segolodi TM, Henderson FL, Pathak SR, Soud FA, Chillag KL, Mutanhaurwa R, Chirwa LI, Kasonde M, Abebe D, Buliva E, Gvetadze RJ, Johnson S, Sukalac T, Thomas VT, Hart C, Johnson JA, Malotte CK, Hendrix CW, Brooks JT. 2012. Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana. *N Engl J Med* 367: 423-34

39. Rees H., Delany-Moretlwe S.A., Lombard C, Baron D, Panchia R, Myer L, Schwartz JL, Doncel GF, Gray G, On behalf of the FACTS 001 Study Team. 23-26 February 2015. FACTS 001 Phase III Trial of Pericoital Tenofovir 1% Gel for HIV Prevention in Women. [Abstract 26LB]. In *Conference on Retroviruses and Opportunistic Infections (CROI)*. Seattle, Washington

40. Van Damme L, Corneli A, Ahmed K, Agot K, Lombaard J, Kapiga S, Malahleha M, Owino F, Manongi R, Onyango J, Temu L, Monedi MC, Mak'Oketch P, Makanda M, Reblin I, Makatu SE, Saylor L, Kiernan H, Kirkendale S, ..., for the FEM-PrEP Study Group. 2012. Preexposure Prophylaxis for HIV Infection among African Women. *New England Journal of Medicine* 367: 411-22

41. Marrazzo JM, Ramjee G, Richardson BA, Gomez K, Mgodi N, Nair G, Palanee T, Nakabiito C, van der Straten A, Noguchi L, Hendrix CW, Dai JY, Ganesh S, Mkhize B, Taljaard M, Parikh UM, Piper J, Mâsse B, Grossman C, ..., Chirenje ZM. 2015. Tenofovir-Based Preexposure Prophylaxis for HIV Infection among African Women. *New England Journal of Medicine* 372: 509-18

42. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noel-Romas L, Grobler A, Westmacott G, Xie IY, Butler J, Mansoor L, McKinnon LR, Passmore JS, Abdool Karim Q, Abdool

Karim SS, Burgener AD. 2017. Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356: 938-45

43. Hillier SL, Meyn LA, Bunge A, Austin M, Moncla BJ, Dezzutti CS, Schwartz J, Marzinke MA, Hendrix C, Rohan LC. 2017. *IMPACT OF VAGINAL MICROBIOTA ON GENITAL TISSUE AND PLASMA CONCENTRATIONS OF TENOFOVIR*. Presented at Conference on Retroviruses and Opportunistic Infections, Seattle, WA
44. Heffron R, McClelland RS, Balkus JE, Celum C, Cohen CR, Mugo N, Bukusi E, Donnell D, Lingappa J, Kiarie J, Fiedler T, Munch M, Fredricks DN, Baeten JM, Partners Pr EPST. 2017. Efficacy of oral pre-exposure prophylaxis (PrEP) for HIV among women with abnormal vaginal microbiota: a post-hoc analysis of the randomised, placebo-controlled Partners PrEP Study. *Lancet HIV*
45. Birdthistle I, Kwaro D, Shahmanesh M, Baisley K, Khagayi S, Chimbindi N, Kamire V, Mthiyane N, Gourlay A, Dreyer J. 2021. Evaluating the impact of DREAMS on HIV incidence among adolescent girls and young women: a population-based cohort study in Kenya and South Africa. *PLoS medicine* 18: e1003837
46. Vandormael A, Cuadros D, Kim H-Y, Bärnighausen T, Tanser F. 2020. The state of the HIV epidemic in rural KwaZulu-Natal, South Africa: a novel application of disease metrics to assess trajectories and highlight areas for intervention. *International journal of epidemiology* 49: 666-75
47. Kharsany AB, Cawood C, Khanyile D, Lewis L, Grobler A, Puren A, Govender K, George G, Beckett S, Samsunder N. 2018. Community-based HIV prevalence in KwaZulu-Natal, South Africa: results of a cross-sectional household survey. *The Lancet HIV* 5: e427-e37
48. Shattock RJ, Moore JP. 2003. Inhibiting sexual transmission of HIV-1 infection. *Nature Reviews Microbiology* 1: 25-34
49. Yeaman GR, Asin S, Weldon S, Demian DJ, Collins JE, Gonzalez JL, Wira CR, Fanger MW, Howell AL. 2004. Chemokine receptor expression in the human ectocervix: implications for infection by the human immunodeficiency virus-type I. *Immunology* 113: 524-33
50. Haynes BF, Shattock RJ. 2008. Critical issues in mucosal immunity for HIV-1 vaccine development. *Journal of Allergy and Clinical Immunology* 122: 3-9

51. Masson L, Passmore JA, Liebenberg LJ, Werner L, Baxter C, Arnold KB, Williamson C, Little F, Mansoor LE, Naranbhai V, Lauffenburger DA, Ronacher K, Walzl G, Garrett NJ, Williams BL, Couto-Rodriguez M, Hornig M, Lipkin WI, Grobler A, Abdool Karim Q, Abdool Karim SS. 2015. Genital inflammation and the risk of HIV acquisition in women. *Clin Infect Dis* 61: 260-9

52. Arnold KB, Burgener A, Birse K, Romas L, Dunphy LJ, Shahabi K, Abou M, Westmacott GR, McCorrister S, Kwatampora J. 2016. Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells. *Mucosal immunology* 9: 194

53. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noël-Romas L, Grobler A, Westmacott G, Xie IY, Butler J. 2017. Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356: 938-45

54. Chen L, Jha P, Stirling B, Sgaier SK, Daid T, Kaul R, Nagelkerke N, Investigators ISoHA. 2007. Sexual risk factors for HIV infection in early and advanced HIV epidemics in sub-Saharan Africa: systematic overview of 68 epidemiological studies. *PloS one* 2: e1001

55. De Oliveira T, Kharsany AB, Gräf T, Cawood C, Khanyile D, Grobler A, Puren A, Madurai S, Baxter C, Karim QA. 2017. Transmission networks and risk of HIV infection in KwaZulu-Natal, South Africa: a community-wide phylogenetic study. *The lancet HIV* 4: e41-e50

56. Maughan-Brown B, George G, Beckett S, Evans M, Lewis L, Cawood C, Khanyile D, Kharsany AB. 2018. HIV risk among adolescent girls and young women in age-disparate partnerships: evidence from KwaZulu-Natal, South Africa. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 78: 155-62

57. Pettifor A, O'Brien K, MacPhail C, Miller WC, Rees H. 2009. Early coital debut and associated HIV risk factors among young women and men in South Africa. *International perspectives on sexual and reproductive health*: 82-90

58. Peltzer K. 2010. Early sexual debut and associated factors among in-school adolescents in eight African countries. *Acta Paediatrica* 99: 1242-7

59. Zuma K, Setswe G, Ketye T, Mzolo T, Rehle T, Mbelle N. 2010. Age at sexual debut: a determinant of multiple partnership among South African youth. *African journal of reproductive health* 14: 47-54

60. Gregson S, Nyamukapa CA, Garnett GP, Mason PR, Zhuwau T, Caraël M, Chandiwana SK, Anderson RM. 2002. Sexual mixing patterns and sex-differentials in teenage exposure to HIV infection in rural Zimbabwe. *The Lancet* 359: 1896-903

61. SS AK, Nkomokazi J. 1991. Sexual behaviour and knowledge of AIDS among urban black mothers. Implications for AIDS intervention programmes. *South African medical journal= Suid-Afrikaanse tydskrif vir geneeskunde* 80: 340-3

62. Simbayi LC, Chauveau J, Shisana O. 2004. Behavioural responses of South African youth to the HIV/AIDS epidemic: a nationwide survey. *AIDS care* 16: 605-18

63. Jewkes RK, Levin JB, Penn-Kekana LA. 2003. Gender inequalities, intimate partner violence and HIV preventive practices: findings of a South African cross-sectional study. *Social science & medicine* 56: 125-34

64. Low N, Chersich MF, Schmidlin K, Egger M, Francis SC, HHM van de Wijgert J, Hayes RJ, Baeten JM, Brown J, Delany-Moretlwe S. 2011. Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. *PLoS medicine* 8: e1000416

65. Cohen MS, Chen YQ, McCauley M, Gamble T, Hosseinipour MC, Kumarasamy N, Hakim JG, Kumwenda J, Grinsztejn B, Pilotto JH, Godbole SV, Mehendale S, Chariyalertsak S, Santos BR, Mayer KH, Hoffman IF, Eshleman SH, Piwowar-Manning E, Wang L, ..., Fleming TR. 2011. Prevention of HIV-1 Infection with Early Antiretroviral Therapy. *N Engl J Med* 365: 493-505

66. U.S. Food and Drug Administration. 2012. FDA approves first drug for reducing the risk of sexually acquired HIV infection. Available from : http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm312210.htm [accessed July 19, 2012]. Silver Spring, MD

67. Karim QA, Archary D, Barre-Sinoussi F, Broliden K, Cabrera C, Chiodi F, Fidler SJ, Gengiah TN, Herrera C, Kharsany AB. 2022. Women for science and science for women: Gaps, challenges and opportunities towards optimizing pre-exposure prophylaxis for HIV-1 prevention. *Frontiers in immunology* 13: 1055042

68. Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany AB, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mlotshwa M, Morris L, Taylor D. 2010. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* 329: 1168-74

69. Marrazzo JM, Ramjee G, Richardson BA, Gomez K, Mgodi N, Nair G, Palanee T, Nakabiito C, van der Straten A, Noguchi L, Hendrix CW, Dai JY, Ganesh S, Mkhize B, Taljaard M, Parikh UM, Piper J, Masse B, Grossman C, Rooney J, Schwartz JL, Watts H, Marzinke MA, Hillier SL, McGowan IM, Chirenje ZM, Team VS. 2015. Tenofovir-based preexposure prophylaxis for HIV infection among African women. *N Engl J Med* 372: 509-18

70. Delany-Moretlwe S, Lombard C, Baron D, Bekker L-G, Nkala B, Ahmed K, Sebe M, Brumskine W, Nchabeleng M, Palanee-Philips T. 2018. Tenofovir 1% vaginal gel for prevention of HIV-1 infection in women in South Africa (FACTS-001): a phase 3, randomised, double-blind, placebo-controlled trial. *The Lancet infectious diseases* 18: 1241-50

71. Nel A, van Niekerk N, Kapiga S, Bekker L-G, Gama C, Gill K, Kamali A, Kotze P, Louw C, Mabude Z. 2016. Safety and efficacy of a dapivirine vaginal ring for HIV prevention in women. *New England Journal of Medicine* 375: 2133-43

72. Delany-Moretlwe S, Hughes JP, Bock P, Ouma SG, Hunidzarira P, Kalonji D, Kayange N, Makhema J, Mandima P, Mathew C. 2022. Cabotegravir for the prevention of HIV-1 in women: results from HPTN 084, a phase 3, randomised clinical trial. *The Lancet* 399: 1779-89

73. Rees H D-MS, Baron D, Lombard C, Gray G, Myer L, Panchia R, Schwartz J, Doncel G. 2015. *FACTS 001 Phase III Trial of Pericoital Tenofovir 1% Gel for HIV Prevention in Women [Abstract 26LB]*. Presented at Conference on Retroviruses and Opportunistic Infections (CROI), Seattle, Washington

74. Bekker L-G, Das M, Abdool Karim Q, Ahmed K, Batting J, Brumskine W, Gill K, Harkoo I, Jaggernath M, Kigozi G. 2024. Twice-yearly lenacapavir or daily F/TAF for HIV prevention in cisgender women. *New England Journal of Medicine* 391: 1179-92

75. Kelley CF, Acevedo-Quiñones M, Agwu AL, Avihingsanon A, Benson P, Blumenthal J, Brinson C, Brites C, Cahn P, Cantos VD. 2024. Twice-Yearly Lenacapavir for HIV Prevention in Men and Gender-Diverse Persons. *New England Journal of Medicine*

76. Van Damme L, Corneli A, Ahmed K, Agot K, Lombaard J, Kapiga S, Malahleha M, Owino F, Manongi R, Onyango J, Temu L, Monedi MC, Mak'Oketch P, Makanda M, Reblin I, Makatu SE, Saylor L, Kiernan H, Kirkendale S, Wong C, Grant R, Kashuba A, Nanda K, Mandala J, Fransen K, Deese J, Crucitti T, Mastro TD, Taylor D, Group FE-PS. 2012. Preexposure prophylaxis for HIV infection among African women. *N Engl J Med* 367: 411-22

77. Mabaso M, Sokhela Z, Mohlabane N, Chibi B, Zuma K, Simbayi L. 2018. Determinants of HIV infection among adolescent girls and young women aged 15–24 years in South Africa: a 2012 population-based national household survey. *BMC public health* 18: 1-7

78. Ramjee G, Daniels B. 2013. Women and HIV in sub-Saharan Africa. *AIDS research and therapy* 10: 1-9

79. McKinnon LR, Liebenberg LJ, Yende-Zuma N, Archary D, Ngcapu S, Sivro A, Nagelkerke N, Garcia Lerma JG, Kashuba AD, Masson L. 2018. Genital inflammation undermines the effectiveness of tenofovir gel in preventing HIV acquisition in women. *Nature medicine* 24: 491-6

80. Hayes R, Watson-Jones D, Celum C, van de Wijgert J, Wasserheit J. 2010. Treatment of sexually transmitted infections for HIV prevention: end of the road or new beginning? *AIDS* 24 Suppl 4: S15-26

81. Sha BE, Zariffard MR, Wang QJ, Chen HY, Bremer J, Cohen MH, Spear GT. 2005. Female genital-tract HIV load correlates inversely with Lactobacillus species but positively with bacterial vaginosis and Mycoplasma hominis. *J Infect Dis* 191: 25-32

82. van de Wijgert JH, Morrison CS, Cornelisse PG, Munjoma M, Moncada J, Awio P, Wang J, Van der Pol B, Chipato T, Salata RA, Padian NS. 2008. Bacterial vaginosis and vaginal yeast, but not vaginal cleansing, increase HIV-1 acquisition in African women. *J Acquir Immune Defic Syndr* 48: 203-10

83. Buve A, Jespers V, Crucitti T, Fichorova RN. 2014. The vaginal microbiota and susceptibility to HIV. *AIDS* 28: 2333-44

84. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, Padavattan N, Ismail N, Moodley A, Sabatini ME, Ghebremichael MS, Nusbaum C, Huttenhower C, Virgin HW, Ndung'u T, Dong KL, Walker BD, Fichorova RN, Kwon DS. 2015. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* 42: 965-76

85. Petrova MI, van den Broek M, Balzarini J, Vanderleyden J, Lebeer S. 2013. Vaginal microbiota and its role in HIV transmission and infection. *FEMS Microbiol Rev* 37: 762-92

86. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. 2011. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 108 Suppl 1: 4680-7

87. Jespers V, Crucitti T, Menten J, Verhelst R, Mwaura M, Mandaliya K, Ndayisaba GF, Delany-Moretlwe S, Verstraelen H, Hardy L, Buve A, van de Wijgert J, Vaginal Biomarkers Study G. 2014. Prevalence and correlates of bacterial vaginosis in different sub-populations of women in sub-Saharan Africa: a cross-sectional study. *PLoS One* 9: e109670

88. Kenyon C, Colebunders R, Crucitti T. 2013. The global epidemiology of bacterial vaginosis: a systematic review. *Am J Obstet Gynecol* 209: 505-23

89. Kumar N, Behera B, Sagiri SS, Pal K, Ray SS, Roy S. 2011. Bacterial vaginosis: Etiology and modalities of treatment-A brief note. *J Pharm Bioallied Sci* 3: 496-503

90. Macklaim JM, Fernandes AD, Di Bella JM, Hammond JA, Reid G, Gloor GB. 2013. Comparative meta-RNA-seq of the vaginal microbiota and differential expression by Lactobacillus iners in health and dysbiosis. *Microbiome* 1: 12

91. Shipitsyna E, Roos A, Datcu R, Hallen A, Fredlund H, Jensen JS, Engstrand L, Unemo M. 2013. Composition of the vaginal microbiota in women of reproductive age--sensitive and specific molecular diagnosis of bacterial vaginosis is possible? *PLoS One* 8: e60670

92. Kenyon C, Colebunders R, Crucitti T. 2013. The global epidemiology of bacterial vaginosis: a systematic review. *American journal of obstetrics and gynecology* 209: 505-23

93. Cohen CR, Lingappa JR, Baeten JM, Ngayo MO, Spiegel CA, Hong T, Donnell D, Celum C, Kapiga S, Delany S. 2012. Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: a prospective cohort analysis among African couples. *PLoS Med* 9: e1001251

94. Verhelst R, Verstraelen H, Claeys G, Verschraegen G, Delanghe J, Van Simaey L, De Ganck C, Temmerman M, Vaneechoutte M. 2004. Cloning of 16S rRNA genes amplified from normal and disturbed vaginal microflora suggests a strong association between Atopobium vaginae, Gardnerella vaginalis and bacterial vaginosis. *BMC microbiology* 4: 1-11

95. Machado D, Castro J, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Cerca N. 2016. Bacterial vaginosis biofilms: challenges to current therapies and emerging solutions. *Frontiers in microbiology* 6: 1528

96. Fredricks DN, Fiedler TL, Thomas KK, Oakley BB, Marrazzo JM. 2007. Targeted PCR for detection of vaginal bacteria associated with bacterial vaginosis. *Journal of clinical microbiology* 45: 3270-6

97. Meltzer MC, Desmond RA, Schwebke JR. 2008. Association of Mobiluncus curtisii with recurrence of bacterial vaginosis. *Sexually transmitted diseases* 35: 611

98. Hilbert DW, Smith WL, Paulish-Miller TE, Chadwick SG, Toner G, Mordechai E, Adelson ME, Sobel JD, Gygax SE. 2016. Utilization of molecular methods to identify prognostic markers for recurrent bacterial vaginosis. *Diagnostic microbiology and infectious disease* 86: 231-42

99. Bradshaw CS, Tabrizi S, Fairley CK, Morton AN, Rudland E, Garland SM. 2006. The association of Atopobium vaginae and Gardnerella vaginalis with bacterial vaginosis and recurrence after oral metronidazole therapy. *The Journal of infectious diseases* 194: 828-36

100. Castro J, Cerca N. 2015. BV and non-BV associated Gardnerella vaginalis establish similar synergistic interactions with other BV-associated microorganisms in dual-species biofilms. *Anaerobe* 36: 56-9

101. Machado A, Cerca N. 2015. Influence of biofilm formation by Gardnerella vaginalis and other anaerobes on bacterial vaginosis. *The Journal of infectious diseases* 212: 1856-61

102. Muzny CA, Taylor CM, Swords WE, Tamhane A, Chattopadhyay D, Cerca N, Schwebke JR. 2019. An updated conceptual model on the pathogenesis of bacterial vaginosis. *The Journal of infectious diseases* 220: 1399-405
103. Swidsinski A, Mendling W, Loening-Baucke V, Ladhoff A, Swidsinski S, Hale LP, Lochs H. 2005. Adherent biofilms in bacterial vaginosis. *Obstetrics & Gynecology* 106: 1013-23
104. Swidsinski A, Loening-Baucke V, Mendling W, Dörffel Y, Schilling J, Halwani Z, Jiang X-f, Verstraelen H, Swidsinski S. 2014. Infection through structured polymicrobial Gardnerella biofilms (StPM-GB). *Histology and histopathology* 29: 567-87
105. Castro J, Machado D, Cerca N. 2019. Unveiling the role of Gardnerella vaginalis in polymicrobial bacterial vaginosis biofilms: the impact of other vaginal pathogens living as neighbors. *The ISME journal* 13: 1306-17
106. Castro J, Jefferson KK, Cerca N. 2018. Innate immune components affect growth and virulence traits of bacterial-vaginosis-associated and non-bacterial-vaginosis-associated Gardnerella vaginalis strains similarly. *Pathogens and disease* 76: fty089
107. Murphy K, Mitchell CM. 2016. The interplay of host immunity, environment and the risk of bacterial vaginosis and associated reproductive health outcomes. *The Journal of infectious diseases* 214: S29-S35
108. Machado A, Jefferson KK, Cerca N. 2013. Interactions between Lactobacillus crispatus and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. *Int J Mol Sci* 14: 12004-12
109. Vaneechoutte M. 2017. The human vaginal microbial community. *Research in microbiology* 168: 811-25
110. Schwebke JR, Muzny CA, Josey WE. 2014. Role of Gardnerella vaginalis in the pathogenesis of bacterial vaginosis: a conceptual model. *The Journal of infectious diseases* 210: 338-43
111. Vaneechoutte M, Guschin A, Van Simaey L, Gansemans Y, Van Nieuwerburgh F, Cools P. 2019. Emended description of Gardnerella vaginalis and description of Gardnerella leopoldii sp. nov., Gardnerella piotii sp. nov. and Gardnerella swidsinskii sp. nov., with delineation of 13 genomic species within the genus Gardnerella. *International Journal of Systematic and Evolutionary Microbiology* 69: 679-87

112. Hill JE, Albert AY, Group VR. 2019. Resolution and Cooccurrence Patterns of Gardnerella leopoldii, G. swidsinskii, G. piotii, and G. vaginalis within the Vaginal Microbiome. *Infection and Immunity* 87: 10.1128/iai. 00532-19

113. Shipitsyna E, Krysanova A, Khayrullina G, Shalepo K, Savicheva A, Guschin A, Unemo M. 2019. Quantitation of all four Gardnerella vaginalis clades detects abnormal vaginal microbiota characteristic of bacterial vaginosis more accurately than putative G. vaginalis sialidase A gene count. *Molecular diagnosis & therapy* 23: 139-47

114. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO. 2011. Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences* 108: 4680-7

115. McClelland RS, Lingappa JR, Srinivasan S, Kinuthia J, John-Stewart GC, Jaoko W, Richardson BA, Yuhas K, Fiedler TL, Mandaliya KN. 2018. Evaluation of the association between the concentrations of key vaginal bacteria and the increased risk of HIV acquisition in African women from five cohorts: a nested case-control study. *The Lancet infectious diseases* 18: 554-64

116. Lennard K, Dabee S, Barnabas SL, Havyarimana E, Blakney A, Jaumdally SZ, Botha G, Mkhize NN, Bekker L-G, Lewis DA. 2018. Microbial composition predicts genital tract inflammation and persistent bacterial vaginosis in South African adolescent females. *Infection and immunity* 86: 10.1128/iai. 00410-17

117. Jespers V, Kyongo J, Joseph S, Hardy L, Cools P, Crucitti T, Mwaura M, Ndayisaba G, Delany-Moretlwe S, Buyze J. 2017. A longitudinal analysis of the vaginal microbiota and vaginal immune mediators in women from sub-Saharan Africa. *Scientific Reports* 7: 1-13

118. Shannon B, Gajer P, Yi T, Ma B, Humphrys M, Thomas-Pavanel J, Chieza L, Janakiram P, Saunders M, Tharao W. 2017. Distinct effects of the cervicovaginal microbiota and herpes simplex type 2 infection on female genital tract immunology. *The Journal of infectious diseases* 215: 1366-75

119. Mueller A, Strange PG. 2004. The chemokine receptor, CCR5. *The international journal of biochemistry & cell biology* 36: 35-8

120. Doerflinger SY, Throop AL, Herbst-Kralovetz MM. 2014. Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human

vaginal epithelia in a species-specific manner. *The Journal of infectious diseases* 209: 1989-99

121. Anton L, Sierra L, DeVine A, Barila G, Heiser L, Brown A, Elovitz M. 2018. Common cervicovaginal microbial supernatants alter cervical epithelial function: mechanisms by which Lactobacillus crispatus contributes to cervical health. Front Microbiol 9: 2181.
122. Selhorst P, Masson L, Ismail SD, Samsunder N, Garrett N, Mansoor LE, Abdool Karim Q, Abdool Karim SS, Passmore J-AS, Williamson C. 2017. Cervicovaginal inflammation facilitates acquisition of less infectious HIV variants. *Clinical Infectious Diseases* 64: 79-82
123. Sharkey DJ, Macpherson AM, Tremellen KP, Mottershead DG, Gilchrist RB, Robertson SA. 2012. TGF-β mediates proinflammatory seminal fluid signaling in human cervical epithelial cells. *The Journal of Immunology* 189: 1024-35
124. Masson L, Mlisana K, Little F, Werner L, Mkhize NN, Ronacher K, Gamieldien H, Williamson C, Mckinnon LR, Walzl G. 2014. Defining genital tract cytokine signatures of sexually transmitted infections and bacterial vaginosis in women at high risk of HIV infection: a cross-sectional study. *Sex Transm Infect*: sextrans-2014-051601
125. Bunker JJ, Erickson SA, Flynn TM, Henry C, Koval JC, Meisel M, Jabri B, Antonopoulos DA, Wilson PC, Bendelac A. 2017. Natural polyreactive IgA antibodies coat the intestinal microbiota. *Science* 358
126. Mantis NJ, Rol N, Corthesy B. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol* 4: 603-11
127. Kawamoto S, Maruya M, Kato LM, Suda W, Atarashi K, Doi Y, Tsutsui Y, Qin H, Honda K, Okada T, Hattori M, Fagarasan S. 2014. Foxp3(+) T cells regulate immunoglobulin a selection and facilitate diversification of bacterial species responsible for immune homeostasis. *Immunity* 41: 152-65
128. Donaldson GP, Ladinsky MS, Yu KB, Sanders JG, Yoo BB, Chou WC, Conner ME, Earl AM, Knight R, Bjorkman PJ, Mazmanian SK. 2018. Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science* 360: 795-800
129. Ansaldo E, Slayden LC, Ching KL, Koch MA, Wolf NK, Plichta DR, Brown EM, Graham DB, Xavier RJ, Moon JJ, Barton GM. 2019. Akkermansia muciniphila induces intestinal adaptive immune responses during homeostasis. *Science* 364: 1179-84

130. Hansen IS, Baeten DLP, den Dunnen J. 2019. The inflammatory function of human IgA. *Cell Mol Life Sci* 76: 1041-55

131. Xu M, Pokrovskii M, Ding Y, Yi R, Au C, Harrison OJ, Galan C, Belkaid Y, Bonneau R, Littman DR. 2018. c-MAF-dependent regulatory T cells mediate immunological tolerance to a gut pathobiont. *Nature* 554: 373-7

132. Hebbandi Nanjundappa R, Ronchi F, Wang J, Clemente-Casares X, Yamanouchi J, Sokke Umeshappa C, Yang Y, Blanco J, Bassolas-Molina H, Salas A, Khan H, Slattery RM, Wyss M, Mooser C, Macpherson AJ, Sycuro LK, Serra P, McKay DM, McCoy KD, Santamaria P. 2017. A Gut Microbial Mimic that Hijacks Diabetogenic Autoreactivity to Suppress Colitis. *Cell* 171: 655-67 e17

133. Yang Y, Torchinsky MB, Gobert M, Xiong H, Xu M, Linehan JL, Alonzo F, Ng C, Chen A, Lin X, Sczesnak A, Liao JJ, Torres VJ, Jenkins MK, Lafaille JJ, Littman DR. 2014. Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. *Nature* 510: 152-6

134. Fluckiger A, Daillere R, Sassi M, Sixt BS, Liu P, Loos F, Richard C, Rabu C, Alou MT, Goubet AG, Lemaitre F, Ferrere G, Derosa L, Duong CPM, Messaoudene M, Gagne A, Joubert P, De Sordi L, Debarbieux L, ..., Zitvogel L. 2020. Cross-reactivity between tumor MHC class I-restricted antigens and an enterococcal bacteriophage. *Science* 369: 936-42

135. Mendoza JL, Fischer S, Gee MH, Lam LH, Brackenridge S, Powrie FM, Birnbaum M, McMichael AJ, Garcia KC, Gillespie GM. 2020. Interrogating the recognition landscape of a conserved HIV-specific TCR reveals distinct bacterial peptide cross-reactivity. *Elife* 9

136. Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR. 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139: 485-98

137. Hegazy AN, West NR, Stubbington MJT, Wendt E, Suijker KIM, Datsi A, This S, Danne C, Campion S, Duncan SH, Owens BMJ, Uhlig HH, McMichael A, Oxford IBDCI, Bergthaler A, Teichmann SA, Keshav S, Powrie F. 2017. Circulating and Tissue-Resident CD4(+) T Cells With Reactivity to Intestinal Microbiota Are Abundant

in Healthy Individuals and Function Is Altered During Inflammation. *Gastroenterology* 153: 1320-37 e16

138. Hand TW, Dos Santos LM, Bouladoux N, Molloy MJ, Pagan AJ, Pepper M, Maynard CL, Elson CO, 3rd, Belkaid Y. 2012. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. *Science* 337: 1553-6

139. Wang Z, Friedrich C, Hagemann SC, Korte WH, Goharani N, Cording S, Eberl G, Sparwasser T, Lochner M. 2014. Regulatory T cells promote a protective Th17-associated immune response to intestinal bacterial infection with C. rodentium. *Mucosal Immunol* 7: 1290-301

140. Nutsch K, Chai JN, Ai TL, Russler-Germain E, Feehley T, Nagler CR, Hsieh CS. 2016. Rapid and Efficient Generation of Regulatory T Cells to Commensal Antigens in the Periphery. *Cell Rep* 17: 206-20

141. Zegarra-Ruiz DF, Kim DV, Norwood K, Kim M, Wu WH, Saldana-Morales FB, Hill AA, Majumdar S, Orozco S, Bell R, Round JL, Longman RS, Egawa T, Bettini ML, Diehl GE. 2021. Thymic development of gut-microbiota-specific T cells. *Nature* 594: 413-7

142. Zhao Q, Duck LW, Huang F, Alexander KL, Maynard CL, Mannon PJ, Elson CO. 2020. CD4(+) T cell activation and concomitant mTOR metabolic inhibition can ablate microbiota-specific memory cells and prevent colitis. *Sci Immunol* 5

143. Nagayama M, Yano T, Atarashi K, Tanoue T, Sekiya M, Kobayashi Y, Sakamoto H, Miura K, Sunada K, Kawaguchi T, Morita S, Sugita K, Narushima S, Barnich N, Isayama J, Kiridooshi Y, Shiota A, Suda W, Hattori M, ..., Honda K. 2020. TH1 cell-inducing Escherichia coli strain identified from the small intestinal mucosa of patients with Crohn's disease. *Gut Microbes* 12: 1788898

144. Tan TG, Sefik E, Geva-Zatorsky N, Kua L, Naskar D, Teng F, Pasman L, Ortiz-Lopez A, Jupp R, Wu HJ, Kasper DL, Benoist C, Mathis D. 2016. Identifying species of symbiont bacteria from the human gut that, alone, can induce intestinal Th17 cells in mice. *Proc Natl Acad Sci U S A* 113: E8141-E50

145. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, ..., Honda K. 2013. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 500: 232-6

146. Rebbapragada A, Howe K, Wachihi C, Pettengell C, Sunderji S, Huibner S, Ball TB, Plummer FA, Jaoko W, Kaul R. 2008. Bacterial vaginosis in HIV-infected women induces reversible alterations in the cervical immune environment. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 49: 520-2
147. Kyongo JK, Jespers V, Goovaerts O, Michiels J, Menten J, Fichorova RN, Crucitti T, Vanham G, Ariën KK. 2012. Searching for lower female genital tract soluble and cellular biomarkers: defining levels and predictors in a cohort of healthy Caucasian women.
148. Brotman RM. 2011. Vaginal microbiome and sexually transmitted infections: an epidemiologic perspective. *The Journal of clinical investigation* 121: 4610-7
149. Anahtar MN, Gootenberg DB, Mitchell CM, Kwon DS. 2018. Cervicovaginal Microbiota and Reproductive Health: The Virtue of Simplicity. *Cell Host Microbe* 23: 159-68
150. Masese L, Baeten JM, Richardson BA, Bukusi E, John-Stewart G, Graham SM, Shafi J, Kiarie J, Overbaugh J, McClelland RS. 2015. Changes in the contribution of genital tract infections to HIV acquisition among Kenyan high-risk women from 1993 to 2012. *Aids* 29: 1077-85
151. Zur Hausen H. 2009. Papillomaviruses in the causation of human cancers—a brief historical account. *Virology* 384: 260-5
152. Kalichman SC, Pellowski J, Turner C. 2011. Prevalence of sexually transmitted co-infections in people living with HIV/AIDS: systematic review with implications for using HIV treatments for prevention. *Sexually transmitted infections* 87: 183-90
153. Bradshaw CS, Vodstrcil LA, Hocking JS, Law M, Pirotta M, Garland SM, De Guingand D, Morton AN, Fairley CK. 2013. Recurrence of bacterial vaginosis is significantly associated with posttreatment sexual activities and hormonal contraceptive use. *Clin Infect Dis* 56: 777-86
154. Swidsinski A, Loening-Baucke V, Mendling W, Dörffel Y, Schilling J, Halwani Z, Jiang XF, Verstraelen H, Swidsinski S. 2014. Infection through structured polymicrobial Gardnerella biofilms (StPM-GB). *Histol Histopathol* 29: 567-87
155. Swidsinski A, Loening-Baucke V, Swidsinski S, Verstraelen H. 2015. Polymicrobial Gardnerella biofilm resists repeated intravaginal antiseptic treatment in a subset of

women with bacterial vaginosis: a preliminary report. *Archives of Gynecology and Obstetrics* 291: 605-9

156. Bradshaw CS, Sobel JD. 2016. Current Treatment of Bacterial Vaginosis—Limitations and Need for Innovation. *The Journal of Infectious Diseases* 214: S14-S20

157. Lev-Sagie A, Goldman-Wohl D, Cohen Y, Dori-Bachash M, Leshem A, Mor U, Strahilevitz J, Moses AE, Shapiro H, Yagel S, Elinav E. 2019. Vaginal microbiome transplantation in women with intractable bacterial vaginosis. *Nature Medicine* 25: 1500-4

158. Bostwick DG, Woody J, Hunt C, Budd W. 2016. Antimicrobial resistance genes and modelling of treatment failure in bacterial vaginosis: clinical study of 289 symptomatic women. *Journal of Medical Microbiology* 65: 377-86

159. Barrons R, Tassone D. 2008. Use of Lactobacillus probiotics for bacterial genitourinary infections in women: a review. *Clin Ther* 30: 453-68

160. Joag V, Obila O, Gajer P, Scott MC, Dizzell S, Humphrys M, Shahabi K, Huibner S, Shannon B, Tharao W, Mureithi M, Oyugi J, Kimani J, Kaushic C, Ravel J, Anzala O, Kaul R. 2019. Impact of Standard Bacterial Vaginosis Treatment on the Genital Microbiota, Immune Milieu, and Ex Vivo Human Immunodeficiency Virus Susceptibility. *Clin Infect Dis* 68: 1675-83

161. Verwijs MC, Agaba SK, Darby AC, van de Wijgert J. 2019. Impact of Oral Metronidazole Treatment on the Vaginal Microbiota and Correlates of Treatment Failure. *Am J Obstet Gynecol*

162. Mtshali A, San JE, Osman F, Garrett N, Balle C, Giandhari J, Onywera H, Mngomezulu K, Mzobe G, de Oliveira T, Rompalo A, Mindel A, Abdool Karim SS, Ravel J, Passmore J-AS, Abdool Karim Q, Jaspan HB, Liebenberg LJP, Ngcapu S. 2021. Temporal Changes in Vaginal Microbiota and Genital Tract Cytokines Among South African Women Treated for Bacterial Vaginosis. *Frontiers in Immunology* 12

163. Swidsinski A, Mendling W, Loening-Baucke V, Swidsinski S, Dörffel Y, Scholze J, Lochs H, Verstraelen H. 2008. An adherent Gardnerella vaginalis biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *American journal of obstetrics and gynecology* 198: 97. e1-. e6

164. Sobel JD. 2007. Vulvovaginal candidosis. *The Lancet* 369: 1961-71

165. Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, Ubeda C, Xavier J, Pamer EG. 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. *Infection and immunity* 80: 62-73

166. Vardakas KZ, Trigkidis KK, Boukouvala E, Falagas ME. 2016. Clostridium difficile infection following systemic antibiotic administration in randomised controlled trials: a systematic review and meta-analysis. *International journal of antimicrobial agents* 48: 1-10

167. Petrina MA, Cosentino LA, Rabe LK, Hillier SL. 2017. Susceptibility of bacterial vaginosis (BV)-associated bacteria to secnidazole compared to metronidazole, tinidazole and clindamycin. *Anaerobe* 47: 115-9

168. Schwebs T, Kieninger A-K, Podpera Tisakova L, Oberbauer V, Berdaguer R, Mtshali A, Mzobe G, Rompalo A, Mindel A, Letsoalo M. 2024. Evaluation of Metronidazole Resistance of Vaginal Swab Isolates from South African Women Treated for Bacterial Vaginosis. *Antibiotics* 13: 1217

169. Mastromarino P, Vitali B, Mosca L. 2013. Bacterial vaginosis: a review on clinical trials with probiotics. *New microbiológica* 36: 229-38

170. Homayouni A, Bastani P, Ziyadi S, Mohammad-Alizadeh-Charandabi S, Ghalibaf M, Mortazavian AM, Mehrabany EV. 2014. Effects of probiotics on the recurrence of bacterial vaginosis: a review. *Journal of lower genital tract disease* 18: 79-86

171. Eriksson K, Carlsson B, Forsum U, Larsson P. 2005. A double-blind treatment study of bacterial vaginosis with normal vaginal lactobacilli after an open treatment with vaginal clindamycin ovules. *Acta dermato-venereologica* 85: 42-6

172. Bradshaw CS, Pirotta M, De Guingand D, Hocking JS, Morton AN, Garland SM, Fehler G, Morrow A, Walker S, Vodstrcil LA. 2012. Efficacy of oral metronidazole with vaginal clindamycin or vaginal probiotic for bacterial vaginosis: randomised placebo-controlled double-blind trial. *PloS one* 7: e34540

173. Larsson P-G, Stray-Pedersen B, Ryttig KR, Larsen S. 2008. Human lactobacilli as supplementation of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study. *BMC women's health* 8: 1-8

174. Anukam K, Osazuwa E, Ahonkhai I, Ngwu M, Osemene G, Bruce AW, Reid G. 2006. Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14: randomized, double-blind, placebo controlled trial. *Microbes and Infection* 8: 1450-4

175. Petricevic L, Witt A. 2008. The role of Lactobacillus casei rhamnosus Lcr35 in restoring the normal vaginal flora after antibiotic treatment of bacterial vaginosis. *BJOG: An International Journal of Obstetrics & Gynaecology* 115: 1369-74

176. Heczko PB, Tomusiak A, Adamski P, Jakimiuk AJ, Stefański G, Mikołajczyk-Cichońska A, Suda-Szczurek M, Strus M. 2015. Supplementation of standard antibiotic therapy with oral probiotics for bacterial vaginosis and aerobic vaginitis: a randomised, double-blind, placebo-controlled trial. *BMC Women's Health* 15: 1-12

177. Swidsinski A, Loening-Baucke V, Swidsinski S, Verstraelen H. 2015. Polymicrobial Gardnerella biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis: a preliminary report. *Archives of gynecology and obstetrics* 291: 605-9

178. Bohbot J, Daraï E, Bretelle F, Brami G, Daniel C, Cardot J. 2018. Efficacy and safety of vaginally administered lyophilized Lactobacillus crispatus IP 174178 in the prevention of bacterial vaginosis recurrence. *Journal of gynecology obstetrics and human reproduction* 47: 81-6

179. Ngugi BM, Hemmerling A, Bukusi EA, Kikuvi G, Gikunju J, Shiboski S, Fredricks DN, Cohen CR. 2011. Effects of bacterial vaginosis-associated bacteria and sexual intercourse on vaginal colonization with the probiotic Lactobacillus crispatus CTV-05. *Sexually transmitted diseases* 38: 1020-7

180. Cohen CR, Wierzbicki MR, French AL, Morris S, Newmann S, Reno H, Green L, Miller S, Powell J, Parks T. 2020. Randomized trial of lactin-V to prevent recurrence of bacterial vaginosis. *New England Journal of Medicine* 382: 1906-15

181. Lev-Sagie A, Goldman-Wohl D, Cohen Y, Dori-Bachash M, Leshem A, Mor U, Strahilevitz J, Moses AE, Shapiro H, Yagel S, Elinav E. 2019. Vaginal microbiome transplantation in women with intractable bacterial vaginosis. *Nat Med* 25: 1500-4

182. Landlinger C, Oberbauer V, Podpera Tisakova L, Schwebs T, Berdaguer R, Van Simaey L, Vaneechoutte M, Corsini L. 2022. Preclinical Data on the Gardnerella-Specific Endolysin PM-477 Indicate Its Potential to Improve the Treatment of Bacterial

Vaginosis through Enhanced Biofilm Removal and Avoidance of Resistance. *Antimicrob Agents Chemother* 66: e0231921

183. Castro J, Sousa LGV, França Â, Podpera Tisakova L, Corsini L, Cerca N. 2022. Exploiting the Anti-Biofilm Effect of the Engineered Phage Endolysin PM-477 to Disrupt In Vitro Single- and Dual-Species Biofilms of Vaginal Pathogens Associated with Bacterial Vaginosis. *Antibiotics* 11: 558
184. Smith SB, Ravel J. 2017. The vaginal microbiota, host defence and reproductive physiology. *The Journal of physiology* 595: 451-63
185. Martín R, Langa S, Reviriego C, Jimínez E, Marín ML, Xaus J, Fernández L, Rodríguez JM. 2003. Human milk is a source of lactic acid bacteria for the infant gut. *The Journal of pediatrics* 143: 754-8
186. Bidlingmaier F, Wagner-Barnack M, Butenandt O, Knorr D. 1973. Plasma estrogens in childhood and puberty under physiologic and pathologic conditions. *Pediatric research* 7: 901-7
187. Pyles RB, Vincent KL, Baum MM, Elsom B, Miller AL, Maxwell C, Eaves-Pyles TD, Li G, Popov VL, Nusbaum RJ. 2014. Cultivated vaginal microbiomes alter HIV-1 infection and antiretroviral efficacy in colonized epithelial multilayer cultures. *PloS one* 9: e93419
188. Cheu RK, Gustin AT, Lee C, Schifanella L, Miller CJ, Ha A, Kim C, Rodriguez VJ, Fischl M, Burgener AD. 2020. Impact of vaginal microbiome communities on HIV antiretroviral-based pre-exposure prophylaxis (PrEP) drug metabolism. *PLoS pathogens* 16: e1009024
189. Baeten JM, Palanee-Phillips T, Brown ER, Schwartz K, Soto-Torres LE, Govender V, Mgodi NM, Matovu Kiweewa F, Nair G, Mhlanga F. 2016. Use of a vaginal ring containing dapivirine for HIV-1 prevention in women. *New England Journal of Medicine* 375: 2121-32
190. Mazibuko-Motau N, Sobia P, Xu J, Elsherbini JA, San JE, Lewis L, Mtshali A, Mzobe G, Ntuli L, Abdool Karim SS. 2022. Vaginal microbial shifts are unaffected by oral pre-exposure prophylaxis in South African women. *Scientific Reports* 12: 16187
191. Maleche A, Day E. 2011. Working Paper for the Third Meeting of the Technical Advisory Group of the Global Commission on HIV and the Law, 7-9 July 2011. *Traditional cultural practices and HIV: reconciling culture and human rights*

192. Hilber AM, Francis SC, Chersich M, Scott P, Redmond S, Bender N, Miotti P, Temmerman M, Low N. 2010. Intravaginal practices, vaginal infections and HIV acquisition: systematic review and meta-analysis. *PloS one* 5: e9119

193. Alcaide ML, Rodriguez VJ, Brown MR, Pallikkuth S, Arheart K, Martinez O, Roach M, Fichorova RN, Jones DL, Pahwa S. 2017. High levels of inflammatory cytokines in the reproductive tract of women with BV and engaging in intravaginal douching: a cross-sectional study of participants in the women interagency HIV study. *AIDS research and human retroviruses* 33: 309-17

194. Hassan WM, Lavreys L, Chohan V, Richardson BA, Mandaliya K, Ndinya-Achola JO, Kiarie J, Jaoko W, Holmes KK, McClelland RS. 2007. Associations between intravaginal practices and bacterial vaginosis in Kenyan female sex workers without symptoms of vaginal infections. *Sexually transmitted diseases* 34: 384-8

195. Smit J, Chersich M, Beksinska M, Kunene B, Manzini N, Hilber AM, Scorgie F. 2011. Prevalence and self-reported health consequences of vaginal practices in KwaZulu-Natal, South Africa: findings from a household survey. *Tropical Medicine & International Health* 16: 245-56

196. Gallo MF, Sharma A, Bukusi EA, Njoroge B, Nguti R, Jamieson DJ, Bell AJ, Eschenbach DA. 2010. Intravaginal practices among female sex workers in Kibera, Kenya. *Sexually transmitted infections* 86: 318-22

197. Myer L, Denny L, De Souza M, Wright Jr TC, Kuhn L. 2006. Distinguishing the temporal association between women's intravaginal practices and risk of human immunodeficiency virus infection: a prospective study of South African women. *American Journal of Epidemiology* 163: 552-60

198. Chen Y, Bruning E, Rubino J, Eder SE. 2017. Role of female intimate hygiene in vulvovaginal health: Global hygiene practices and product usage. *Women's Health* 13: 58-67

199. Crann SE, Cunningham S, Albert A, Money DM, O'Doherty KC. 2018. Vaginal health and hygiene practices and product use in Canada: a national cross-sectional survey. *BMC women's health* 18: 1-8

200. Sabo MC, Balkus JE, Richardson BA, Srinivasan S, Kimani J, Anzala O, Schwebke J, Feidler TL, Fredricks DN, McClelland RS. 2019. Association between vaginal washing and vaginal bacterial concentrations. *PLoS One* 14: e0210825

201. van De Wijgert JH, Morrison CS, Cornelisse PG, Munjoma M, Moncada J, Awio P, Wang J, Van der Pol B, Chipato T, Salata RA. 2008. Bacterial vaginosis and vaginal yeast, but not vaginal cleansing, increase HIV-1 acquisition in African women. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 48: 203-10

202. Hawes SE, Hillier SL, Benedetti J, Stevens CE, Koutsky LA, Wølner-Hanssen P, Holmes KK. 1996. Hydrogen peroxide—producing lactobacilli and acquisition of vaginal infections. *Journal of Infectious Diseases* 174: 1058-63

203. Hutchinson KB, Kip KE, Ness RB. 2007. Vaginal douching and development of bacterial vaginosis among women with normal and abnormal vaginal microflora. *Sexually transmitted diseases* 34: 671-5

204. McClelland RS, Richardson BA, Graham SM, Masese LN, Gitau R, Lavreys L, Mandaliya K, Jaoko W, Baeten JM, Ndinya-Achola JO. 2008. A prospective study of risk factors for bacterial vaginosis in HIV-1-seronegative African women. *Sexually transmitted diseases* 35: 617-23

205. Myer L, Denny L, Telerant R, de Souza M, Wright Jr TC, Kuhn L. 2005. Bacterial vaginosis and susceptibility to HIV infection in South African women: a nested case-control study. *The Journal of infectious diseases* 192: 1372-80

206. Nagot N, Ouedraogo A, Defer M-C, Vallo R, Mayaud P, Van de Perre P. 2007. Association between bacterial vaginosis and Herpes simplex virus type-2 infection: implications for HIV acquisition studies. *Sexually transmitted infections* 83: 365-8

CHAPTER 2

Vaginal microbial shifts are unaffected by oral pre-exposure prophylaxis in South African women

Pre-exposure prophylaxis (PrEP) strategies, including tenofovir alone or in combination with emtricitabine, have proven to be safe and effective in preventing HIV acquisition. Recent research has proposed that vaginal microbiota may influence the effectiveness of topical tenofovir in HIV prevention, although no such effect has been observed with oral tenofovir-based PrEP. The impact of oral PrEP on vaginal microbiota remains unclear.

Chapter 2 investigates the longitudinal use of tenofovir disoproxil fumarate (TDF) combined with emtricitabine (FTC) or Truvada® on vaginal microbiota composition in women enrolled in the CAPRISA 082 and CAPRISA 084 daily PrEP demonstration projects. The study emphasizes the importance of understanding the role of vaginal microbiota in HIV prevention, particularly for high-risk populations such as South African women. Previous studies suggested that vaginal microbiota might influence the efficacy of topical PrEP (Tenofovir gel), but this study shows that oral PrEP does not alter vaginal microbiome composition. Therefore, oral PrEP can be administered without concerns regarding its impact on vaginal microbial health. The study also highlights the need for concurrent management of bacterial vaginosis (BV) and other vaginal infections in HIV prevention efforts. Future research could focus on identifying geographically relevant *Lactobacillus crispatus* strains that could be used in immunoregulatory products and enhanced BV treatments, potentially improving sexual and reproductive health worldwide.

This study was conceptualised and designed by Dr. Ngcapu and myself. I conducted DNA extraction and PCR pooling of DNA amplicons for sequencing under the supervision of Dr. Sinaye Ngcapu. Dr S Ngcapu and I contributed to the analysis and wrote the manuscript. The manuscript has been published in *Scientific Reports* and can be accessed at https://doi.org/10.1038/s41598-022-20486-z (Article number: 16187, 2022).

scientific reports

Check for updates

OPEN

Vaginal microbial shifts are unaffected by oral pre-exposure prophylaxis in South African women

Noluthando Mazibuko-Motau[1,2,8], Parveen Sobia[1,2,8], Jiawu Xu[3], Joseph Ahmed Elsherbini[3], James E. San[4], Lara Lewis[1], Andile Mtshali[1,2], Gugulethu Mzobe[1,2], Lungelo Ntuli[1,2], Salim S. Abdool Karim[1,5], Leila E. Mansoor[1], Quarraisha Abdool Karim[1,5], Douglas S. Kwon[3,6,7,9], Derseree Archary[1,2,9] & Sinaye Ngcapu[1,2,9]✉

Vaginal microbiota have been shown to be a modifier of protection offered by topical tenofovir in preventing HIV infection in women, an effect not observed with oral tenofovir-based pre-exposure prophylaxis (PrEP). It remains unclear whether PrEP can influence the vaginal microbiota composition. This study investigated the impact of daily oral tenofovir disoproxil fumarate in combination with emtricitabine for PrEP on the vaginal microbiota in South African women. At baseline, *Lactobacillus iners* or *Gardnerella vaginalis* dominant vaginal communities were observed in the majority of participants. In cross sectional analysis, vaginal microbiota were not affected by the initiation and use of PrEP. Longitudinal analysis revealed that *Lactobacillus crispatus*-dominant "cervicotypes 1 (CT1)" communities had high probability of remaining stable in PrEP group, but had a higher probability of transitioning to *L. iners*-dominant CT2 communities in non-PrEP group. *L. iners*-dominant communities were more likely to transition to communities associated with bacterial vaginosis (BV), irrespective of PrEP or antibiotic use. As expected, BV-linked CTs had a higher probability of transitioning to *L. iners* than *L. crispatus* dominant CTs and this shift was not associated with PrEP use.

Over the past decade, studies have demonstrated the efficacy of prophylactic antiretrovirals (ARVs) for the prevention of HIV, with oral tenofovir-based treatment showing markedly reduced rates of HIV infection among men who have sex with men, transgender women, injection drug users, serodiscordant couples, and heterosexual men and women[1–5]. Pre-exposure prophylaxis[6] for men using either daily or intermittent dosing strategies has consistently demonstrated protective efficacy ranging from 44 to 96%[1–5,7]. Despite adherence, PrEP for women in Southern Africa has yielded mixed results, underscoring a plausible role for other biological factors that mitigate PrEP efficacy[6,8,9].

Previous studies have suggested that the vaginal microbiome can significantly modulate the efficacy of topical tenofovir-based PrEP[10,11], an effect not observed with oral tenofovir-based PrEP[12]. In addition, proteomics analyses of vaginal specimens of participants from the CAPRISA 004 1% tenofovir gel trial showed a significantly reduced risk of HIV acquisition in women with *Lactobacillus* dominant compared to non-*Lactobacillus* dominant microbiota[8,10]. Furthermore, the tenofovir levels were reduced in vaginal specimens from women with a *Gardnerella vaginalis* dominant microbial community, suggesting that the depletion of tenofovir was secondary to drug metabolism by *G. vaginalis*[8,10]. These findings are supported by high tenofovir levels in cervical tissue of women with a Nugent score of ≤ 3 (*Lactobacillus* dominant) and reduced tenofovir levels in women with bacterial

[1]Centre for the AIDS Programme of Research in South Africa, 2nd Floor, Doris Duke Medical Research Institute, 719 Umbilo Road, Durban 4041, South Africa. [2]Department of Medical Microbiology, University of KwaZulu-Natal, Durban, South Africa. [3]Ragon Institute of MGH, MIT, and Harvard, Massachusetts General Hospital, Cambridge, MA, USA. [4]KwaZulu-Natal Research Innovation and Sequencing Platform, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa. [5]Department of Epidemiology, Columbia University, New York City, NY, USA. [6]Division of Infectious Diseases, Massachusetts General Hospital, Boston, MA, USA. [7]Harvard Medical School, Boston, MA, USA. [8]These authors contributed equally: Noluthando Mazibuko-Motau and Parveen Sobia. [9]These authors jointly supervised this work: Douglas S. Kwon, Derseree Archary and Sinaye Ngcapu. ✉email: sinaye.ngcapu@caprisa.org

vaginosis (BV) (*G. vaginalis* dominant[11]). However, none of these studies have characterized the potential impact of PrEP on the vaginal microbial communities of healthy women in sub-Saharan Africa. To address this gap, we investigated the impact of longitudinal oral tenofovir disoproxil fumarate + emtricitabine (TDF-FTC) PrEP on the vaginal microbiome of healthy South African women.

Materials and methods

Study design, participants, and sample collection. This is a retrospective study within the CAPRISA 082[13] and CAPRISA 084 (https://www.caprisa.org/Pages/CAPRISAStudies) studies. CAPRISA 082 was a prospective observational cohort study of HIV risk factors and prevention choices in young women aged 18–30 years old from an urban and rural population in KwaZulu-Natal, South Africa. CAPRISA 084 was a PrEP demonstration project that assessed the feasibility, acceptability, uptake and patterns of daily oral TDF/FTC PrEP provided as part of sexual reproductive health services to young women and men (aged 18 years and older) at risk of acquiring HIV in eThekwini, Vulindlela and Umlazi in KwaZulu-Natal, South Africa. Women living with HIV and those who had other factors contraindicated for PrEP use were excluded from both studies. Consenting participants in both studies were followed monthly for up to 3 months, then quarterly for approximately 18 months. Some CAPRISA 082 women agreed to take PrEP while others disagreed, and all were reimbursed for attending clinic visits. All CAPRISA 084 participants were provided with PrEP at all visits and were not reimbursed for attending clinic visits. For this study, we only included cervicovaginal swab samples collected at baseline, 3, and 6 months. At each visit, cervicovaginal swabs collected from the posterior fornix and lateral vaginal walls from each participant were tested for vaginal pH using pH indicator strips, and for *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma genitalium* and *Trichomonas vaginalis* using PCR. Gram staining was performed to confirm a diagnosis of BV based on Nugent score ≥ 7. CAPRISA 082 and 084 participants were treated with regimens recommended in the South African STI treatment guidelines for the following diagnoses: *Chlamydia trachomatis* (azithromycin 1 g oral), *Neisseria gonorrhoeae* (ceftriaxone 250 mg intramuscular and azithromycin 1 g oral), *Trichomonas vaginalis* (metronidazole 2 g orally) and candidiasis (clotrimazole 500 mg pessary and clotrimazole 1% cream). Women who had a Nugent score ≥ 4 were treated with metronidazole (2 g oral single dose). At follow-up visits, CAPRISA 082 participants were managed syndromically, however CAPRISA 084 participants were screened and treated for STIs and BV. All participants provided written informed consent. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC number: BE603/18). All the experimental procedures are in the accordance with the relevant ethical guidance and regulations.

Bacterial DNA extraction, 16S rRNA gene sequencing, taxonomic assignment and CTs assignment. Total nucleic acid was extracted using phenol–chloroform from stored vaginal swabs and the V4 region of the 16S rRNA gene was amplified and sequenced using the Illumina MiSeq as previously described[14]. Divisive Amplicon Denoising Algorithm (DADA) 2[15] was used to filter for quality, trim, and identify inference of amplicon sequence variant (ASV). ASVs were taxonomically classified to genus or higher levels using a Naïve Bayes classification approach[16] and SILVA ribosomal RNA database[15]. The ASVs for *Lactobacillus, Prevotella, Sneathia* and *Mobiluncus* were further refined to the species level with speciateIT (version 1.0, http://ravel-lab.org/speciateIT). A phyloseq object containing a phylogenetic tree, ASV table, taxonomic table, and sample metadata was created using the phyloseq R package[17]. Based on composition and diversity at species-level where possible and genus-level, bacterial communities clustered into 4 basic groups, which we referred to as "cervicotypes"[14].

Statistical analysis. Descriptive statistics for quantitative variables were summarized using medians and interquartile ranges (IQRs) whilst categorical data were summarized with both frequency counts and percentages. Mann–Whitney U test was used to compare continuous variables and Fisher's Exact test for categorical comparisons. α-diversity including Richness (Chao1), evenness (Simpson's E) and phylogenetic diversity (Faith's PD) estimates were calculated using the *R vegan library*. β-diversity between samples was estimated by Bray–Curtis distances. Non-metric Multidimensional scaling (NMDS) was performed to assess differences in taxonomy profiles among samples based on the Bray–Curtis distance of ASV relative abundance using the *metaMDS function* in the *vegan R package*. The transition probabilities are calculated as proportions of the samples (stratified by antibiotic and PrEP use) shifting from one CT to the next. Permutational multivariate analysis of variance (PERMANOVA) was performed with *Adonis* function in the *vegan R package* with a similarity index using 9999 permutations to measure the differences in beta diversity between PrEP and non-PrEP groups. All statistical calculations were carried out using R and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

Data availability. The datasets generated or analysed during the current study are publicly available in the NCBI BioProject respository, (http://www.ncbi.nlm.nih.gov/bioproject/858949) under accession number PRJNA858949.

Results

Study participants. A total of 100 women were included in this sub-study, of whom 60 (60%) were from CAPRISA 082 and 40 (40%) from CAPRISA 084. All CAPRISA 084 participants were provided with PrEP. For CAPRISA 082 participants, 24 (40%) were provided with PrEP and the other 36 (60%) were not (Table 1). Most women were below 30 years (median age 24 years (IQR 21.5–28) of age, but compared with non-PrEP group, PrEP group was older [median age 26.5 years (IQR 22.5–34) vs 22 years (20.5–25); $p = < 0.0001$]. Although majority of women reported having a stable partner (84/100, 84%), the proportion of women in the PrEP group was lower than that of non-PrEP group [49/64 (76.6%) vs 35/36 (97.2%); $p = 0.0087$]. A greater proportion of

Variables		Overall (N = 100) n (%)	PrEP group (N = 64) n (%)	Non-PrEP group (N = 36) n (%)	p-value
Study	CAPRISA 082	60	24	36	
	CAPRISA 084	40	40	0	
Age (years)	Median (IQR)	24 (21.5–28)	26.5 (22.5–34)	22 (20.5–25)	< 0.0001
	≤ 21	25 (25.0)	10 (15.6)	15 (41.7)	0.0073
	22–25	33 (33.0)	17 (26.6)	16 (44.4)	0.0796
	26–30	22 (22.0)	17 (26.6)	5 (13.9)	0.2086
	31–40	8 (12.5)	8 (12.5)	0 (0.0)	0.0479
	40–68	12 (18.8)	12 (18.8)	0 (0.0)	0.0037
Living site	Urban	36 (36.0)	19 (29.7)	17 (47.2)	0.0878
	Rural	64 (64.0)	45 (70.3)	19 (52.8)	
Education	Less than secondary	30 (30.0)	22 (34.4)	8 (22.2)	0.2581
	Secondary or higher	70 (70.0)	42 (65.6)	28 (77.8)	
	Married	12 (12.0)	12 (18.8)	0 (0.0)	0.0037
Partner status	Stable	84 (84.0)	49 (76.6)	35 (97.2)	0.0087
	Casual	5 (5.0)	4 (6.3)	1 (2.8)	0.6514
	No partner	3 (3.0)	3 (4.7)	0 (0.0)	0.5512
Partner HIV positive	Yes	16 (16.0)	16 (26.2)	0 (0.0)	0.0004
	No	61 (61.0)	34 (55.7)	27 (75.0)	0.0815
	Don't know	20 (20.0)	11 (18.0)	9 (25.0)	0.4441
Vaginal sex in last 30 days	Yes	80 (80.0)	49 (77.8)	31 (86.1)	0.4282
Contraceptive	None	27 (27)	16 (25.0)	11 (30.6)	0.5954
	Depo-Provera	49 (49.0)	31 (48.4)	18 (50.0)	1
	Oral contraceptive	4 (4.0)	4 (6.3)	0 (0.0)	0.2936
	Nur-isterate	5 (5.0)	4 (6.3)	1 (2.8)	0.6514
	Implant	10 (10)	6 (9.4)	4 (11.1)	0.7438
	IUCD	3 (3.0)	1 (1.6)	2 (5.6)	0.2935
	Other	2 (2.0)	2 (3.1)	0 (0.0)	0.5345

Table 1. Demographic and behavioural characteristics of study participants at baseline.

participants in the PrEP group were married (12/64, 18.8%) compared to those in the non-PrEP group (0/36; $p = 0.0037$). A high number of women in the PrEP group (16/64, 26.2%) reported having a partner who was HIV positive compared to those in the non-PrEP group (0/36; $p = 0.001$). Study site, education level, vaginal sex within 30 days and contraceptive use were similar between PrEP and non-PrEP groups. Depo medroxyprogesterone acetate (49%, 49/100) was the most common contraceptive used in both groups.

Prevalence of BV and STIs in PrEP and non-PrEP group. In the 100 participants, three CAPRISA 084 women at baseline, two at 3 months and four at 6 months had missing BV data. For women in the CAPRISA 082 study, BV and STIs data were performed at baseline but not at follow-up visits. At baseline, 31.6% (31/97) of women had intermediate BV (Nugent score 4–6) and 21.4% (21/97) had BV (Nugent score ≥ 7) (Table 2). Compared with PrEP group, there was a trend for non-PrEP group to be more likely to have BV ($p = 0.0576$). The proportion with BV in the PrEP group was 14.8% compared to 36.1% in the non-PrEP group ($p = 0.0231$) At 3 months follow-up, 36.8% (14/38) of CAPRISA 084 women had intermediate BV and two of them 5.3% (2/38) had BV. At 6 months, both intermediate BV rate 22.2% (8/36) and BV rate 16.7% (6/36) were almost the same. For STIs, 12% women (12/100) had *C. trachomatis*, 3.1% (3/98) had *T. vaginalis* and 2% (2/100) had *N. gonorrhoea* at baseline, and no difference was observed between PrEP and non-PrEP group. At the 3-month and 6-month visit, 38 and 37 of those in the PrEP group had STI data, respectively, of which 5.3% (2/38) were infected with *C. trachomatis* at 3 months and remained the same 5.3% (2/38) at 6 months. No women had *T. vaginalis* and *N. gonorrhoea* at 3 months and at follow-up visits (Table 2).

Composition of the vaginal microbiota at baseline. Based on the composition and relative abundance of bacterial species, we assigned bacterial communities into four CTs (CT1-4, Fig. 1)[14,18]. Samples with the relative majority of sequences (≥ 50%) assigned to non-*iners Lactobacillus* species were defined as CT1 and more than > 97% were *Lactobacillus crispatus*. The communities that had the highest relative abundance of *Lactobacillus iners* and *Gardnerella vaginalis* were classified as CT2, and CT3, respectively. CT4 had a mixed dominant bacterial taxon. Both PrEP and non-PrEP groups had similar vaginal microbial composition at all timepoints (Supplementary Fig. 1). At baseline, 11% (11/100) of women were assigned to CT1, 47% (47/100) to CT2, 26% (26/100) to CT3, and 16% (16/100) to CT4. Of women with intermediate BV (Nugent Score 4–6) and BV (Nugent Score > 7), 18% (2/11) were found in CT1, 36% (17/47) in CT2, 73% (19/26) in CT3 and 88% (14/16) in

Variables		Overall (N = 100) n (%)	PrEP group (N = 64) n (%)	Non-PrEP group (N = 36) n (%)	p-value
Baseline[a]					
Bacterial vaginosis (Nugent score)	No BV (0–3)	45 (46.4)	31 (50.8)	13 (36.1)	0.2063
	Intermediate BV (4–6)	31 (32.0)	21 (34.4)	10 (27.8)	0.6527
	BV (7–10)	21 (21.6)	9 (14.8)	13 (36.1)	0.0231
Trichomoniasis	Positive	3 (3.1)	1 (1.6)	2 (5.6)	0.5524
Gonorrhoea	Detected	2 (2.0)	2 (3.1)	0 (0.0)	0.5345
Chlamydia	Detected	12 (12.0)	6 (9.4)	6 (16.7)	0.3416
Month 3 follow-up[b]					
Bacterial Vaginosis (Nugent score)	No BV (0–3)	22 (57.9)	22 (57.9)	–	–
	Intermediate BV (4–6)	14 (36.8)	14 (36.8)	–	–
	BV (7–10)	2 (5.3)	2 (5.3)	–	–
Trichomoniasis	Positive	0 (0.0)	0 (0.0)	–	–
Gonorrhoea	Detected	0 (0.0)	0 (0.0)	–	–
Chlamydia	Detected	2 (5.3)	2 (5.3)	–	–
Month 6 follow-up[c]					
Bacterial Vaginosis (Nugent score)	No BV (0–3)	22 (61.1)	22 (61.1)	–	–
	Intermediate BV (4–6)	8 (22.2)	8 (22.2)	–	–
	BV (7–10)	6 (16.7)	6 (16.7)	–	–
Trichomoniasis	Positive	0 (0.0)	0 (0.0)	–	–
Gonorrhoea	Detected	0 (0.0)	0 (0.0)	–	–
Chlamydia	Detected	2 (5.3)	2 (5.3)	–	–

Table 2. Prevalence of BV and STIs in PrEP and Non-PrEP groups. [a]Three participants missing BV data and two missing *Trichomoniasis* data at baseline, [b]Two missing BV, *Trichomoniasis, Gonorrhoea* and *Chlamydia* results at 3 months, [c]Four missing BV and *Trichomoniasis* and three missing *Gonorrhoea* and *Chlamydia* results at 6 months. [b & c] STI follow-up data not collected in CAP082.

Figure 1. Heat map of the most abundant vaginal bacteria identified by 16S rRNA sequencing in 100 women at baseline. Relative abundance of key taxa across all of the samples was used to determine each of the four CTs: *L. crispatus* (CT1), *L. iners* (CT2), *G. vaginalis* (CT3), and mixed communities not dominated by other taxa (CT4). BV and STI statuses were also depicted.

CT4 (Fig. 1); X^2 (3, N = 100) = 21.395, $p = 0.871$. Of women with STI infection, 10% (1/11) were found in CT1, 13% (6/47) in CT2, 23% (6/26) in CT3 and 25% (4/16) in CT4; Fisher's Exact test $p = 0.568$.

Similar cervicotype composition and transition patterns among groups. We stratified the composition and structure of vaginal microbiota by PrEP use overtime (Fig. 2A). At baseline, among *L. crispatus* dominated CT1, 8% (3/36) and 13% (8/64) were non-PrEP and PrEP groups, respectively. Of women who were CT1 at baseline, 38% (3/8) transitioned to CT2 and 13% (1/8) to CT3 in the PrEP group while 67% (2/3) non-

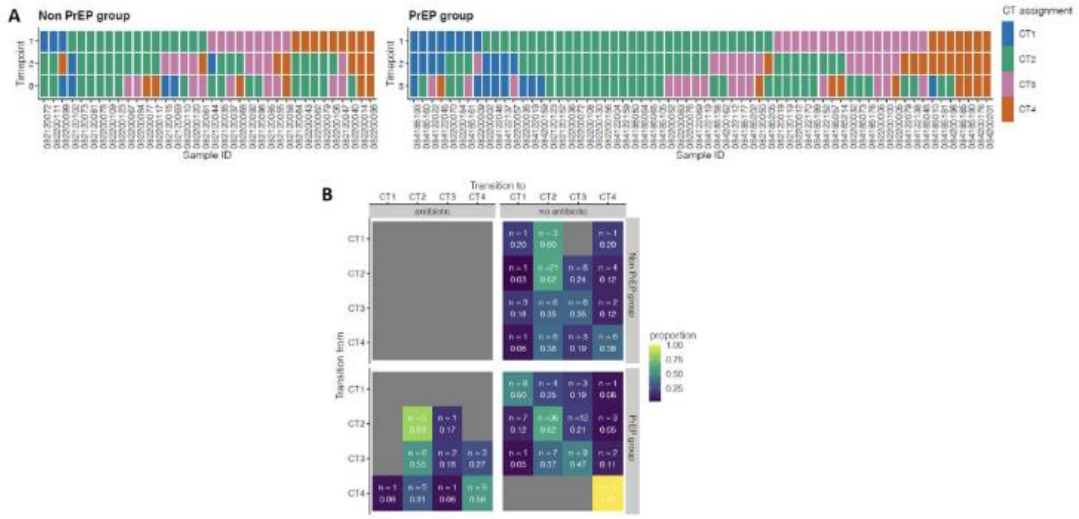


Figure 2. Composition and transitions between cervicotype in PrEP (n = 64) and non-PrEP groups (n = 36). (**A**) Box plots showing CTs assigned to individual participants over 3 visits at baseline, 3 months and 6 months. Blue colour depicts CT1, green CT2, pink CT3, and orange CT4. PID number that starts with 082 refers to women enrolled in CAPRISA 082 and 084 refers to CAPRISA 084 cohort. (**B**) Transition probabilities between CT's from one visit to another in PrEP and non-PrEP groups who did not use or used antibiotics during the study.

PrEP group transitioned to CT2 and 33% (1/3) to CT4 at the next time point. In addition, of the four women in the PrEP group who remained stable at 3 months, three women (38%, 3/8) transitioned to new CTs at 6 months. The proportions of *L. iners* dominated CT2 were different between PrEP (n = 32) and non-PrEP (n = 15) groups at baseline; X^2 (1, N = 45) = 0.188, $p = 0.665$. At 3 months, 34% (11/32) of women with *L. iners* dominated CT2 in the PrEP group transitioned between CTs, with 13% (4/32) to CT1, 19% (6/32) to CT3 and 3% (1/32) to CT4] and 6 non-PrEP group transitioned to new CTs [7% (1/15) CT1, 27% (4/15) to CT3 and 7% (1/15) to CT4]. Furthermore, women with stable CT2 over two visits transitioned to new CTs at 6 months; PrEP group [14% (3/21) transitioned to CT1 and 24% (5/21) to CT3] and non-PrEP group [22% (2/9) to CT3 and 22% (2/9) to CT4]. More than a third of PrEP (33%, 8/24) and non-PrEP (50%, 9/18) groups with BV-associated at baseline transitioned to *Lactobacillus* dominated CTs at 3 months. PrEP (46%, 6/13) and non-PrEP (50%, 3/6) groups with stable BV-associated CTs over two visits transitioned to *Lactobacillus* dominated CTs at 6 months. No major shifts in microbial communities were detected in PrEP compared to non-PrEP group.

We further assessed the transition probabilities between CTs in PrEP and non-PrEP groups, respectively (Fig. 2B). CT1 had an increased probability of remaining stable (0.5) in PrEP groups while were more likely to transition to CT2 (0.6) in non-PrEP group. In both groups, those with CT2 had a higher probability of remaining (0.62, respectively) compared to those with other CTs. CT3 in PrEP group without using antibiotics were less likely (0.47) to transition while those who used antibiotics were most likely to transition to CT2 (0.55). In PrEP group, CT4 had an increased probability of remaining (0.56 and 1.00 in antibiotic and non-antibiotic users, respectively) rather than transitioning to other states, while favours transitioning to CT2 (0.38) in non-PrEP group. BV-linked CTs had an increased probability of remaining rather than transitioning to *Lactobacillus* dominant CTs and the observed shifts were not associated with PrEP.

PrEP or antibiotics did not influence spartial segregation. To evaluate the possible impact of PrEP on vaginal microbial communities, we perfomed Non-metric Multidimensional scaling (NMDS) on Bray–Curtis distance at all timepoints. No clustering was detected based on PrEP or antibiotic use (Fig. 3). We further explored the relative contribution of PrEP to the diversity relative to other unknown factors by perfoming a permutational multivariate analysis of variance (PERMANOVA) in adonis. We found no significant differences between the two study groups (PrEP and non-PrEPgroups) at all timepoints ($P = 0.467$, $R^2 = 0.0087$, $P = 0.080$, $R^2 = 0.0092$ and $P = 0.344$, $R^2 = 0.0108$) respectively.

Discussion

The World Health Organization recommends offering PrEP as an additional prevention choice for persons at increased risk of HIV infection as part of combination HIV prevention approaches[19]. There is a paucity of data on the impact of PrEP on the vaginal microbial communities of healthy women in sub-Saharan Africa. Here, we longitudinally assessed vaginal microbiota in South African women to understand whether PrEP (TDF/FTC) can influence the vaginal microbiome. We found fewer women with a high relative abundance of *L. crispatus*

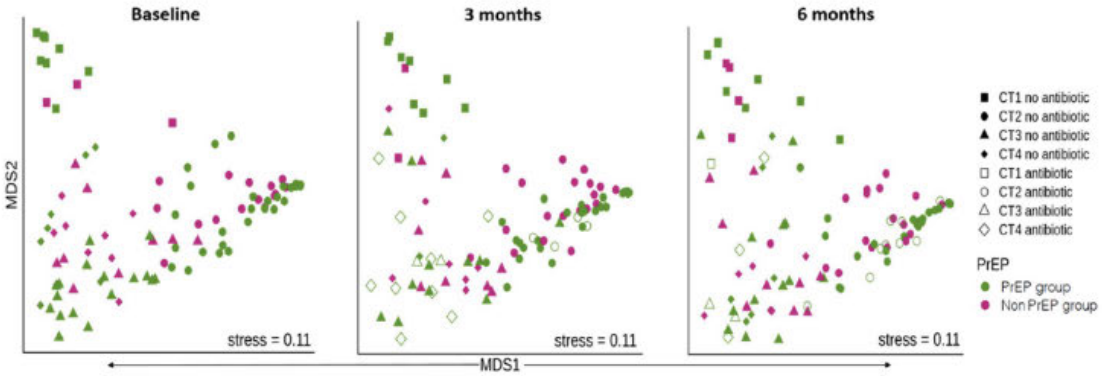


Figure 3. Non-metric Multidimensional scaling (NMDS) of Bray–Curtis showing no clustering dissimilarity of species level relative abundances in PrEP and non-PrEP groups at baseline, 3 months and 6 months. The pink colour represents non-PrEP group and green colour represents PrEP group. Open shape represents participants on antibiotics.

compared to that of *L. iners* and *G. vaginalis* dominant communities. Furthermore, we also observed transitions between CTs in PrEP and non-PrEP groups, with few women with stable *L. crispatus* over time.

Studies have revealed that European-American vaginal microbiota have high *Lactobacillus* abundance, while those in women of African descent is more diverse in nature[14,18,20–25]. In addition, Gosmann et al., (2017) demonstrated that 10% of a cohort (N = 236) of South African Black women aged 18–23 years lacked *L. crispatus* dominance in their cervical vaginal microbiome over time[18]. Another study found that only 37% of South African women had *Lactobacillus*-dominant communities[26]. However, these studies solely characterised vaginal microbial communities in women residing in South Africa and no other part of Africa. Therefore, there is a need to understand geographical and lifestyle impact on vaginal microbiome. Consistent with these studies, we demonstrate that only 8%, (3/36, non-PrEP) and 13% (8/64, PrEP group) of women had *L. crispatus*-dominated communities and this number was reduced to one participants (PrEP group) at the 6 month visit. In agreement with previous studies[18,23,26,27], majority of South African women had vaginal community with high abundance of *L. iners*. *L. iners*-dominated communities are known to be unstable with high probability of transitioning to a high diversity community state associated with BV. Several factors have been posited to play a dominant role in shaping the vaginal microbiota, including racial lines, vaginal hygiene practices, contraceptive use, sexual behaviour, rectal colonization and host genetics. However, their individual or collective contributions to the shifts in the vaginal microbiome remains unresolved[18,28].

We explored transition patterns between the observed microbial communities over time. There was no difference in changes in the microbial communities overtime between PrEP and non-PrEP groups. Although *L. iners*-dominant communities were less likely to transition, once they did, they favoured communities associated with BV and increased HIV risk, irrespective of PrEP or antibiotic use. This is consistent with previous studies that showed women with *L. iners*-dominant communities transition more frequently to ecological niche with other bacterial taxa, such as *G. vaginalis, A. vaginae*, and other strict and facultative anaerobes[23,27,29–31]. Furthermore, Munoz et al.[27] showed that transitions away from *L. crispatus* and towards *L. iners*-dominant communities are less likely to revert to *L. crispatus* communities but most likely to further transition to BV-associated communities. This is particularly concerning and may explain high rates of diverse microbial communities and BV occurrence among women of African descent. These data affirm that there is a microbiome footprint that dictates the colonization of certain bacteria together more strongly, perhaps due to the synergy and mutually beneficial effects for these species to survive together. In addition, viable alternatives such as probiotics that shift transition from *L. iners* to *L. crispatus* leading to curative or preventive vaginal dysbiosis interventions are needed.

We also observed that *G. vaginalis*-dominant communities remained stable over time and more transitions to *L. iners* than *L. crispatus*-dominant communities, irrespective of PrEP or antibiotic use. In this study, some of the PrEP group participants diagnosed with BV were treated with metronidazole at baseline (CAPRISA 082 and CAPRISA 084), 3 months and 6 months (CAPRISA 084 only). Previous studies have demonstrated that BV treatments were associated with temporal changes in the vaginal microbiota[23,32]. Therefore, the observed shifts away from BV-associated communities in this study is more likely attributed to BV treatment rather than PrEP use[23,29,30]. This is confirmed by high numbers of observed transitions from BV associated CTs to *L iners* dominated CT in women actively taking antibiotics.

One of the strengths of this study is the longitudinal follow-up and sampling before and after PrEP initiation in at risk South African women. However, this study had limitations which could have influenced the composition of vaginal microbiota. Firstly, PrEP adherence data relied on pill count and self-reporting, and systemic or genital PrEP levels were measured only in a sub-set of women. Secondly, the lack of longitudinal data for STI and BV in the CAPRISA 082 trial that precluded a detailed analyses of the effect of PrEP and BV on the vaginal microbiota. Additionally, we could not measure in detail the impact of BV treatment on vaginal microbiota, instead BV scores (particularly in the CAPRISA 084 cohort- PrEP group) were used as a surrogate of treatment

adherence. Furthermore, we did not investigate the impact of other potential co-factors such as STIs, human papillomavirus, herpes simplex virus, hormonal contraceptives, diets, hormonal status, and other vaginal disorders (e.g., aerobic vaginitis), although these may have different biological effects on microbial communities.

In conclusion, our results showed no major differences in vaginal microbial communities between PrEP and non-PrEP groups, suggesting that PrEP initiation does not alter the vaginal microbial communities of HIV-negative sub-Saharan African women. These findings reaffirm that PrEP rollout can be delivered in women with optimal and/or sub-optimal vaginal microbiota. Regular screening for STIs and BV during PrEP care remains an ideal option, particularly in regions with rates of HIV infection.

Received: 7 July 2022; Accepted: 14 September 2022
Published online: 28 September 2022

References

1. Baeten, J. M. *et al.* Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N. Engl. J. Med.* **367**, 399–410. https://doi.org/10.1056/NEJMoa1108524 (2012).
2. Grant, R. M. *et al.* Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N. Engl. J. Med.* **363**, 2587–2599. https://doi.org/10.1056/NEJMoa1011205 (2010).
3. McCormack, S., Dunn, D. & On behalf of the PROUD Study Group. in *Conference on Retroviruses and Opportunistic Infections (CROI)*, 23–26 February (Seattle, Washington, 2015).
4. Molina, J.-M. *et al.* in *Conference on Retroviruses and Opportunistic Infections.*
5. Thigpen, M. C. *et al.* Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana. *N. Engl. J. Med.* **367**, 423–434 (2012).
6. Van Damme, L. *et al.* Preexposure prophylaxis for HIV infection among African women. *N. Engl. J. Med.* **367**, 411–422 (2012).
7. Choopanya, K. *et al.* Antiretroviral prophylaxis for HIV infection in injecting drug users in Bangkok, Thailand (the Bangkok Tenofovir Study): a randomised, double-blind, placebo-controlled phase 3 trial. *The Lancet* **381**, 2083–2090 (2013).
8. Abdool Karim, Q. *et al.* Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science* **329**, 1168–1174 (2010).
9. Rees H. *et al.* in *Conference on Retroviruses and Opportunistic Infections (CROI)* (Seattle, Washington, 2015).
10. Klatt, N. R. *et al.* Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* **356**, 938–945. https://doi.org/10.1126/science.aai9383 (2017).
11. Hillier, S. L. *et al.* in *Conference on Retroviruses and Opportunistic Infections.*
12. Heffron, R. *et al.* Efficacy of oral pre-exposure prophylaxis (PrEP) for HIV among women with abnormal vaginal microbiota: a post-hoc analysis of the randomised, placebo-controlled partners PrEP study. *Lancet HIV* https://doi.org/10.1016/S2352-3018(17)30110-8 (2017).
13. Mansoor, L. E. *et al.* Prospective study of oral pre-exposure prophylaxis initiation and adherence among young women in KwaZulu-Natal, South Africa. *J. Int. AIDS Soc.* **25**, e25957. https://doi.org/10.1002/jia2.25957 (2022).
14. Anahtar, M. N. *et al.* Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* **42**, 965–976. https://doi.org/10.1016/j.immuni.2015.04.019 (2015).
15. Callahan, B. J. *et al.* DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583. https://doi.org/10.1038/nmeth.3869 (2016).
16. McDonald, D. *et al.* An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* **6**, 610–618. https://doi.org/10.1038/ismej.2011.139 (2012).
17. McMurdie, P. J. & Holmes, S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**, e61217. https://doi.org/10.1371/journal.pone.0061217 (2013).
18. Gosmann, C. *et al.* Lactobacillus-deficient cervicovaginal bacterial communities are associated with increased HIV acquisition in young South African women. *Immunity* **46**, 29–37. https://doi.org/10.1016/j.immuni.2016.12.013 (2017).
19. World Health Organization. Guideline on when to start antiretroviral therapy and on pre-exposure prophylaxis for HIV (2015).
20. Fettweis, J. M. *et al.* Differences in vaginal microbiome in African American women versus women of European ancestry. *Microbiology* **160**, 2272–2282. https://doi.org/10.1099/mic.0.081034-0 (2014).
21. Ravel, J. *et al.* Vaginal microbiome of reproductive-age women. *Proc. Natl. Acad. Sci. U.S.A.* **108**(Suppl 1), 4680–4687. https://doi.org/10.1073/pnas.1002611107 (2011).
22. Lennard, K. *et al.* Microbial composition predicts genital tract inflammation and persistent bacterial vaginosis in South African adolescent females. *Infect. Immun.* https://doi.org/10.1128/IAI.00410-17 (2018).
23. Mtshali, A. *et al.* Temporal changes in vaginal microbiota and genital tract cytokines among South African women treated for bacterial vaginosis. *Front. Immunol.* https://doi.org/10.3389/fimmu.2021.730986 (2021).
24. Riganelli, L. *et al.* Structural variations of vaginal and endometrial microbiota: Hints on female infertility. *Front. Cell Infect. Microbiol.* **10**, 350. https://doi.org/10.3389/fcimb.2020.00350 (2020).
25. Gajer, P. *et al.* Temporal dynamics of the human vaginal microbiota. *Sci. Transl. Med.* **4**, 132ra152-132ra152 (2012).
26. Anahtar, M. N., Gootenberg, D. B., Mitchell, C. M. & Kwon, D. S. Cervicovaginal microbiota and reproductive health: The virtue of simplicity. *Cell Host Microbe* **23**, 159–168. https://doi.org/10.1016/j.chom.2018.01.013 (2018).
27. Munoz, A. *et al.* Modeling the temporal dynamics of cervicovaginal microbiota identifies targets that may promote reproductive health. *Microbiome* **9**, 163. https://doi.org/10.1186/s40168-021-01096-9 (2021).
28. Muzny, C. A. & Schwebke, J. R. Pathogenesis of bacterial vaginosis: Discussion of current hypotheses. *J. Infect. Dis.* **214**(Suppl 1), S1-5. https://doi.org/10.1093/infdis/jiw121 (2016).
29. Joag, V. *et al.* Impact of standard bacterial vaginosis treatment on the genital microbiota, immune milieu, and ex vivo human immunodeficiency virus susceptibility. *Clin. Infect. Dis.* **68**, 1675–1683. https://doi.org/10.1093/cid/ciy762 (2019).
30. Verwijs, M. C., Agaba, S. K., Darby, A. C. & van de Wijgert, J. Impact of oral metronidazole treatment on the vaginal microbiota and correlates of treatment failure. *Am. J. Obstet. Gynecol.* https://doi.org/10.1016/j.ajog.2019.08.008 (2019).
31. Ravel, J. *et al.* Daily temporal dynamics of vaginal microbiota before, during and after episodes of bacterial vaginosis. *Microbiome* **1**, 29. https://doi.org/10.1186/2049-2618-1-29 (2013).
32. Ahrens, P. *et al.* Changes in the vaginal microbiota following antibiotic treatment for *Mycoplasma genitalium*, *Chlamydia trachomatis* and bacterial vaginosis. *PLoS ONE* **15**, e0236036. https://doi.org/10.1371/journal.pone.0236036 (2020).

Acknowledgements

We would like to thank all CAPRISA 082 and CAPRISA 084 study participants and the study team for their contributions to this research. This research was partially supported by the Department of Science and

Innovation – National Research Foundation Centre of Excellence in HIV Prevention (UID 96354) and the Bill & Melinda Gates Foundation (BMGF, INV-018075). NMM was funded by Talent Excellence Scholarship, University of KwaZulu-Natal. DA was funded through the SAMRC Self-Initiated Grant and the NRF of South Africa Thuthuka (grant#TTK160517165310), the NRF Research Career Advancement Fellowship (grant#RCA13101656388), and a European & Developing Countries Clinical Trials Partnership (EDCTP) senior fellowship (grant#TMA2017SF-1960). SN was supported by Columbia University-Southern African Fogarty AITRP Programme (grant#D43TW00231), National Research Fund Thuthuka Research Grant (grant# TTK160510164586), and Poliomyelitis Research Foundation Research Grant (grant#16/17). P.S. received support from the South African Medical Research Council (SAMRC) Special Initiative (Grant no. 96151) and S&F Scarce Skills Postdoctoral Fellowships (Grant no.132714).The CAPRISA 082 observational study was made possible by the generous support of the American people through the United States Agency for International Development (USAID) under the Cooperation Agreement No. AID-OAA-A-15-00040 and a grant funded by FHI360 under Cooperative Agreement/Grant No. AID-674-A-14-00009 funded by USAID Southern Africa. CAPRISA 084 PrEP Demonstration project was supported by FHI360 under Cooperative Agreement/Grant No. AID-674-A-14-00009 funded by USAID Southern Africa.

Author contributions

N.M.M., P.S., D.A., D.S.K. and S.N. conceived and designed the study. N.M.M. and J.X. carried out experiments and A.M., G.M. and L.N. provided extra support during laboratory experiments. N.M.M., S.N., J.A.E. & S.E.J. performed the analysis and L.L. provided statistical support. L.E.M. and Q.A.K. were the principal investigators of the CAPRISA 082 and CAPRISA 084 studies, respectively. N.M.M., P.S., S.E.J., J.X., L.L., A.M., G.M., L.N., L.E.M., Q.A.K., S.S.A.K., D.A., D.S.K. and S.N. contributed to the interpretation and discussion of the results and writing of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-20486-z.

Correspondence and requests for materials should be addressed to S.N.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

CHAPTER 3

Lactobacillus-Dominant and Polymicrobial Vaginal Microbiomes not associated with High or Low levels of 1% Tenofovir Gel

Tenofovir is a commonly used component of antiretroviral therapy and has been thoroughly studied in efficacy trials for PrEP. However, PrEP results in women have been inconsistent, with some studies showing limited to no protection for HIV. Recent studies suggested that the highly diverse vaginal microbiota may affect the mucosal concentrations of topically applied tenofovir microbicides. The majority of African women consist of highly diverse vaginal microbiota, which is also associated with an increased risk of HIV acquisition. This study evaluated whether vaginal microbiota can diminish the prophylactic efficacy of topical tenofovir.

Chapter 3: This study investigated whether vaginal microbiota influences the concentration of tenofovir (TFV) in cervicovaginal fluid among HIV-uninfected women using 1% TFV gel in the CAPRISA 008 cohort. The study found no significant differences in TFV levels between women with *Lactobacillus*-dominated CSTs and those with polymicrobial communities. TFV concentration was unaffected by specific bacterial taxa, including *Gardnerella vaginalis* or *Lactobacillus species*. Factors such as days since gel use, age, and sexual frequency influenced TFV levels. Overall, vaginal microbiota alone did not impact TFV concentrations or its effectiveness, underscoring other factors in influencing TFV gel outcomes. This study is relevant since several clinical trials are currently testing PrEP, including (CAB-LA, Dapiverine ring, and an intravaginal ring containing TFV (IVR)) on African women. Therefore, this study will provide a basis for PrEP rollout in women with both dysbiotic and optimal vaginal microbiota.

For this study, I was centrally involved in conceptualizing and designing the project, in collaboration with Dr. Sinaye Ngcapu. I led the laboratory work, including DNA extraction, PCR amplification, and pooling of samples for 16S rRNA sequencing. I performed the data analysis and interpretation, integrating microbiome outputs with clinical metadata. My analyses were validated by our statistician, Marothi Peter Letsoalo, and our bioinformatician, Olona Asavela Kama, ensuring methodological rigor. I drafted the manuscript, which was subsequently reviewed and refined with input from all co-authors: Olona Asavela Kama, Jiawu Xu, Katya Govender, Andile Mtshali, Gugulethu Mzobe, Lungelo Ntuli, Leila Mansoor, Quarraisha Abdool Karim, Salim S. Abdool Karim, and Douglas S. Kwon. All co-authors contributed to the interpretation and discussion of the results and provided critical feedback on manuscript revisions. In summary, my contributions encompassed experimental execution, primary data analysis, interpretation, and manuscript drafting, forming a substantial and independent component of my PhD training, while the co-authors provided complementary support, oversight, and expert guidance throughout the study

Lactobacillus-Dominant and Polymicrobial Vaginal Microbiomes not associated with high or Low levels of 1% tenofovir gel

Noluthando Mazibuko-Munonde[1,2], Olona Asavela Kama[1], Marothi Peter Letsoalo[1], Jiawu Xu[3], Katya Govender[4], Andile Mtshali[1,2,], Gugulethu Mzobe[1,2], Lungelo Ntuli[1,2], Leila Mansoor[1], Quarraisha Abdool Karim[1,6], Salim S. Abdool Karim[1,6], Douglas S Kwon[3,7,8†], Sinaye Ngcapu[1,2†*].

[1]Centre for the AIDS Programme of Research in South Africa, Durban, South Africa; [2]School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal, Durban, South Africa; [3]Ragon Institute of MGH, MIT, and Harvard, Massachusetts General Hospital, Cambridge, Massachusetts, United States of America; [4]Africa Health Research Institute, Durban, South Africa; [5]National Health Laboratory Service, Cape Town, South Africa; [6]Department of Epidemiology, Columbia University, New York City, NY, USA; [7]Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts, United States of America; [8]Harvard Medical School, Boston, Massachusetts, United States of America.

[†]These authors share senior authorship.

*** Correspondence:** Dr. Sinaye Ngcapu, sinaye.ngcapu@caprisa.org

Keywords: Vaginal Microbiota, pre-exposure prophylaxis, Bacterial vaginosis, sexually transmitted infection

Abstract

Tenofovir, commonly used as a component of ARVs, has been studied extensively in efficacy trials for PrEP, with studies in women showing inconsistent outcomes. Recent evidence suggests that the vaginal microbiota may alter mucosal TFV concentrations of topically delivered microbicides. This study aimed to determine whether the changes in the vaginal microbiota could lower cervicovaginal fluid 1% TFV gel concentration. We utilised cervicovaginal swabs and aspirates at 1 to 3 time-points from 69 HIV-uninfected women who used 1% tenofovir gel in the CAPRISA 008 cohort and sequenced the V4 region of the 16S rRNA gene. We measured the concentration of TFV in the cervicovaginal (CV) aspirate using LC-MS/MS technique. The participants were stratified into two groups (High TFV: TFV >100ng/ml and Low TFV: TFV <100ng/ml). Four major community state types (CSTs) were identified based on predominant taxa, including *Lactobacillus crispatus* (CST I, 18.3%), *Lactobacillus iners* CST III, 38.2%), *Gardnerella vaginalis* (CST IV-B, 7.6%) and polymicrobial (CST IV-C, 35.9%). The frequency of the bacterial taxa and CSTs was distributed equally between high and low TFV groups. There was no significant difference in the median log TFV concentration (ng/mL) between women with CST IV versus those with *Lactobacillus*-dominated CSTs. The TFV concentration did correlate with the relative abundances of any species, including *Gardnerella vaginalis*, *Prevotella* species, *L. iners, L. crispatus and L. jensenii*. Although a slight increase in TFV concentrations was observed among STI-negative women, this difference was not statistically significant ($p > 0.05$). Our results suggest that TFV concentrations are associated with days since gel use, age and the frequency of vaginal sex in the last 30 days. In conclusion, vaginal microbiota did not differ women with either High TFV or Low TFV groups. Vaginal microbiota alone did not influence TFV concentration or its effectiveness in vivo in the CAPRISA 008 trial.

3.1. INTRODUCTION

Although the global incidence of new HIV infections has declined over recent years due to advancements in prevention and treatment strategies, young women, particularly in sub-Saharan Africa, continue to bear a disproportionate burden of the epidemic (1). In sub-Saharan Africa, women and girls represent 62% of all new HIV infections. Each week, approximately 4,000 adolescent girls and young women (AGYW) aged 15–24 acquire HIV, with 3,100 of these cases occurring in sub-Saharan Africa. AGYW in this region are three times more likely to contract HIV than their male counterparts (2).

Since 2010, the outcomes of numerous clinical trials have highlighted the preventive benefits of antiretroviral (ARV) drugs in reducing the risk of sexually acquired HIV infection, offering renewed hope for HIV prevention. These PrEP trials, evaluating tenofovir alone or in combination with emtricitabine (Truvada®), have consistently demonstrated safety and efficacy in preventing HIV acquisition among diverse populations, including men who have sex with men, transgender women, people who inject drugs, serodiscordant couples, and heterosexual men and women (3-8). Importantly, no significant safety concerns have been identified. A critical finding from these trials is that PrEP's effectiveness is strongly dependent on adherence, consistent use is essential for achieving optimal protection. While data from men across trials show a protective benefit ranging from 44% to 96% with both daily and intermittent dosing strategies (3-8), results among women in southern Africa have been more variable (9-16). Trials evaluating oral and topical PrEP in this population have produced mixed outcomes, with effectiveness limited by low-risk perception and challenges with adherence to the daily regimen, particularly among adolescent girls and young women (17). Recent evidence suggests that vaginal microbiota may play a role in the reduced efficacy of topical tenofovir (TFV) in protecting women from HIV infection (18, 19), a phenomenon not observed with oral TFV-based PrEP (20, 21).

Proteomic analysis of vaginal specimens from participants in the CAPRISA 004 trial demonstrated that 1% tenofovir gel significantly reduced the risk of HIV acquisition in women with a *Lactobacillus*-dominant vaginal microbiota compared to those with a non-*Lactobacillus*-dominant microbiota (18). Additionally, tenofovir levels were lower in vaginal specimens from

women with a *Gardnerella vaginalis*-dominant microbial community, suggesting that high levels of *G. vaginalis* may rapidly deplete tenofovir (18). Similar findings were observed through Gram stain analysis, further corroborating this relationship (19). Women with a Nugent score ≤3, indicative of *Lactobacillus* dominance, exhibited higher cervical tissue tenofovir levels, while those diagnosed with bacterial vaginosis, reflecting increased *G. vaginalis* levels, had reduced tenofovir concentrations (19). While tenofovir biodegradation by bacteria was shown *in vitro*, these findings may not fully replicate *in vivo* conditions. Thus, this study aims to longitudinally evaluate the impact of the vaginal microbiota on concentrations of genital tract tenofovir *in vivo*. A deeper understanding of how the vaginal microbiome influences PrEP efficacy could inform strategies to prevent bacterial vaginosis and enhance HIV prevention efforts.

3.2. MATERIALS AND METHODS

3.2.1. Study design, participants, and sample collection:

This retrospective sub-study was conducted within the CAPRISA 008 trial (Ref: BFC237/010), an open-label, randomized controlled trial aimed at evaluating the implementation effectiveness and safety of 1% tenofovir gel provision through family planning services in KwaZulu-Natal, South Africa, at both rural Vulindlela and urban eThekwini Research Clinics (22). The study enrolled women aged 18 and older who were sexually active, HIV-uninfected, had a negative urine pregnancy test and provided written consent to initiate non-barrier contraception. Participants received single-use, prefilled applicators of 1% tenofovir gel, with instructions to use the first dose within 12 hours before anticipated coitus and a second dose as soon as possible, but no later than 12 hours after intercourse, with a maximum of two doses within 24 hours. Women in the intervention arm (family planning clinics) attended monthly visits during the first three months, after which gel provision and monitoring aligned with routine family planning visits (typically every 2-3 months). Women in the control arm (CAPRISA clinics) had monthly visits at either the CAPRISA urban or rural clinic for gel provision. Participants were required to attend standard visits at enrollment, months 6, 12, 18, 24, and at study exit for blood and genital specimen collection. This sub-study included cervicovaginal swabs and aspirates from 69 participants across one to three consecutive time points. Swabs collected at each visit were tested for bacterial vaginosis (BV) using Gram stain

based on Nugent's criteria (0-3 considered BV negative, 4-6 intermediate, and 7-10 BV positive)(23). Common STI pathogens such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, and *Trichomonas vaginalis* were detected using Multiplex PCR amplification with the Fast-track Diagnostics STD9 detection kit. The study was approved by the Ethics Review Committee of the University of KwaZulu-Natal (BREC number: BE603/18).

3.2.2. Bacterial DNA extraction, sequencing, taxonomic assignment and CST assignment

Total nucleic acid was extracted from stored vaginal swabs collected from 69 participants using the phenol-chloroform method. Bacterial 16S rRNA gene sequencing and analysis were performed as described previously (24). The V4 hypervariable region of the 16S rRNA gene was amplified using 515F/806 universal primers. The resulting amplicons were pooled into libraries and sequenced on an Illumina MiSeq platform. The Divisive Amplicon Denoising Algorithm (DADA) 2 (25), was used to filter, trim, and identify amplicon sequence variants (ASVs). Only samples with ≥2000 reads were included in further analyses. A phyloseq object was created, containing the ASV table, taxonomic table, and sample metadata, using the phyloseq R package (26). ASVs were taxonomically classified to the genus or higher levels using the SILVA ribosomal RNA database (27). Bacterial communities were categorized into four major community state types (CSTs) using the VALENCIA algorithm (28), with reference centroids available at github.com/ravel-lab/VALENCIA. Briefly, women were classified into CST I if their genital microbiota was dominated by *Lactobacillus crispatus*, or CST III if dominated by *Lactobacillus iners*. CST IV-B was characterised by a higher abundance of the BV-associated bacterium *Gardnerella vaginalis*, and CST IV-C consisted of a wider range of facultative and strictly anaerobic bacteria.

3.2.3. Detection of Tenofovir concentrations in CVL samples by LC-MS/MS

Sample analysis was conducted using an Agilent High-Pressure Liquid Chromatography (HPLC) system coupled to an AB Sciex 5500 triple quadrupole mass spectrometer, equipped with an electrospray ionization TurboIonSpray source. The LC-MS/MS method, adapted and optimized for quantifying tenofovir was used (29). Cervicovaginal liquid (CVL) samples were processed using a protein precipitation extraction method with methanol. To prepare the

samples, 50 µl of internal standard (ISTD) solution was added to 50 µl of CVL, 50 µl of a blank clinical sample, and 50 µl of a Quality Control (QC) sample. The mixture was briefly mixed, and 200 µl of 70% methanol was added for protein precipitation. This was followed by sonication and centrifugation at 16,000 rpm at 4°C for 10 minutes. The clear supernatant (300 µl) was transferred to a microcentrifuge tube and dried overnight using a SpeedVac at 35°C. The dried samples were reconstituted in 50 µl of 70% acetonitrile in 0.1% formic acid water, vortexed, sonicated, and centrifuged at 16,000 rpm at 4°C for 10 minutes. The clear supernatant was transferred to an amber vial and placed in an auto-sampler rack, where 2 µl of each sample was injected for LC-MS/MS analysis. Analytes were separated on a Luna Amino (NH2) column (Phenomenex, Torrance, CA) at a flow rate of 0.2 mL/min using gradient elution. The autosampler syringe and injection valve were washed with a water:acetonitrile (30:70, v/v) solution post-injection to minimize carryover. Data acquisition and quantitative analysis were performed using Analyst software, version 1.6.2. The assay's lower limit of quantification for CVF (aspirate) tenofovir was 2 ng/mL.

3.2.4. Statistical analysis

Descriptive statistics for quantitative variables were summarized using medians and interquartile ranges (IQRs), while categorical data were summarized with frequency counts and percentages. The Wilcoxon rank sum test was used to compare continuous variables, and Pearson's Chi-squared test and Fisher's Exact test were applied for categorical comparisons. α-diversity, including Richness (Chao1) and Evenness (Simpson's E), was calculated using the R vegan library. β-diversity between samples was estimated using Bray–Curtis distances. Non-metric Multidimensional Scaling (NMDS) was performed to visualize differences in taxonomic profiles among samples based on Bray–Curtis distances of ASV relative abundance, using the metaMDS function in the vegan R package. Linear and logistic regression models were used to estimate the unadjusted and adjusted effects of *Lactobacillus* dominance status, days since the last gel insertion, age, and sexually transmitted infections on tenofovir concentration. All statistical calculations were performed using R.

3.3. RESULTS

3.3.1. Study participants

The study analyzed data from 69 women who had one to three follow-up visits during the CAPRISA 008 vaginal microbicide trial, with 39/69 (57%) from the family planning arm (intervention) and 30/69 (43%) from the CAPRISA arm (control). Among 131 samples with detectable cervicovaginal (CV) aspirate tenofovir (TFV) levels (>2 µg/mL), 74/131 (56.5%) were categorized as High TFV (>100 ng/mL) and 57/131(43.5%) as Low TFV (<100 ng/mL), based on prior findings from the CAPRISA 004 study, which showed that a CV aspirate TFV concentration of 100 ng/mL provided 65% protection against HIV-1 acquisition, while concentrations above 1,000 ng/mL conferred 75% protection with TFV vaginal gel use(30, 31).

The overall median age of participants was 30 years (interquartile range [IQR]: 26–33 years). There were no significant differences between the High TFV and Low TFV groups in terms of age at sexual debut, age at first menstruation, educational attainment, partner HIV status, partner circumcision, cohabitation with a regular partner, or lifetime number of sexual partners at study enrollment (Table 3.1). Participants reported a median of 6 (IQR: 3–8) sex acts in the past 30 days, with 91.3% indicating they were in stable relationships. The majority (57.1%, 32/56) reported consistent condom use, and Depo-Provera (DMPA) was the most commonly used contraceptive method in both groups. The prevalence of bacterial vaginosis (BV) was similar between the groups and only seven participants were diagnosed with at least one sexually transmitted infection (STIs), such as *Chlamydia, Gonorrhea,* and *Trichomonas.*

Table 3.1: Baseline Participant Characteristics and Prevalence of BV and STIs by TFV Group

VARIABLE	OVERALL (N=69)	HIGH TFV (N=21)	LOW TFV (N=48)	P-VALUE
	n (%)			
STUDY ARM				
CAP	30 (43.5%)	11 (36.7%)	19 (63.3%)	-
FP	39 (56.5%)	11 (28.2%)	28 (71.8%)	-
AGE (YEARS; Median (IQR)	30 (26–33)	30 (26–37)	28 (25–32)	0.191
VAGINAL SEX IN THE LAST 30 DAYS	6 (3-8)	4.5 (3–8)	6 (3–9)	0.523
NUMBER OF LIFETIME PARTNERS	2 (1-3)	3 (1–3)	2 (1–3)	0.154
PARTNER HIV STATUS				0.999
Negative	63 (96.9%)	20 (100%)	43 (96.0%)	
Positive	1 (1.5%)	0 (0.0%)	1 (2.2%)	
Unknown	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Not Applicable	1 (1.5%)	0 (0.0%)	1 (2.2%)	
CONDOM USE				0.452
Always	32 (57.1%)	9 (47.0%)	23 (62.0%)	
Sometimes	16 (28.6%)	6 (32.0%)	10 (27.0%)	
Never	8 (14.3%)	4 (21.0%)	4 (11.0%)	
CONTRACEPTIVE USE				0.378
Depo-Provera	34 (50.0%)	9 (43.0%)	25 (53.0%)	
Oral Contraceptives	8 (11.8%)	4 (19.0%)	4 (8.5%)	
Nur-isterate	13 (19.1%)	3 (14.0%)	10 (21.0%)	
Other	12 (17.6%)	4 (19.0%)	8 (17.0%)	
Not Applicable	1 (1.5%)	1 (4.8%)	0 (0.0%)	
BACTERIAL VAGINOSIS (Nugent Score)				0.556
No BV (0–3)	52 (76.5%)	18 (86.0%)	34 (72.0%)	
Intermediate BV (4–6)	8 (11.8%)	2 (9.5%)	6 (13.0%)	
BV (7–10)	8 (11.8%)	1 (4.8%)	7 (15.0%)	
ANY STI (*Chlamydia, Gonorrhoea and Trichomononas)*				0.334
At least one STI	7 (10.1%)	1 (4.8%)	6 (13.0%)	
None	13 (18.8%)	6 (29.0%)	7 (15.0%)	
Not Assessed	49 (71.0%)	14 (67.0%)	35 (73.0%)	

[1]median (IQR); n (%) [2]Wilcoxon rand sum test; Pearson's Chi-square test; Fisher's exact test. [3]some participants had missing demographics and clinical data.

3.3.2. Characterisation of the vaginal microbiota in 131 samples

Hierarchical clustering of the cervical microbiota, based on the types and relative abundances of bacterial taxa, identified three distinct community state types (CSTs): CST I, CST III, and CST IV (Figure 3.1). CST I, dominated by *L. crispatus*, was observed in 24 samples (24/131, 18.3%). CST III, characterized by *L. iners*, was present in 50 samples (50/131, 38.2%). The most prevalent CST, CST IV, occurred in 57 samples (57/131, 43.5%) and featured a diverse and complex array of facultative and strictly anaerobic BV-associated bacteria, including. Within CST IV, a continuum of bacterial taxa relative abundances was observed, revealing two distinct sub-clusters: CST IV-B dominated by *Shuttleworthia*-dominant (10/57, 17.5%), and CST IV-C dominated by *Gardnerella*-dominant (47/57, 82.5%), subsequently, metadata associated with the women in each CST were compared.

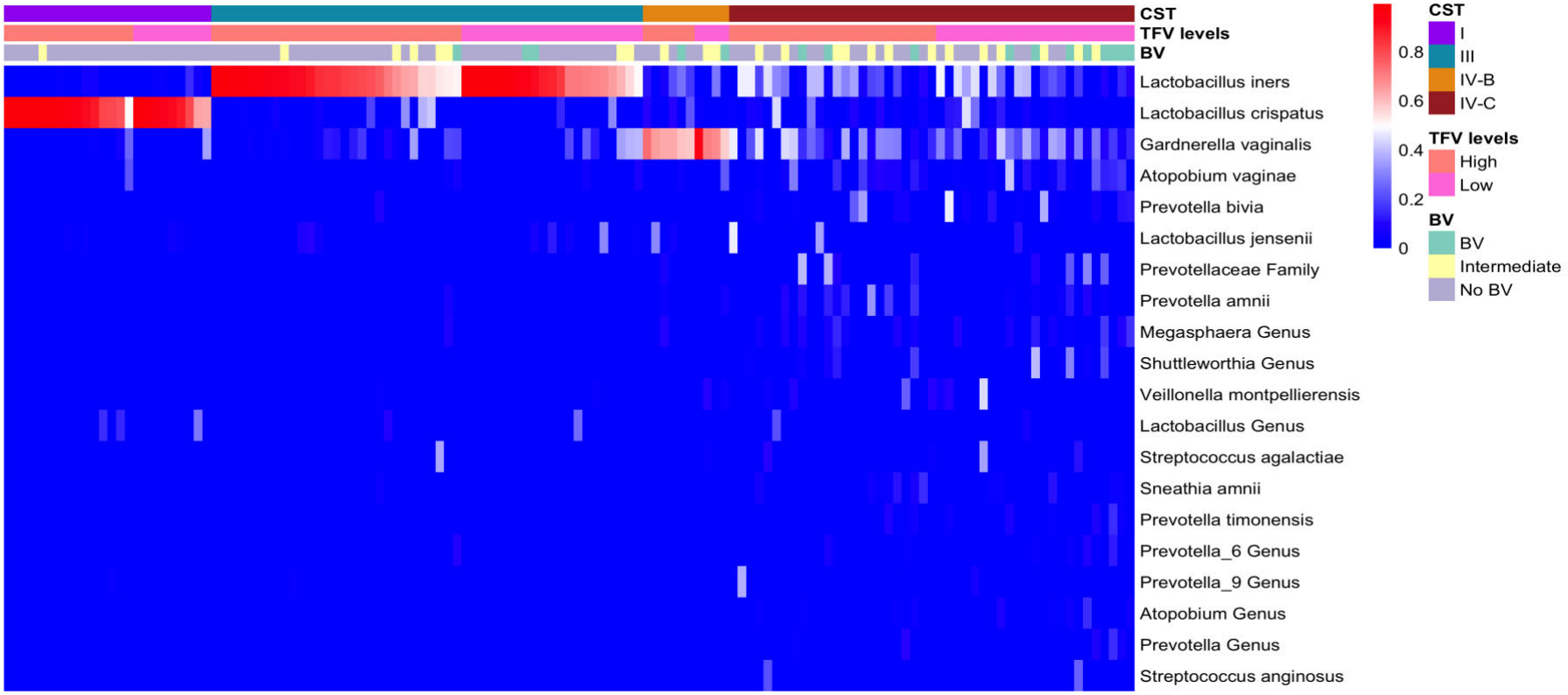


Figure 3.1. Vaginal community clusters in South African women using 1% tenofovir gel. Heat map of the most abundant vaginal bacteria identified by 16S rRNA sequencing at 131 available samples. The samples were grouped by community state type (CST) into the most abundant species in each CST (CST I: *L. crispatus*, CST III: *L. iners*, CST IV-B: *G. Vaginalis* and CST IV-C: mixed bacterial taxa). BV statuses represented by (BV: lime green, intermediate BV: Yellow and No BV: grey) and Tenofovir levels are also depicted by (High TFV levels: Orange colour and Low TFV levels: pink).

3.3.3. Comparison of alpha diversity across CSTs,

Alpha diversity of the cervical microbiota was assessed using multiple indices, including Shannon, Chao1 and Inverse Simpson (Figure 3.2 A-C). CST I exhibited the lowest diversity (Shannon index) in both high and low TFV groups [median (IQR): 0.23 (0.10 - 0.57) high vs 0.51 (0.33-0.70) low p=0.434], while CST III [median (IQR) 0.80 (0.55 - 1.18) high vs 0.77 (0.35 - 0.93) low, p= 0.268] and CST IV-B [median (IQR) 1.28 (1.07 - 1.47) high vs 1.15 (0.92 - 1.41) low, p=0.314; Table S3.2 & Figure 3.2] found no significant differences. In contrast, Group IV-C demonstrated the highest diversity, though the differences were not statistically significance between samples that had high and Low TFV concentration levels in Shannon index, Chao1 index (species richness) and Inverse Simpson index (species evenness), respectively [median (IQR):2.13 (1.93-2.51) high vs 2.23 (1.88 - 2.32) low, p=0.831; 49 (29 - 66) high vs 38 (31- 58) low, p=0.157; 5.50 (4.45 - 7.41) high vs 5.78 (4.46 - 6.27) low, p=0.886); Table S3.2 & Figure 3.2: A-C]. Additionally, no significant differences were observed in the InvSimpson index and Chao1 index between high and low TFV levels for CST I (p=603) and III (p=0.111) (Table S3.2 and Figure 3.2: B-C). However, after adjusting for the effects of site and days, the Inverse Simpson diversity index showed a significant association with TFV concentration [median (IQR) 2.56 (2.03-3.02) vs 2.23 (1.80 - 3.32), p=0.02; Table S3.2, Table S3.3 and Figure 3.2], suggesting that higher diversity may be linked to either high or low TFV levels. Additionally, TFV levels were significantly associated with species richness in CST IV-B [median (IQR)18 (16 - 21) vs 13 (12 - 21), p=0.009: Table S3.3 and Figure 3.2].

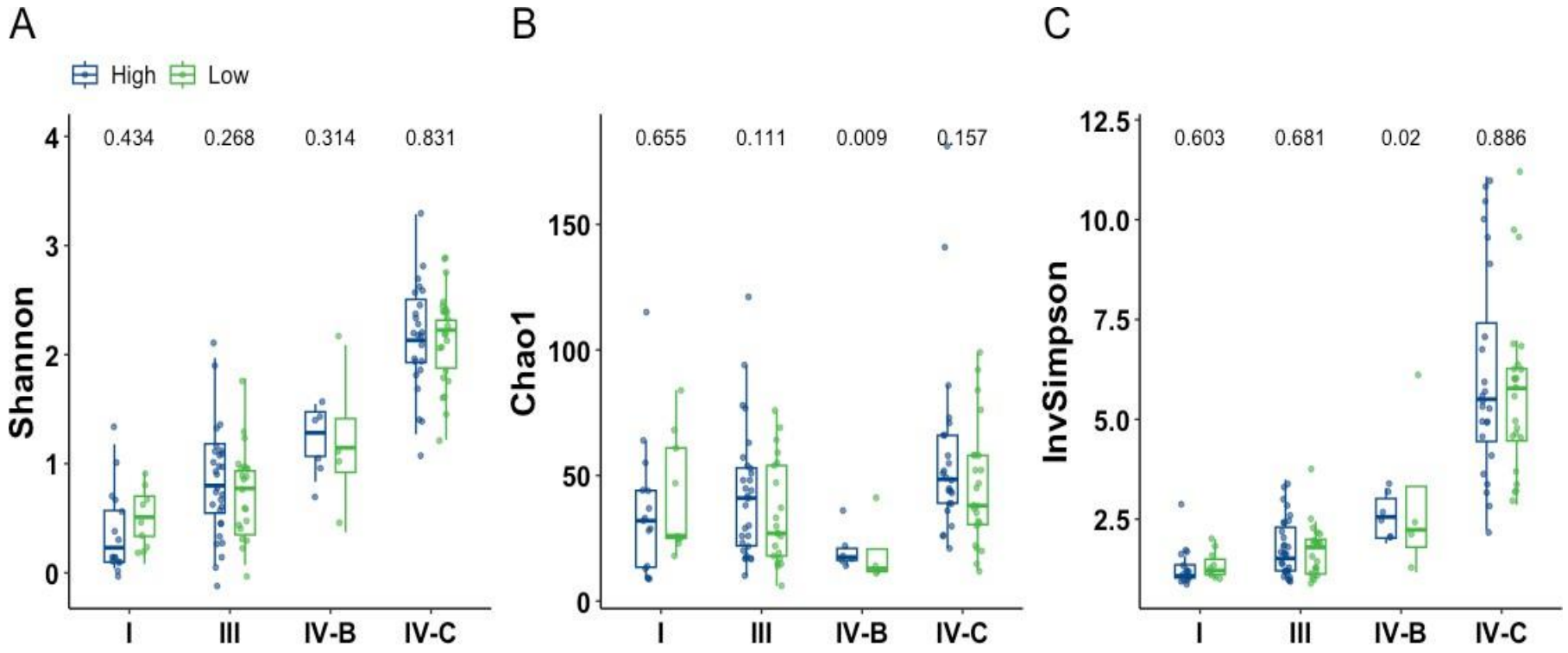


Figure 3.2: Alpha diversity indices: A-C. a subgroup analysis was performed within each CST, comparing Shannon, Chao1 and InvSimpson index of samples that had high conc to those with low concentration. Shannon diversity, Chao1 richness, and Inverse Simpson indices were calculated to assess the diversity and structure of microbial communities. The Shannon index reflects both species richness and evenness, with higher values indicating a more diverse and balanced community. Chao1 estimates total species richness, accounting for rare and potentially undetected species. The Inverse Simpson index emphasizes evenness and dominance, with higher values when species are more evenly distributed. Statistical comparisons between groups were performed using the Kruskal–Wallis test followed by post-hoc Dunn's correction.

3.3.4. The overall microbiota diversity not associated with tenofovir levels.

To evaluate the impact of vaginal microbiota on tenofovir (TFV) levels, bacterial diversity was assessed using the Shannon diversity index (within-sample diversity) and Bray–Curtis distances (between-sample diversity). First, we determined the number of days since the last gel insertion. The samples ranged from 0 to 120 days after gel use, with most collected within 0–15 days. TFV concentration declined over time following the last gel application (Figure S3.2). No significant association was observed between *Prevotella bivia* abundance and log10 TFV concentration (R=–0.077, p=0.87). Similarly, no correlation was found between TFV levels and the relative abundances of specific bacterial taxa (Lactobacillus *crispatus; R= 0.0079, p=0.93; Lactobacillus iners; R =0.014, p=0.87; Lactobacillus jensenii; R = 0.015, p=0.87; Gardnerella vaginalis; R= -0.019, p=0.83,* and *Veillonella montpellierensis; p=0.74*), nor with diversity indices (R= <0.11, p= >0.23; Figure 3.3).

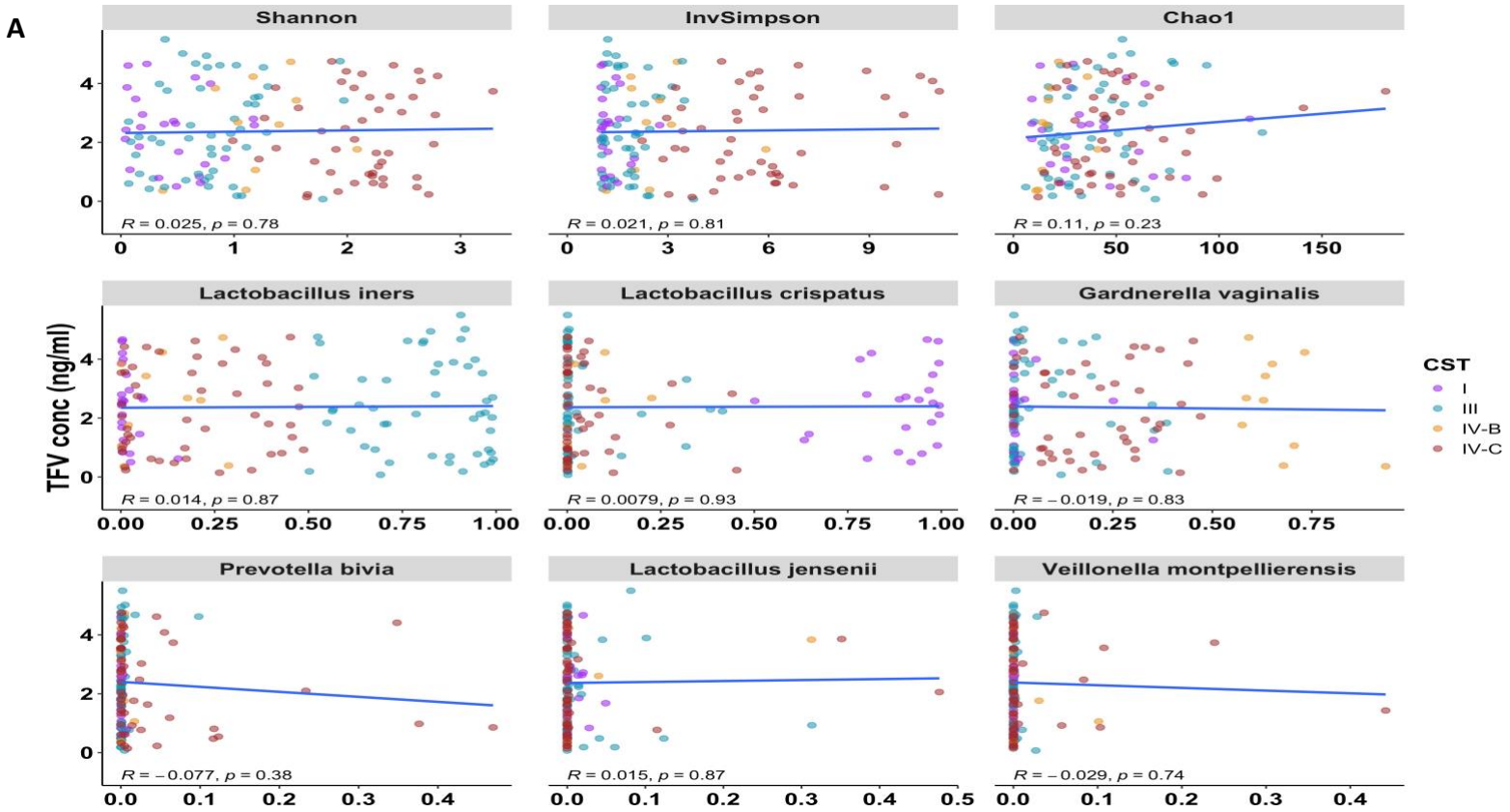
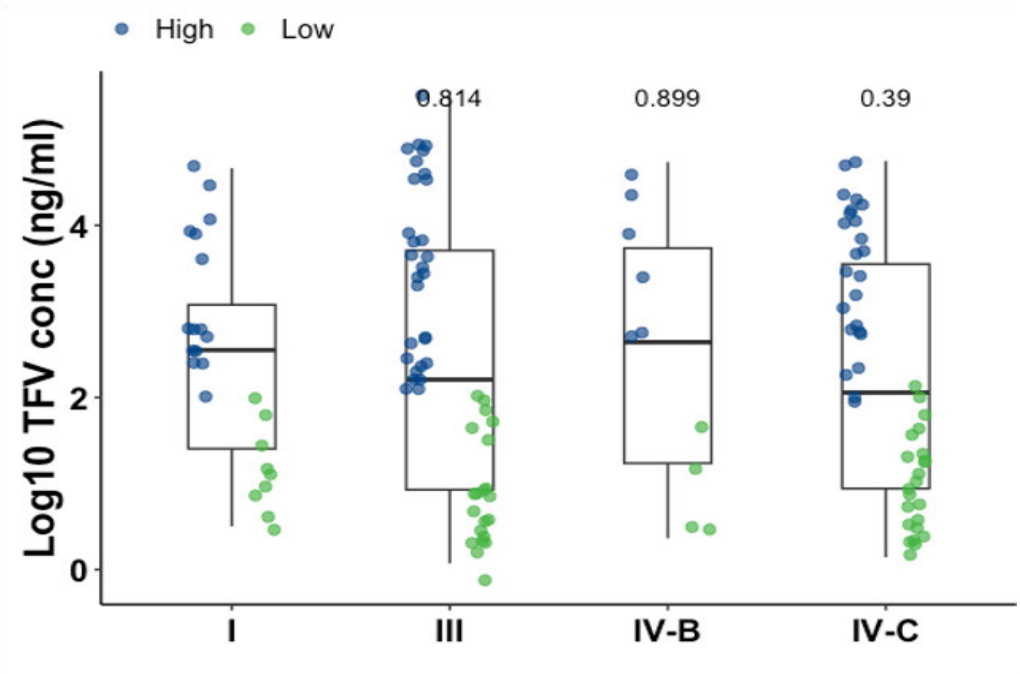


Figure 3.3: Illustrates the association between Alpha diversity indices and the Relative abundance of the top 6 bacterial taxa with tenofovir (TFV) levels. Panel (a) highlights alpha diversity indices, including Shannon, Inverse Simpson, and Chao1, which measure within-sample species diversity. Linear regression analyses depict the relationship between bacterial taxa and TFV concentrations, with statistical significance determined at a p-value threshold of 0.05.

Additionally, there were no significant differences in log10 tenofovir concentrations between CSTs [median (IQR): CST1: 2.55ng/ml (1.40ng/ml-3.08ng/ml); CST III: 2.21ng/ml (0.93ng/ml-3.71ng/ml); CST IV-B: 2.64ng/ml (1.24ng/ml-3.73ng/ml); CST IV-C: 2.06ng/ml (0.94ng/ml-3.55ng/ml); p=0.930; Table S3.4, Figure 3.4]. Furthermore, samples without STIs had a slightly high vaginal TFV concentration [median (IQR): 3.75ng/ml (2.13ng/ml-4.12ng/ml) in comparison to those that had at least one STI 2.23ng/ml (1.46ng/ml-3.55ng/ml), p=0.201], however, it was not statistically significant (Figure 3.4, Figure S3.4 & Table S3.4). Non-metric Multidimensional Scaling (NMDS) analysis of Bray–Curtis distances, based on beta diversity across 131 samples, revealed no clustering associated with TFV levels ($F=0.3795$, $R^2=0.0012$, $p=0.868$) or sexually transmitted infections ($F=1.1701$, $R^2=0.00739$, $p=0.300$; Figure 3.4B).

A



B

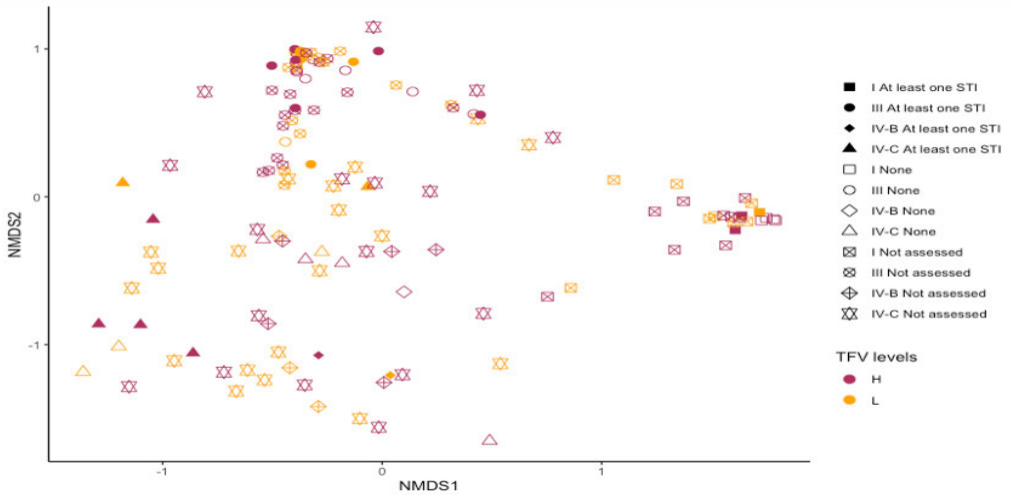


Figure 3.4: Overall microbiota diversity with use of tenofovir. (A) Demonstrate the association between community state types (CSTs), and log10-transformed tenofovir (TFV) levels. The boxplots display the median, quartiles, and variability beyond the interquartile range, revealing no significant differences across CSTs. Panel (B) presents Non-Metric Multidimensional Scaling (NMDS) based on Bray–Curtis distances, illustrating no clustering of species-level relative abundances between high (red) and low (orange) TFV groups. Shapes

denote CSTs, while design patterns represent the presence of sexually transmitted infections (STIs).

3.3.5. Impact of Cervicovaginal Tenofovir Concentrations on Vaginal Microbiota Structure

Here, we examined the association between the structure of the vaginal microbiota, BV, and STIs with cervicovaginal (CV) aspirate TFV concentrations. By analyzing the microbial CSTs and their relation to BV and STIs, we aimed to understand how these factors influence TFV levels in the vaginal environment. Among the 131 samples with detectable TFV in cervicovaginal (CV) aspirates and microbiota data, the vaginal microbiota community state types (CSTs) were grouped into *Lactobacillus*-dominant (LD; CST I and III) and non-*Lactobacillus*-dominant (nLD; CST IV-B and IV-C) categories to align with previous TFV pharmacokinetics (PK) and microbiota studies (18, 19). Of these samples, 74 exhibited *Lactobacillus*-dominated microbiota, while 57 had CST IV microbiota (Table 3.2 & Table S3.1). When CSTs were grouped as LD versus nLD, women with LD had slightly higher mean [(2.423ng/ml:LD vs 2.307ng/ml:nLD) and median (2.313 vs 2.100); p=0.7] of log-transformed TFV concentrations, though it was not significantly different (Table 3.2). Similarly, combining intermediate bacterial vaginosis and BV categories in comparison with No BV, revealed no significant differences in CV aspirate TFV concentrations [mean; 2.278 vs 2.414 and median; 1.908 vs 2.382; p=0.6]. While the proportion of samples with sexually transmitted infections (STIs) was similar between high and low TFV groups (Figure S3.4), women without STIs demonstrated a marginally higher TFV concentration (median (IQR): 3.75 (2.13-4.12) than those with at least one STI 2.23 (1.46-3.54), though this difference was not statistically significant (p=0.2, Figure S3.4, Table 3.2 & Table S3.4).

Table 3.2: Vaginal fluid TFV concentrations (ng/ml) based on BV status, *Lactobacillus* dominated (LD or non-LD) microbiota and STI status.

Characteristics		Tenofovir Concentration (ng/ml)			
		N	Mean (SD)	Median (IQR)	P value
BV status	Intermediate BV and BV	40	2.278 (1.574)	1.908 (2.610)	0.6
	No BV	91	2.414 (1.408)	2.382 (2.593)	
***Lactobacillus* status**	*Lactobacillus* dominant	74	2.423 (1.465)	2.313 (2.504)	0.7
	Non-*Lactobacillus* dominant	57	2.307 (1.454)	2.100 (2.599)	
STIs status	At leat one STI	19	2.52 (1.48)	2.23 (2.08)	0.2
	None STI	20	3.20 (1.34)	3.75 (1.99)	

[1]Wilcoxon rank sum test, [2]Wilcoxon rank sum exact test.

3.3.6. Clinical and Behavioural Factors Associated with TFV Concentrations

We evaluated the influence of clinical and behavioural factors on tenofovir (TFV) concentrations. A significant decrease in TFV levels was observed with increasing time since the last TFV use (β = -1.0, 95% CI: -1.4 to -0.71, $p < 0.001$), with women being more likely to have low TFV levels as the interval increased (odds ratio [OR]: 0.34, 95% CI: 0.17–0.71, p = 0.004). Older women exhibited slightly higher TFV levels (OR: 1.09, 95% CI: 1.00–1.18, p = 0.040), while a higher frequency of vaginal intercourse was associated with lower TFV concentrations (OR: 0.92, 95% CI: 0.85–1.00, p = 0.044) (Table 3.3). Although the presence of sexually transmitted infections (STIs) appeared to influence TFV concentrations, this association was not statistically significant (p = 0.080, Table 3.3). When categorized by *Lactobacillus*-dominant (LD) or non-*Lactobacillus*-dominant (non-LD) microbiota, no significant differences were observed in TFV concentrations or the likelihood of high/low TFV levels. Similarly, the presence of *Gardnerella vaginalis* showed no significant association with TFV concentration or the odds of high TFV levels (OR: 1.11, 95% CI: 0.14–8.92, $p > 0.9$). Overall, there was no significant relationship between TFV concentration and the vaginal microbiota, whether assessed by LD dominance, the presence of *G. vaginalis*, or bacterial community state types (Table 3.3).

Table 3.3: Clinical and Behavioral Factors Associated with TFV Levels

		Linear regression (TFV concentration)			Logistic Regression (High/Low)		
Characteristic	**N**	**Beta**	**95% CI[1]**	**p-value**	**OR[1]**	**95% CI[1]**	**p-value**
Days since TFV administration		-1.0	-1.4, -0.71	**<0.001**	0.34	0.17, 0.71	**0.004**
site				0.079			0.5
eThekwini		0.00	—		1.00	—	
Vulindlela		0.40	-0.05, 0.85		1.37	0.58, 3.24	
Age		0.05	0.01, 0.09	**0.011**	1.09	1.00, 1.18	**0.040**
Vaginal sex in the last 30 days		-0.05	-0.08, -0.01	**0.005**	0.92	0.85, 1.00	**0.044**
Any STI				**0.001**			0.080
At least one STI		0.00	—		1.00	—	
None		0.65	-0.04, 1.3		2.77	0.68, 11.3	
Not assessed		-0.48	-1.1, 0.12		0.63	0.21, 1.88	
Frequency of condom use				0.2			0.3
Always		0.00	—		1.00	—	
Never		-0.09	-0.72, 0.54		1.30	0.40, 4.31	
Sometimes		-0.43	-0.90, 0.04		0.54	0.22, 1.32	
Lacto Dominance (Adjusted)							
LD		0.00	—		1.00	—	
Non-LD		0.01	-0.41, 0.43	>0.9	0.89	0.39, 2.00	0.8
CST (Adjusted)	131						
I		0.00	—		1.00	—	
III		0.10	-0.41, 0.62	0.7	0.95	0.32, 2.82	>0.9
IV-B		0.59	-0.26, 1.4	0.2	2.30	0.37, 14.5	0.4
IV-C		0.06	-0.43, 0.55	0.8	0.82	0.26, 2.59	0.7
Gardnerella vaginalis (Adjusted)	131	0.46	-0.66, 1.6	0.4	1.11	0.14, 8.92	>0.9

[1]CI = Confidence Interval, OR = Odds Ratio

3.4. DISCUSSION

While it is recognized that vaginal microbiota can influence the pharmacokinetics of topical antiretrovirals, particularly TFV in single-agent, peri-coitally dosed products (18, 32), the *in vitro* biodegradation of TFV may not accurately reflect *in vivo* conditions. This study utilized 16S rRNA gene sequencing to investigate the impact of the vaginal microbiota on genital tract TFV concentrations *in vivo*.

Our study revealed no differences in vaginal microbiota composition between women with high and low genital tract TFV levels. This finding was further supported by the observation that CSTs, categorized for this population, clustered similarly between the two groups, suggesting that TFV use did not influence the vaginal microbiota composition. Consistent with prior research (33), our study aligned with findings demonstrating high and comparable local concentrations of TFV and TFV-DP among intravaginal ring users with *Lactobacillus*-dominated microbiota compared to those with BV-related microbiota. Additionally, we observed no correlation between genital TFV concentrations and any of the most abundant individual bacterial strains, consistent with previous observations (33). In contrast, earlier studies (18, 19) reported correlations between TFV-diphosphate (TFV-DP) levels in genital tissues or TFV levels in plasma and markers of bacterial vaginosis, such as Nugent scores and increased quantitative PCR detection of *G. vaginalis* and *A. vaginae*. Furthermore, we found no significant differences in mean log-transformed TFV concentrations between women with *Lactobacillus*-dominated microbiota and those with non-*Lactobacillus*-dominated microbiota. Although previous studies identified a negative correlation between tenofovir levels and bacterial vaginosis-associated bacteria, their limitations included data collected at single, random time points and the use of bacterial proteins (CAPRISA 004 study) or Nugent scores (FAME program) to classify vaginal bacterial communities. In contrast, our analysis relied on CST groupings. Our study, along with the FAME 04 sub-analysis (19) and the CONRAD A13-128 (33), reutilized direct quantification of genital tract TFV-DP concentrations, a method not feasible in the CAPRISA 004 cohort (18). Future studies with larger cohorts are necessary to validate these findings, including investigations leveraging comparative genomic approaches to identify subspecies associated with variations in TFV levels.

Although not the primary focus of this study, our findings suggest that TFV concentrations are associated with factors such as days since gel use, age, STIs, and the frequency of vaginal sex within the last 30 days. These observations are consistent with prior studies demonstrating that the timing of TFV gel application relative to sexual activity significantly affects drug level (34), likely due to semen-induced leakage, displacement, and gel dilution. In contrast, Kashuba et al., (2015) reported a poor correlation between the time of the last dose and drug levels in the CAPRISA 004 trial, suggesting potential bias in self-reported timing of gel insertion (31). Additionally, our study found that older women had increased odds of achieving high TFV concentrations. This aligns with findings from the Depivirine ring studies, which showed greater protective efficacy in older women compared to younger participants (14, 35). These findings highlight the complexity of factors influencing TFV concentrations and suggest that future studies should consider these variables, including the timing of gel use, age, and sexual activity, to optimize the effectiveness of TFV-based interventions.

This study has several important limitations that should be considered when interpreting the findings. First, the study was not designed to evaluate the primary outcomes of the parent study, which could limit the scope and focus of our analysis. Additionally, since all participants used the TFV gel and there was no placebo arm, we were unable to compare the effects of TFV gel use against a non-treatment control, which could provide more robust conclusions about the observed relationships. The study design also involved coitally dependent, unscheduled application of the tenofovir gel, which may not have coincided with the timing of clinic visits when PK samples were collected. This mismatch in timing could introduce variability in the assessment of drug concentrations, as the pharmacokinetics of TFV may be influenced by the time elapsed between gel use and sample collection. Moreover, due to the nature of the trial, we were unable to assess the longitudinal impact of the vaginal microbiota on TFV concentrations. While some participants had multiple visits, the vaginal microbiome can change over time, and our cross-sectional approach did not capture these dynamic changes. Longitudinal data would have allowed for a more comprehensive understanding of how fluctuations in the vaginal microbiota might influence TFV concentrations over time. Another limitation is the reliance on self-reported data for the timing and frequency of gel use. While self-reporting is commonly used in clinical studies, it is subject to recall bias, and participants may not accurately report the exact timing or frequency of gel application. This could lead to

inaccuracies in the assessment of the relationship between gel use and TFV concentrations. Finally, there was limited availability of comprehensive STI data, which could have influenced the accuracy of our correlations between TFV levels and the vaginal microbiome composition. The presence of STIs can significantly alter the vaginal microbiota and may also affect the pharmacokinetics of TFV. Without more detailed STI data, it is challenging to account for the full range of factors that might influence the observed relationships between microbiome composition and drug concentrations.

In conclusion, our study found no significant differences in vaginal microbiota composition between women with high and low genital tract TFV levels, suggesting that TFV use does not influence the vaginal microbiome. These findings align with prior research demonstrating high and comparable local concentrations of TFV and TFV-DP in users with *Lactobacillus*-dominated microbiota compared to BV-related microbiota. Furthermore, we observed no correlation between genital TFV concentrations and individual bacterial strains, consistent with earlier studies. However, our results contrast with studies that reported correlations between TFV levels and markers of bacterial vaginosis, highlighting differences in study design and analytical methods. These findings underscore the need for future studies with larger cohorts and advanced methodologies to further elucidate the relationships between TFV pharmacokinetics, vaginal microbiota, and other influencing factors.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions: NMM, DSK and SN conceived and designed the analysis and NMM, JX, AM, GM & LN carried out experiments. AOK, MPL, & SN performed the analyses and provided statistical support. QAK was the principal investigator, and LM & SSAK were the co-principal investigators of the CAPRISA 008 study. NMM and SN prepared the first draft. NMM, AOK, MPL, JX, LL, AM, GM, LN, LM, JSP, QAK, SSAK, DA, DSK and SN contributed to the final interpretation and discussion of the results and writing of the manuscript.

Funding: This research was partially supported by the Department of Science and Innovation – National Research Foundation Centre of Excellence in HIV Prevention (UID 96354) and the Bill & Melinda Gates Foundation (BMGF, INV-018075). NMM was funded by the Poliomyelitis Research Foundation Research Grant (grant# 21/59). SN was supported by Columbia University-Southern African Fogarty AITRP Programme (grant# D43TW00231), National Research Fund Thuthuka Research Grant (grant# TTK160510164586), and Poliomyelitis Research Foundation Research Grant (grant# 16/17).

Acknowledgements: We thank all the CAPRISA 008 study participants and the study team for their contributions to this research.

Availability of Data and Materials:

The raw 16S rRNA gene sequence, along with all the experimental and procedural metadata such as unrarefied OTU tables, and R scripts used in this study data will be deposited in the sequenced Read archive (SRA) at NCBI and GitHub (https://github.com).

References

1. (UNAIDS) JUNPoHA. 2023. *Global HIV & AIDS statistics — Fact sheet*
2. UNAIDS. 2024. The urgency of now: AIDS at a crossroads. Geneva: Joint United Nations Programme on HIV/AIDS; 2024.
3. Baeten JM, Donnell D, Ndase P, Mugo NR, Campbell JD, Wangisi J, Tappero JW, Bukusi EA, Cohen CR, Katabira E, Ronald A, Tumwesigye E, Were E, Fife KH, Kiarie J, Farquhar C, John-Stewart G, Kakia A, Odoyo J, ..., Celum C. 2012. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med* 367: 399-410
4. Choopanya K, Martin M, Suntharasamai P, Sangkum U, Mock PA, Leethochawalit M, Chiamwongpaet S, Kitisin P, Natrujirote P, Kittimunkong S, Chuachoowong R, Gvetadze RJ, McNicholl JM, Paxton LA, Curlin ME, Hendrix CW, Vanichseni S. 2013. Antiretroviral prophylaxis for HIV infection in injecting drug users in Bangkok, Thailand (the Bangkok Tenofovir Study): a randomised, double-blind, placebo-controlled phase 3 trial. *The Lancet* 381: 2083-90
5. Grant RM, Lama JR, Anderson PL, McMahan V, Liu AY, Vargas L, Goicochea P, Casapia M, Guanira-Carranza JV, Ramirez-Cardich ME, Montoya-Herrera O, Fernandez T, Veloso VG, Buchbinder SP, Chariyalertsak S, Schechter M, Bekker LG, Mayer KH, Kallas EG, ..., Glidden DV. 2010. Preexposure chemoprophylaxis for HIV prevention in men who have sex with men. *N Engl J Med* 363: 2587-99
6. McCormack S, Dunn D, On behalf of the PROUD Study Group. 2015. Pragmatic Open-Label Randomised Trial of Preexposure Prophylaxis: The PROUD Study. [Abstract 22LB]. In *Conference on Retroviruses and Opportunistic Infections (CROI), 23-26 February*. Seattle, Washington
7. Molina J-M, Capitant C, Spire B, Pialoux G, Chidiac C, Charreau I, Tremblay C, Meyer L, Delfraissy J-F, On behalf of the ANRS Ipergay Study Group. 23-26 February 2015. On Demand PrEP with Oral TDF-FTC in MSM: Results of the ANRS Ipergay Trial. [Abstract 23LB]. In *Conference on Retroviruses and Opportunistic Infections (CROI)*. Seattle, Washington
8. Thigpen MC, Kebaabetswe PM, Paxton LA, Smith DK, Rose CE, Segolodi TM, Henderson FL, Pathak SR, Soud FA, Chillag KL, Mutanhaurwa R, Chirwa LI, Kasonde M, Abebe D, Buliva E, Gvetadze RJ, Johnson S, Sukalac T, Thomas VT, ..., Brooks JT.

2012. Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana. *N Engl J Med* 367: 423-34

9. Abdool Karim Q, Abdool Karim SS, Frohlich JA, Grobler AC, Baxter C, Mansoor LE, Kharsany ABM, Sibeko S, Mlisana KP, Omar Z, Gengiah TN, Maarschalk S, Arulappan N, Mlotshwa M, Morris L, Taylor D, on behalf of the CAPRISA 004 Trial Group. 2010. Effectiveness and Safety of Tenofovir Gel, an Antiretroviral Microbicide, for the Prevention of HIV Infection in Women. *Science* 329: 1168-74
10. Rees H., Delany-Moretlwe S.A., Lombard C, Baron D, Panchia R, Myer L, Schwartz JL, Doncel GF, Gray G, On behalf of the FACTS 001 Study Team. 23-26 February 2015. FACTS 001 Phase III Trial of Pericoital Tenofovir 1% Gel for HIV Prevention in Women. [Abstract 26LB]. In *Conference on Retroviruses and Opportunistic Infections (CROI)*. Seattle, Washington
11. Van Damme L, Corneli A, Ahmed K, Agot K, Lombaard J, Kapiga S, Malahleha M, Owino F, Manongi R, Onyango J, Temu L, Monedi MC, Mak'Oketch P, Makanda M, Reblin I, Makatu SE, Saylor L, Kiernan H, Kirkendale S, Wong C, Grant R, Kashuba A, Nanda K, Mandala J, Fransen K, Deese J, Crucitti T, Mastro TD, Taylor D, for the FEM-PrEP Study Group. 2012. Preexposure Prophylaxis for HIV Infection among African Women. *New England Journal of Medicine* 367: 411-22
12. Marrazzo JM, Ramjee G, Richardson BA, Gomez K, Mgodi N, Nair G, Palanee T, Nakabiito C, van der Straten A, Noguchi L, Hendrix CW, Dai JY, Ganesh S, Mkhize B, Taljaard M, Parikh UM, Piper J, Mâsse B, Grossman C, Rooney J, Schwartz JL, Watts H, Marzinke MA, Hillier SL, McGowan IM, Chirenje ZM. 2015. Tenofovir-Based Preexposure Prophylaxis for HIV Infection among African Women. *New England Journal of Medicine* 372: 509-18
13. Baeten JM, Donnell D, Ndase P, Mugo NR, Campbell JD, Wangisi J, Tappero JW, Bukusi EA, Cohen CR, Katabira E, Ronald A, Tumwesigye E, Were E, Fife KH, Kiarie J, Farquhar C, John-Stewart G, Kakia A, Odoyo J, Mucunguzi A, Nakku-Joloba E, Twesigye R, Ngure K, Apaka C, Tamooh H, Gabona F, Mujugira A, Panteleeff D, Thomas KK, Kidoguchi L, Krows M, Revall J, Morrison S, Haugen H, Emmanuel-Ogier M, Ondrejcek L, Coombs RW, Frenkel L, Hendrix C, Bumpus NN, Bangsberg D, Haberer JE, Stevens WS, Lingappa JR, Celum C. 2012. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med* 367: 399-410

14. Nel A, van Niekerk N, Kapiga S, Bekker L-G, Gama C, Gill K, Kamali A, Kotze P, Louw C, Mabude Z. 2016. Safety and efficacy of a dapivirine vaginal ring for HIV prevention in women. *New England Journal of Medicine* 375: 2133-43
15. Rees H., Delany-Moretlwe S.A., Lombard C, Baron D, Panchia R, Myer L, Schwartz JL, Doncel GF, Gray G, On behalf of the FACTS 001 Study Team. 2015. FACTS 001 Phase III Trial of Pericoital Tenofovir 1% Gel for HIV Prevention in Women. [Abstract 26LB]. In *Conference on Retroviruses and Opportunistic Infections (CROI)*. Seattle, Washington
16. Thigpen MC, Kebaabetswe PM, Paxton LA, Smith DK, Rose CE, Segolodi TM, Henderson FL, Pathak SR, Soud FA, Chillag KL, Mutanhaurwa R, Chirwa LI, Kasonde M, Abebe D, Buliva E, Gvetadze RJ, Johnson S, Sukalac T, Thomas VT, Hart C, Johnson JA, Malotte CK, Hendrix CW, Brooks JT. 2012. Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana. *N Engl J Med* 367: 423-34
17. Mansoor LE, Lewis L, Naicker CL, Harkoo I, Dawood H, Naidoo K, Gengiah TN, Samsunder N, Beesham I, Abdool Karim SS. 2022. Prospective study of oral pre-exposure prophylaxis initiation and adherence among young women in KwaZulu-Natal, South Africa. *Journal of the International AIDS Society* 25: e25957
18. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noel-Romas L, Grobler A, Westmacott G, Xie IY, Butler J, Mansoor L, McKinnon LR, Passmore JS, Abdool Karim Q, Abdool Karim SS, Burgener AD. 2017. Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356: 938-45
19. Hillier SL, Meyn LA, Bunge A, Austin M, Moncla BJ, Dezzutti CS, Schwartz J, Marzinke MA, Hendrix C, Rohan LC. 2017. *IMPACT OF VAGINAL MICROBIOTA ON GENITAL TISSUE AND PLASMA CONCENTRATIONS OF TENOFOVIR*. Presented at Conference on Retroviruses and Opportunistic Infections, Seattle, WA
20. Heffron R, McClelland RS, Balkus JE, Celum C, Cohen CR, Mugo N, Bukusi E, Donnell D, Lingappa J, Kiarie J, Fiedler T, Munch M, Fredricks DN, Baeten JM, Partners Pr EPST. 2017. Efficacy of oral pre-exposure prophylaxis (PrEP) for HIV among women with abnormal vaginal microbiota: a post-hoc analysis of the randomised, placebo-controlled Partners PrEP Study. *Lancet HIV*

21. Mazibuko-Motau N, Sobia P, Xu J, Elsherbini JA, San JE, Lewis L, Mtshali A, Mzobe G, Ntuli L, Abdool Karim SS. 2022. Vaginal microbial shifts are unaffected by oral pre-exposure prophylaxis in South African women. *Scientific Reports* 12: 16187
22. Mansoor LE, Yende-Zuma N, Baxter C, Mngadi KT, Dawood H, Gengiah TN, Samsunder N, Schwartz JL, Doncel GF, Karim QA. 2019. Integrated provision of topical pre-exposure prophylaxis in routine family planning services in South Africa: a non-inferiority randomized controlled trial. *African Journal of Reproduction and Gynaecological Endoscopy* 22
23. Nugent RP, Krohn MA, Hillier SL. 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of clinical microbiology* 29: 297-301
24. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, Padavattan N, Ismail N, Moodley A, Sabatini ME, Ghebremichael MS, Nusbaum C, Huttenhower C, Virgin HW, Ndung'u T, Dong KL, Walker BD, Fichorova RN, Kwon DS. 2015. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* 42: 965-76
25. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 13: 581-3
26. McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8: e61217
27. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO. 2011. Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences* 108: 4680-7
28. France MT, Ma B, Gajer P, Brown S, Humphrys MS, Holm JB, Waetjen LE, Brotman RM, Ravel J. 2020. VALENCIA: a nearest centroid classification method for vaginal microbial communities based on composition. *Microbiome* 8: 1-15
29. Govender K, Gibhard L, Du Plessis L, Wiesner L. 2015. Development and validation of a lc–ms/ms method for the quantitation of lumefantrine in mouse whole blood and plasma. *Journal of Chromatography B* 985: 6-13

30. Karim SSA, Kashuba AD, Werner L, Karim QA. 2011. Drug concentrations after topical and oral antiretroviral pre-exposure prophylaxis: implications for HIV prevention in women. *The Lancet* 378: 279-81

31. Kashuba AD, Gengiah TN, Werner L, Yang K-H, White NR, Karim QA, Karim SSA. 2015. Genital tenofovir concentrations correlate with protection against HIV infection in the CAPRISA 004 trial: importance of adherence for microbicide effectiveness. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 69: 264-9

32. Hillier S, Meyn L, Bunge K, Austin M, Moncla B, Dezzutti C, Rohan L. 2017. *Impact of vaginal microbiota on genital tissue and plasma concentrations of tenofovir*. Presented at Conference on retroviruses and opportunistic infections (CROI)

33. Thurman AR, Schwartz JL, Ravel J, Gajer P, Marzinke MA, Yousefieh N, Anderson SM, Doncel GF. 2019. Vaginal microbiota and mucosal pharmacokinetics of tenofovir in healthy women using tenofovir and tenofovir/levonorgestrel vaginal rings. *PLoS One* 14: e0217229

34. Herold BC, Chen BA, Salata RA, Marzinke MA, Kelly CW, Dezzutti CS, McGowan I, Galaska B, Levy L, Piper JM. 2016. Impact of sex on the pharmacokinetics and pharmacodynamics of 1% tenofovir gel. *Clinical infectious diseases* 62: 375-82

35. Baeten JM, Palanee-Phillips T, Brown ER, Schwartz K, Soto-Torres LE, Govender V, Mgodi NM, Matovu Kiweewa F, Nair G, Mhlanga F. 2016. Use of a vaginal ring containing dapivirine for HIV-1 prevention in women. *New England Journal of Medicine* 375: 2121-32

Supplementary

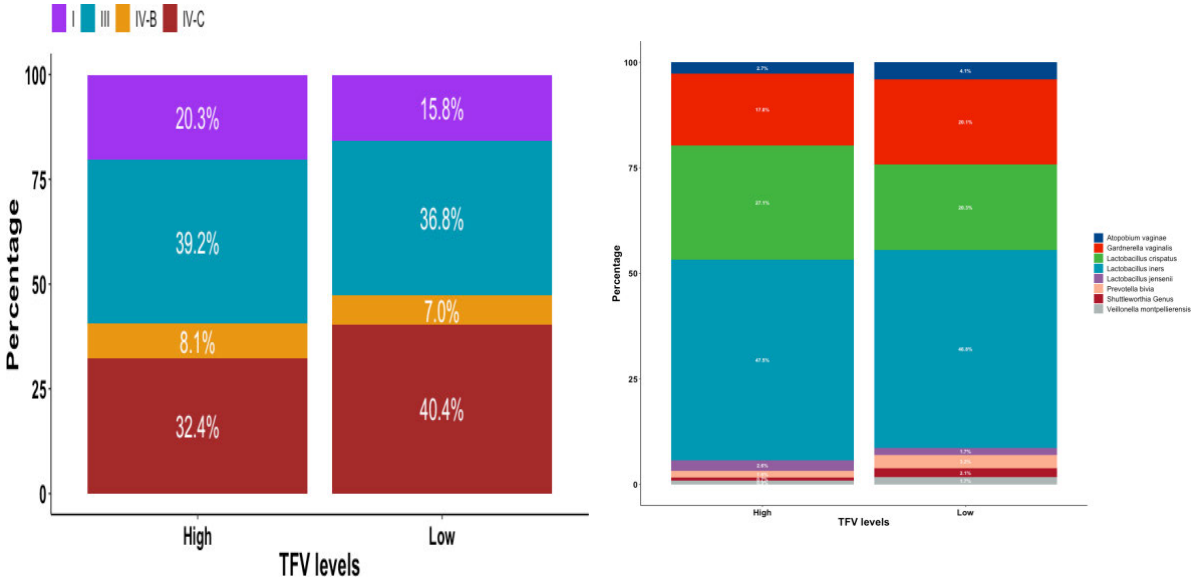


Figure S3.1: Community state types (CSTs) by tenofovir levels. *Lactobacillus crispatus* dominant; CST1, *L. iners* dominant; CST III, *Gardnerella vaginalis* dominant; CST IV-B and mix bacterial taxa; CST IV-C

Table S3.1: BV, intermediate BV and no BV in 131 visits dominated with *Lactobacillus* and non-*Lactobacillus*

	***Lactobacillus* dominance, N=74**		**Non-*Lactobacillus* dominance, N= 57**	
Characteristic	CST, N = 24	CST III, N = 50	CST IV-B, N = 10	CST IV-C, N = 47
Visit code				
1280			1 (10%)	
2040			1 (10%)	
2060			1 (10%)	
2100			2 (20%)	
2110			1 (10%)	
2130			2 (20%)	
2150			1 (10%)	
2170			1 (10%)	
TFV Conc ng/ml				
Median (IQR)	2,090 (1,273, 2,110)	2,080 (1,260, 2,100)		2,080 (1,240, 2,105)
BV category				
Intermediate	1 (4.2%)	7 (14%)	3 (30%)	13 (28%)
No BV	23 (96%)	40 (80%)	5 (50%)	23 (49%)
BV		3 (6.0%)	2 (20%)	11 (23%)
strt				
population	24 (100%)	50 (100%)	10 (100%)	47 (100%)

Table S3.2: Bacterial diversity was assessed using the Shannon diversity index between samples that had high and low TFV concentration.

Characteristics		Median (IQR)			
		CST I	CST III	CST IV-B	CST IV-C
Shannon Index	High TFV	0.23(0.10-0.57)	0.80(0.5.5-1.18)	1.28(1.07-1.47)	2.13(1.93-2.51)
	Low TFV	0.51(0.33-0.70)	0.77(0.35-0.93)	1.15(0.92-1.41)	2.23(1.88-2.32)
	p-value	0.238	0.249	0.914	0.858
Chao1	High TFV	32(14-44)	41(22-53)	18(16-21)	49(29-66)
	Low TFV	26(25-61)	27(18-54)	13(12-21)	38(31-58)
	p-value	0.550	0.366	0.285	0.221
InvSimpson	High TFV	1.08(1.03-1.36)	1.52(1.21-2.30)	2.56(2.03-3.02)	5.50(4.45-7.41)
	Low TFV	1.22(1.11-1.50)	1.79(1.13-1.99)	2.23(1.80-3.32)	5.78(4.46-6.27)
	p-value	0.238	0.612	0.914	0.891

Table S3.3: Association of TFV concentrations with days since TFV administration

Characteristic	OR	95% CI	p-value	OR	95% CI	p-value
		Chao1			InvSimpson	
Chao1/InvSimpson	1.01	1.0 - 1.02	0.3	0.94	0.82 - 1.09	0.4
Days since the TFV administration	0.40	0.20 - 0.77	0.006	0.38	0.19 - 0.73	0.004
Site			0.6			0.7
eThekwini	1.00	—		1.00	----	
Vulindlela	0.84	0.40 - 1.76		0.86	0.41 - 1.80	

[1]OR = Odds Ratio, CI = Confidence Interval

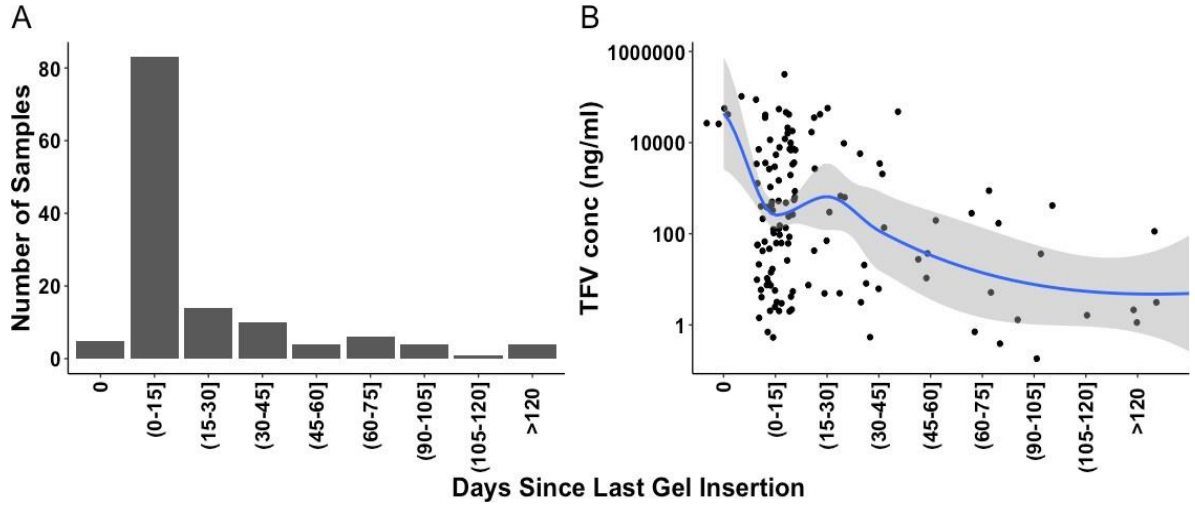


Figure S3.2: Individual measurements of TFV concentration at various times since the last gel insertion. Line: A smooth line fitted through the data points, showing the trend in TFV concentration over time. TFV concentration decreases over time after the last gel insertion. There is an initial rapid decrease followed by a more gradual decline. The shaded area represents the confidence interval, indicating the variability of the data around the smooth line.

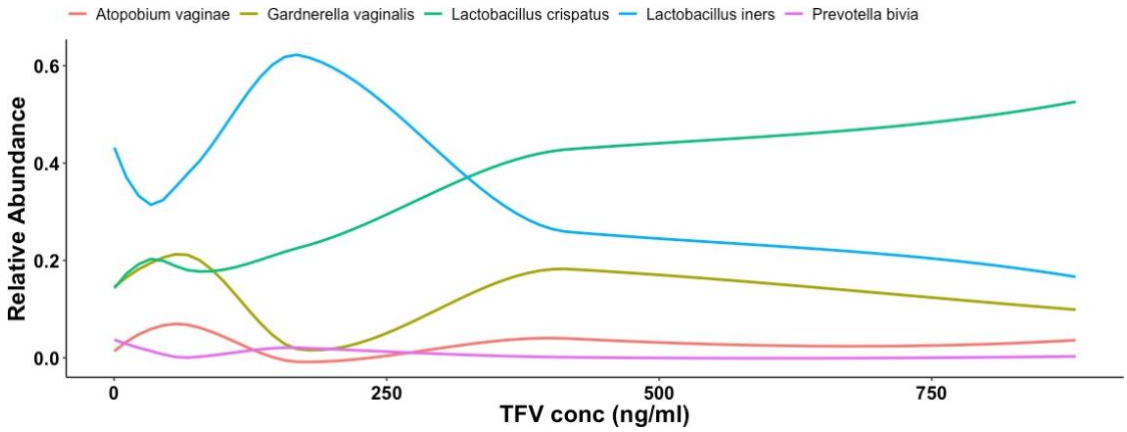


Figure S3.3: Correlation of tenofovir (TFV) vaginal fluid concentration with the relative abundance of *Gardnerella vaginalis*, *Atopobium vaginae, Lactobacillus crispatus, Lactobacillus iners* and *Prevotella bivia.*

Table S3.4: Association between CSTs and STIs with log_{10} transformed tenofovir concentrations.

Characteristics		N	Tenofovir Concentration (ng/ml)	
		131	Median (IQR)	P value
CSTs	CST I	24	2.55(1.40-3.08)	0.930
	CST III	50	2.21(0.93-3.71)	
	CST IV-B	10	2.64(1.24-3.73)	
	CST IV-C	47	2.06(0.94-3.55)	
STIs presence	At least one STI	19	2.23(1.46-3.54)	0.017
	None	20	3.75(2.13-4.12)	
	Not assessed	92	2.08(0.80-3.34)	

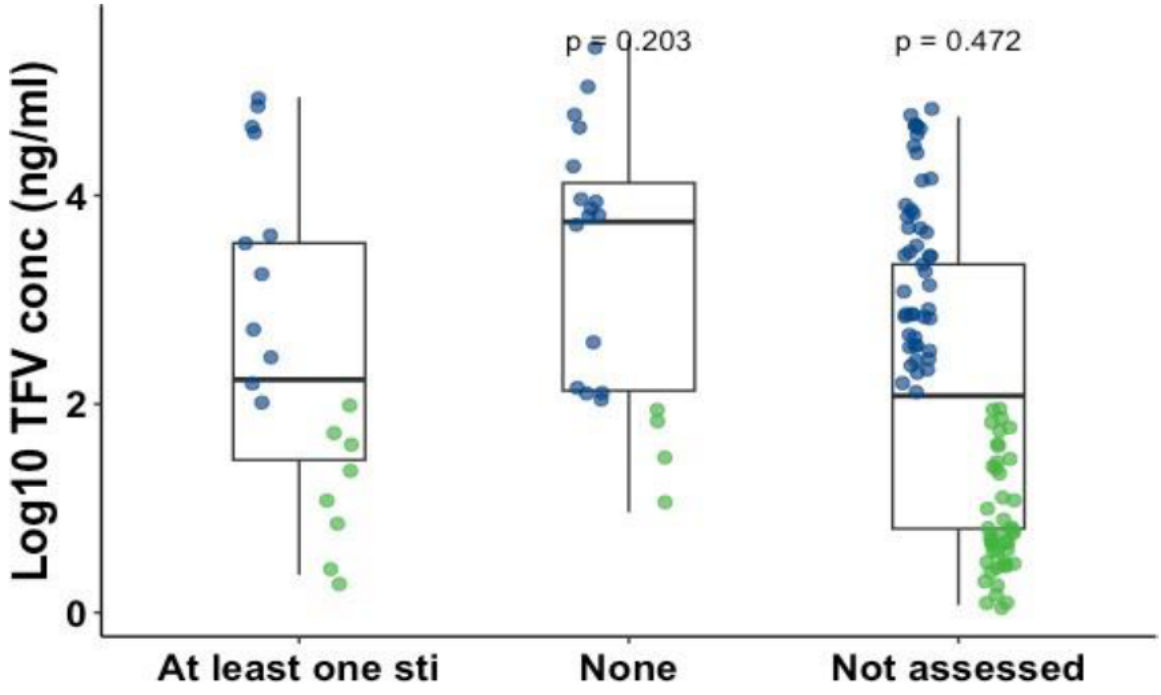


Figure. S3.4: Association of STIs with log10 TFV concentrations (ng/ml)

CHAPTER 4

Impact of Vaginal Insertion Products, Sexual Activity, and Vaginal Microbiome Dynamics on Genital Inflammation Markers Among South African Women

The preceding chapters established that PrEP does not affect the vaginal microbiota and that vaginal dysbiosis does not compromise the efficacy of PrEP. However, a thorough investigation into the impact of intravaginal practices (IVPs) on the vaginal microbiota remains critical, particularly due to the widespread use of vaginal insertion products (VIPs) and their potential associations with altered vaginal microbiota and increased HIV susceptibility among women in sub-Saharan Africa. In Chapter 4, we hypothesised that VIP use would lead to alterations in the vaginal microbiome and that these microbial changes would influence concentrations of cellular and cytokine markers of genital inflammation. Contrary to our hypothesis, the findings revealed that VIP use did not affect the vaginal microbiota or immune modulation. This manuscript, entitled *Effects of Sexual Activity and Vaginal Insertion Product Use on Vaginal Microbiota and Genital Inflammation Markers,* has been submitted for peer review in BioMed Central: Microbiome

Contribution: For this study, I was centrally involved in the conception and design of the analysis, working closely with Dr. Sinaye Ngcapu. I led and participated in the data analyses alongside Dr. Ngcapu, Dr. Olona Asavela Kama, and Dr. Marothi Letsoalo, performing microbiome data processing, statistical analyses, and integration with clinical metadata. The CAPRISA 090 study was overseen by principal investigators LGB, JSP, LM, and HBJ, who provided oversight and access to study samples. All other co-authors, including SN, PR, PG, CML, NR, AGA, SS, RH, MTM, BM, HA, DP, WH, LJPL, DA, AS, QAK, LGB, JSP, HH, LM, HBJ, and SN, contributed to the interpretation and discussion of results and provided critical input during manuscript preparation. I drafted the initial manuscript and coordinated revisions with all co-authors, incorporating feedback to finalize the publication. In summary, my primary contributions encompassed study conceptualization, analysis, interpretation of findings, and manuscript drafting, forming a substantial and independent component of my PhD training, while co-authors provided complementary guidance, supervision, and critical review throughout the study.

Impact of Vaginal Insertion Products, Sexual Activity, and Vaginal Microbiome Dynamics on Genital Inflammation Markers Among South African Women

Mazibuko-Munonde N[1,2]*, Kama OA [1]*, Letsoalo M[1]*, Ngcobo S[1], Radebe P[3], Gumbi P[1,3] Mehou-Loko C[4], Bunjun R, Mtshali A[1,2], Mzobe GF[1,2], Magini SN[1,2], Ntuli L[1,2], Maphumulo N[1], Mntambo N[1], Radzey N[4], Abrahams AG[4], Sibeko S[5], Harryparsad R[4], Manhanzva MT[4], Meyer B[4], Ahmed N[6], Potloane D,[1] Horsnell W[4], Liebenberg LJP[1,2], Archary D[1,2], Sivro A[1,2,7,8], Abdool Karim Q[1], Bekker LG[4,6], Passmore JS[1,3,9], Humphries H[10,11], Masson L[1,4,13,14], Jaspan HB[4,10#], Ngcapu S[1,2#]

[1]Centre for the AIDS Programme of Research in South Africa, Durban, South Africa
[2]Department of Medical Microbiology, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, South Africa
[3]Biochemistry Department, School of Life Sciences, University of KwaZulu-Natal, South Africa
[4]Institute of Infectious Disease and Molecular Medicine and Department of Pathology, University of Cape Town, South Africa
[5]Faculty of Medicine and Health Sciences, University of Stellenbosch, South Africa
[6]Desmond Tutu HIV Centre, Cape Town, University of Cape Town, South Africa
[7]JC Wilt Infectious Disease Research Centre, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada
[8]Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, Manitoba, Canada
[9]National Health Laboratory Service, Cape Town, South Africa
[10]Seattle Children's Research Institute and University of Washington, USA
[11]Centre for Community-Based Research, Human Science Research Council, South Africa
[12]Department of Psychology, University of KwaZulu-Natal, Pietermaritzburg, South Africa
[13]Life Sciences Discipline, Disease Elimination Program, Burnet Institute, Melbourne, Australia
[14]Central Clinical School, Monash University, Melbourne, Australia

*Co-first: these authors share first authorship
[#]Co-senior: these authors share senior authorship

Abstract

The use of vaginal insertion products (VIPs) is widespread among women in Southern Africa, with many seeking a dry, tight vaginal environment during sexual intercourse. Research suggests that certain VIPs can shift the *Lactobacillus*-dominant vaginal microbiota toward a more diverse, anaerobic community associated with bacterial vaginosis. This study aimed to characterise the vaginal microbiome in South African adolescent girls and adult women, focusing on the effects of sexual activity and VIP use on microbial dynamics and genital inflammation markers using 16S rRNA gene sequencing, multiplex bead arrays, and flow cytometry. This study found that while all women at the Western Cape (WC) site primarily used soap and water for genital hygiene, a significant proportion of participants at the KwaZulu-Natal (KZN) site used a variety of VIPs for both hygiene and sexual enhancement, with 58% (68/117) of adolescent girls and 65% (38/56) of adult women reporting VIP use. The vaginal microbiome of adolescent girls was primarily dominated by *Lactobacillus iners* and BV-associated communities, similar to adult women. VIP users had significantly higher levels of *Sneathia* and *L. iners* over time compared to non-users. Non-users had higher concentrations of pro-inflammatory cytokines (MIP-1β, MIP-1α, IFNα, IL-6, G-CSF) and lower concentrations of IP-10, GM-CSF, and immune cell markers. Sexual activity timing influenced immune responses, with a significant decrease in cytokines (MIP-1β, IL-8, IL-17, TRAIL, IL-1β, IL-6, IL-10) 10 days post-coitus. VIP users showed increased levels of IFN-α and G-CSF. The study concluded that vaginal microbiota diversity and sexual activity timing significantly influenced immune responses, while VIP use had a subtle effect on microbiota or immune modulation. These findings highlight the importance of microbial diversity and sexual activity timing in shaping vaginal health and immune function.

4.1. Introduction

Vaginal practices are common among women worldwide for various purposes, including cleansing, tightening, drying, or warming the vaginal area, often aimed at enhancing hygiene, health, or sexual pleasure (1). The World Health Organisation (WHO) categorizes these practices into seven classifications: external washing, intravaginal cleansing, external application, intravaginal insertion, oral ingestion, vaginal streaming or smoking, and anatomical modification. Intravaginal practices specifically include intravaginal cleansing, utilizing cloth or fingers to clean the interior of the vagina; and intravaginal insertion, which involves placing substances such as powders, creams, herbs, antiseptics, detergents, or tissues inside the vagina (1, 2). Understanding these practices is crucial, particularly in the context of their prevalence and potential health implications in various regions, including Africa.

Research from Africa indicates that these intravaginal practices are widespread in areas with high HIV prevalence (3-10), especially in South Africa, where approximately 60–90% of female sex workers in KwaZulu-Natal province reported using intravaginal substances to achieve a dry and tight vagina, prevent unintended pregnancies, and address sexually transmitted infections and vaginal discharge (7, 8). Studies conducted among women attending family planning clinics in Zambia and Zimbabwe further demonstrate a preference for intravaginal practices aimed at sexual enhancement and hygiene, with participants utilizing leaves and powders for drying and tightening (9, 10). Additionally, a cross-sectional study among Malawian women identified the use of traditional substances to tighten the vagina prior to sexual intercourse, with the intention of enhancing the partner's sexual enjoyment and treating vaginal discharge and itching (5). While the use of certain vaginal cleansing products, particularly after sexual intercourse, has been associated with the clearance of transmitted HPV and a reduction in infection risks (11), intravaginal practices have been informally associated with an increased susceptibility to HIV infection (12-14). However, the precise mechanisms by which vaginal practices may influence women's susceptibility to HIV remain unclear.

Studies demonstrate that the intravaginal insertion of certain products can lead to increased inflammatory responses in the vaginal and cervical mucosa (6, 10, 13, 15). Additionally, specific intravaginal practices may cause physical abrasions or disrupt the vaginal epithelium (16). Research has hypothesized that the use of soaps, detergents, and antiseptics for internal

vaginal washing can disrupt the vaginal protective microenvironment, resulting in a vaginal flora with a reduced proportion or abundance of lactobacilli (17, 18). This alteration can lead to vaginal dysbiosis, characterized by colonization by a diverse spectrum of primarily anaerobic bacteria (1, 9, 19-23). Clinically, this dysbiosis may present with symptoms such as vaginal discharge, a fish-like odor, vaginal discomfort, and urinary symptoms, along with elevated genital inflammation that is linked to an increased risk of HIV and higher incidences of preterm birth and cervical dysplasia (24-31). While the literature indicates significant associations between vaginal practices and BV, some studies report no significant associations (32, 33), underscoring the complexity of this relationship.

Given the widespread use of vaginal practices, particularly vaginal insertion products (VIP), and the anecdotal associations with altered vaginal microbiota and increased susceptibility to HIV infection among women, there is a critical need for a comprehensive investigation into the impact of these intravaginal practices on vaginal microbiota. The aim of this study was to longitudinally characterize the composition of the vaginal microbiome in South African adolescent girls and adult women. Additionally, we assessed whether intravaginal practices among these populations lead to alterations in the vaginal microbiome and examined the correlation between these microbial changes and cellular and cytokine markers of genital inflammation. Furthermore, we investigated the influence of sexual activity on the composition and stability of the vaginal microbiota over time.

4.2. METHODS

4.2.1. Study design, participants and sample collection:

This study was conducted as part of the MIST (Mucosal Injury from Sexual Trauma) longitudinal cohort study, which enrolled HIV-uninfected adolescent girls (14-19 years) and adult women (25-35 years) from two sites in South Africa: the first site was located at the Vulindlela Clinical Research Site (VCRS) at the Centre for the AIDS Programme of Research in South Africa (CAPRISA) in Mafakathini, KwaZulu-Natal (KZN), and the second site was within the Desmond Tutu Health Foundation (DTHF) Adolescent Clinic in Philippi East, Western Cape (WC). The MIST study aimed to investigate socio-behavioural, anatomical, and biological characteristics related to sexual debut in adolescent girls, focusing on factors such as early sexual exposure, exposure to male semen, mucosal trauma, and wound healing,

comparing these with data from adult women. Participants were instructed to abstain from using vaginal insertion products (VIPs) and refrain from sexual activity for at least two weeks prior to their baseline visit.

The study collected the following genital samples: cervicovaginal fluid obtained by placing a disposable menstrual cup (Softcup) over the cervix for one hour, vaginal swabs for sexually transmitted infection (STI) testing, pH, Nugent scoring, and microbiota analysis. Additionally, the cervical cytobrushes were collected exclusively at the VCRS-CAPRISA site to isolate cervical T cells for flow cytometry analysis. Written informed consent was obtained from all participants, and for minors aged 14-17 years, both assent and parental informed consent were sought. Ethical approval for the study protocol was obtained from the Biomedical Research Ethics Committee (BREC) of the University of KwaZulu-Natal (BF504/17), the Human Research Ethics Committee (HREC) at the University of Cape Town (HREC/REF 696/2017), and the Institutional Review Board of the Seattle Children's Research Institute (REF STUDY00000462).

4.2.2. Testing for STIs and BV:

At the Vulindlela Clinical Research Site (VCRS-CAPRISA), urine samples were analyzed for *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) infections using the Cepheid Xpert CT/NG assay. Meanwhile, at the Desmond Tutu Health Foundation (DTHF) site, vaginal swabs were utilized to test for CT, NG, and *Trichomonas vaginalis* (TV) infections using PrimerdesignTM genesig® kits at the Bio Analytical Research Corporation (BARC) reference laboratory located in Johannesburg, South Africa. Gram staining was employed to detect the presence of yeast and fungal hyphae, as well as to diagnose bacterial vaginosis (BV) using the Nugent score criterion of ≥7.

4.2.3. Cytokine measurements in Softcup supernatants

Stored cervicovaginal Softcup supernatants were thawed overnight on ice and subsequently filtered using Spin X filter columns (Merck KGaA, Darmstadt, Germany). The filtered samples were then use to quantify the concentrations of 28 different cytokines by Human XL Cytokine Luminex® Performance Panel Premixed Kit (R&D systems Inc, Minneapolis, MN, USA) and ran on Bio-Plex MagPix Array Reader (Bio-Rad Laboratories, CA, USA) multiplex bead array.

These include Monocyte Chemoattractant Protein-1 (MCP-1), Macrophage Inflammatory Protein-1beta (MIP-1β), Macrophage Inflammatory Protein-3alpha (MIP-3α) , Interferon Gamma-Induced Protein-10 (IP-10), Granulocyte-macrophage Colony-stimulation Factor (GM-CSF), Interferon-gamma (INF-γ), Interleukin-1beta (IL-1β), Interleukin-2 (IL-2), Interleukin-5 (IL-5), Interleukin-8 (IL-8), Interleukin-12p70 (IL-12p70), Interleukin-17 (IL-17), Transforming Growth Factor-alpha (TGF-α), Vascular Endothelial Growth Factor (VEGF), Macrophage Inflammatory Protein-1alpha (MIP-1α), Eotaxin, CXC motif chemokine ligand-1 (CXCL-1), Granulocyte Colony-stimulating Factor (G-CSF), Interferon alpha (INF-α), Interleukin-1alpha (IL-1α), Interleukin-1 Receptor Antagonist (IL-1Ra), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-13 (IL-13), Platelet Derived Growth Factor AA (PDGF-AA), Tumor Necrosis Factor-alpha (TNF-α), Chemokine Ligand-5 (CCL5). The data generated by the Bio-Plex system were used to calculate sample concentrations based on standard curves. Cytokine levels that fell below the lower limit of detection of the assay were reported as the midpoint between the lowest measurable concentration for each cytokine and zero.

4.2.4. Cervical cytobrush Staining and flow cytometry

Cervical cytobrush cell pellets were used for evaluation of T cell immune activation status using a BD LSRFortessa™ X-20 Cell Analyzer (BD Immunocytometry Systems). The cells were stained with antibodies by incubating with antibody cocktail containing CD3-Allophycocyanin-H7 (APC-H7), CD4-Phycoerythrin cyanine 5.5 (PECy5.5), CD8-Brilliant Violet 711 (BV711), HLA-DR-Alexa-fluor-700, CD38-Phycoerythrin cyanine 7 (PE-Cy7), CCR5-Alkaline Phosphatase Conjugate (APC), CD14-Pacific Blue (Monocyte for exclusion), CD19 -Pacific Blue (B-cell for exclusion), and Live/Dead cells-Pacific Blue. The cells were washed with 2% FBS/PBS and at least 500000-1000000 events were acquired. Gates differentiating negative and positive populations were set by Fluorescence Minus One (FMO) staining(34). Cell doublets were excluded using forward scatter-area versus forward scatter-height parameters and dead cells were excluded from the analyses. FMO controls were used to set gates and data were analyzed using FlowJo version 10.7.1 (Treestar; Ashland, OR).

4.2.5. Amplification and sequencing of the V3-V4 region of the 16S rRNA gene

Genomic DNA was extracted from each vaginal swab using a 96-well PowerSoil DNA Isolation kit. A fragment of the 16S rRNA gene spanning the V3-V4 variable region was amplified using universal primers 319F (forward) and 806R (reverse) and purified libraries consisting of ~120 pooled samples will be sequenced with Illumina MiSeq platform (paired-end with v3 chemistry). FastQC was used to perform data quality control of raw sequences and infer parameters for downstream processing. Divisive Amplicon Denoising Algorithm (DADA) 2 was employed to infer amplicon sequence variants (ASVs) (35) Taxonomic assignment was performed using a reference taxa file obtained from the Silva database which contained both genus and species-level annotations. The ASV table, and taxonomic table combined with relevant metadata were consolidated into a phyloseq object using the phyloseq R package (36). Community state types were inferred using VALENCIA (VAginaL community state typE Nearest CentroId clAssifier)(37), a nearest centroid classification method for vaginal microbial communities based on composition, implemented in python (version 3.6) and has the pandas module as a dependency (38). VALENCIA uses distance matrices to classify similarity of bacterial community structures between individual samples based on species proportions to each reference centroid were calculated using the Yue-Clayton θ (39).

4.2.6. Statistical Analysis

All downstream statistical analyses were performed using R software version 4.3.1 (www.r-project.org). Descriptive statistics were used to compare demographic characteristics. The fishers exact test was used to compare characteristics between age groups within each site. Clinical characteristics that were assessed include STI, and Bacterial vaginosis status. Age, usage of enhancers and vaginal hygiene information was also compared across age classes within each site. These comparisons were tabulated and Mann-Whitney U test was applied for non-parametric independent sample comparisons. Alpha diversity measures, Chao1, Shannon's and inverse simpson's index were calculated using the R-vegan package. Beta diversity was measured using bray-curtis distances using the same package. To visualise the spatial segregation of CSTs, Non-metric multidimensional scaling (NMDS) was used. We used linear mixed models (LMMs) and generalized estimating equation (GEE) models to analyze the clustered outcomes of this study. For alpha diversity measures (using LMM) and cytokines with logarithm transformation of continuous outcomes (using GEE), we employed a Gaussian link function. For cytokine binary outcomes and cell frequency proportions, we used a GEE with a logit link function. Post-modeling comparisons were conducted, and multiple

comparisons were adjusted using the false discovery rate (FDR) method. Results for alpha diversity models are presented as mean differences. For models with a logarithm of continuous outcome, results are presented as geometric means and as percentage change relative to the reference group. Binary outcome models are presented as odds ratios. All comparisons include 95% confidence intervals and p-values. Statistical significance was defined as $p < 0.05$.

4.3. RESULTS

4.3.1. Baseline participant demographic and clinical characteristics

A total of 326 adolescent girls and adult women from South Africa participated in the MIST study. Among these, 153 participants (102 adolescents aged 17-19 years and 51 adult women aged 25-29 years) were recruited from the peri-urban communities of Philippi, Cape Town, WC South Africa. Additionally, 173 participants (117 adolescents aged 16-18 years and 56 adult women aged 27-31 years) were recruited from the rural communities of Vulindlela, KZN, South Africa. Baseline demographic and clinical characteristics for the participants are provided in Table 4.1 and detailed in Mkhize et al., 2024 (40). At the WC site, adolescent girls and adult women reported using only soap and water to wash their female genital tracts (FGTs), both internally and externally, solely for hygiene reasons. In contrast, at the KZN site, adolescent girls and adult women reported using a variety of VIP to wash their FGTs, both internally and externally, for both hygiene and sexual enhancement purposes. The different types of VIP utilized by adolescent girls and adult women in KZN are outlined in Gumbi et al., 2024 (40). There were no significant differences in the prevalence of BV and intermediate BV between adolescent girls and adult women at either the Western Cape or KZN sites. There was a significant difference in the prevalence of STIs between age groups, with higher rates of *C. trachomatis* observed in adolescent girls compared to adult women at both sites ($p<0.001$). In the WC, there was a trend towards a higher prevalence of *N. gonorrhoeae* in adolescents compared to adult women, but this trend was not observed in KZN.

Table 4.1: Baseline participant demographic and clinical characteristics

	Cape Town N = 153				Kwa-Zulu Natal N = 173			
Characteristic	**N**	**Adolescent Girls** N = 102[1]	**Adult Women** N = 51[1]	**p-value**[2]	**N**	**Adolescent Girls** N = 117[1]	**Adult Women** N = 56[1]	**p-value**[2]
Age (Years)	153	18.0 (17.0, 19.0)	28.0 (25.0, 29.0)	<0.001	173	18.0 (16.0, 18.0)	28.0 (27.0, 31.0)	<0.001
Using Sexual Enhancers	153	0 (0%)	3 (5.9%)	0.036	173	68 (58%)	38 (68%)	0.2
Vaginal Hygiene Use-Inside	153	102 (100%)	51 (100%)	>0.9	173	24 (21%)	15 (27%)	0.4
Using Vaginal Hygiene-Outside	153	100 (98%)	51 (100%)	0.6	173	115 (98%)	54 (96%)	0.6
Any Vaginal Product	153	102 (100%)	51 (100%)	>0.9	173	116 (99%)	55 (98%)	0.5
BV by Nugent Score	134			0.5	173			0.6
BV		41 (46%)	24 (55%)			50 (43%)	24 (43%)	

		Cape Town N = 153				Kwa-Zulu Natal N = 173		
Characteristic	**N**	**Adolescent Girls** N = 102[1]	**Adult Women** N = 51[1]	**p-value**[2]	**N**	**Adolescent Girls** N = 117[1]	**Adult Women** N = 56[1]	**p-value**[2]
Intermediate		15 (17%)	4 (9.1%)			24 (21%)	8 (14%)	
No BV, NS		34 (38%)	16 (36%)			43 (37%)	24 (43%)	
Neisseria Gonorrhoeae	130	14 (16%)	2 (4.4%)	0.053	173	10 (8.5%)	2 (3.6%)	0.3
Chlamydia Trachomatis	150	52 (51%)	10 (21%)	<0.001	173	40 (34%)	4 (7.1%)	<0.001
Trichomonas Vaginalis	147	11 (11%)	5 (10%)	>0.9	171	10 (8.7%)	3 (5.4%)	0.5
At least one STI	152	62 (61%)	14 (28%)	<0.001	173	50 (43%)	8 (14%)	<0.001

[1]Median (IQR); n (%),[2]Fisher's exact test

4.3.2. Microbial Community Structures Across Age Groups

To characterise the vaginal community state types (CSTs), we sequenced the V3-V4 region of the 16S rRNA gene from bacterial DNA extracted from swabs collected from adolescents and adult women from KZN and CT. This analysis yielded a total of 329 OTUs, which were assigned to 123 different taxa. Utilizing the VALENCIA tool, we identified five distinct CSTs based on the composition and relative abundance of bacterial species (Figure 4.1). Our results indicated that at baseline, the population was primarily dominated by either diverse anaerobic bacteria (CST IV, n = 172/326; 52.76%) or *Lactobacillus iners* (CST III, n = 106/326; 32.51%). Additionally, we observed a modest representation of *Lactobacillus crispatus* (CST I, n = 47/326; 14.41%). One adult was dominated by *Lactobacillus gasseri* (CST II) at baseline (n=1, 0.3%). CST IV was further subdivided into three subtypes: CST IV-A, characterized by high to moderate relative abundance of BVAB1 and *Gardnerella vaginalis*; CST IV-B, marked by the moderate to high relative abundance of *G. vaginalis* and *Atopobium vaginae*; and CST IV-C, which is characterised by the low relative abundances of *G. vaginalis*, BVAB1, and *Lactobacillus* species. CST IV-C samples observed either had relatively even distributions of *Prevotella species* or the dominance of *Streptococcus agalactiae*. The distribution of CSTs was consistent among adolescents from KZN and CT compared to adult women, suggesting that the microbial community structures may remain stable across different age groups, with no significant differences detected among the cohorts.

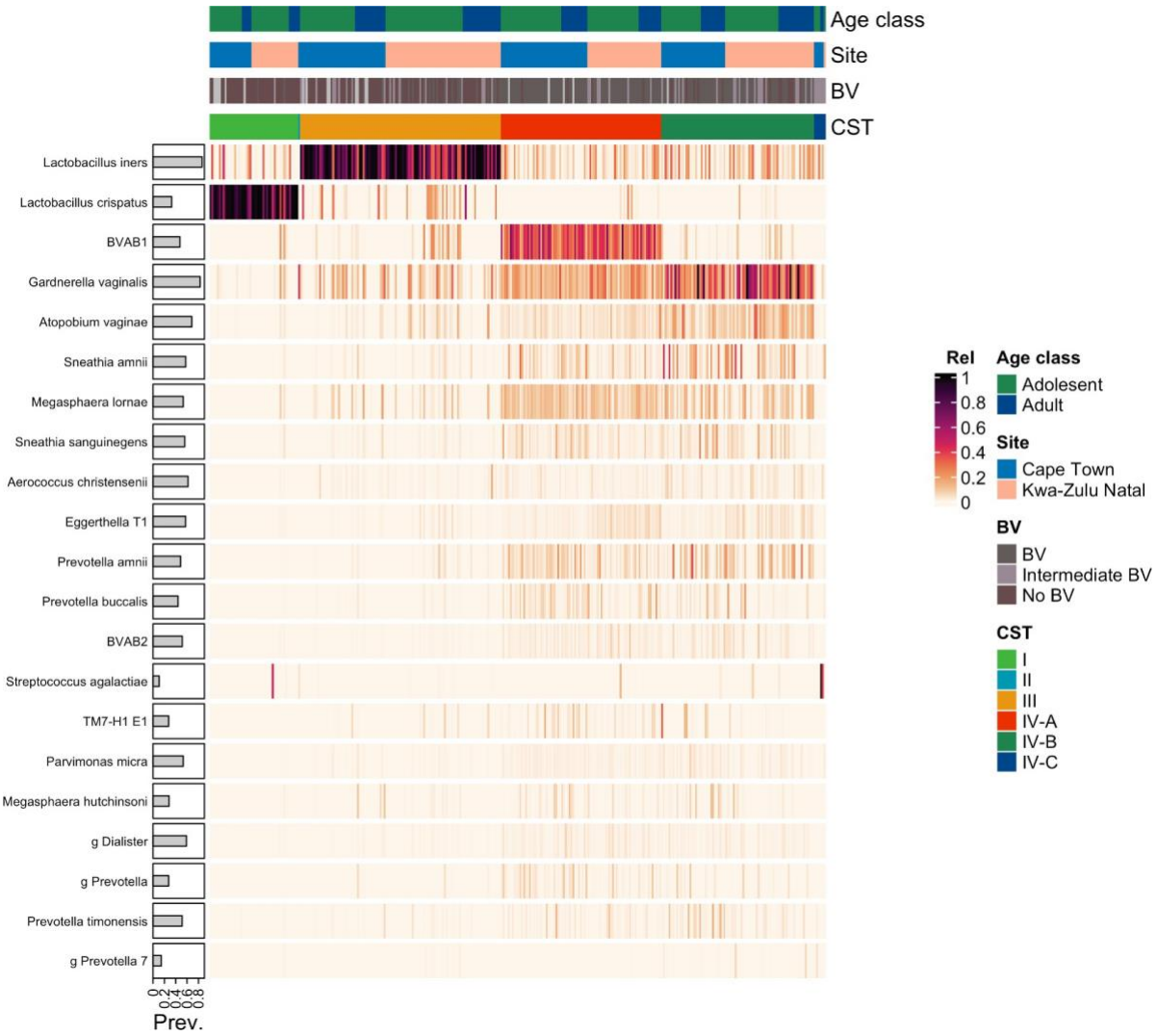


Figure 4.1: Heat map showing the 20 most abundant vaginal bacteria identified by 16S rRNA gene sequencing in South African adolescent girls and adult women. The relative abundance of key bacterial taxa across the samples was used to categorize them into five community state types (CSTs): *Lactobacillus crispatus* (CST I), *Lactobacillus gasseri* (CST II), *Lactobacillus iners* (CST III), BVAB1 and *G.vaginalis* (CST IV-A), *Gardnerella vaginalis* and *Atopobium vaginae* (CST IV-B), and a diverse mixture of species (CST IV-C). Additional information, including age class (top bar), study site (upper middle bar), and BV status based on Nugent scoring (lower middle bar), is also displayed.

Next, we assessed the alpha diversity of the microbiome at the species level using the Shannon Diversity Index and examined the effects of age class (adolescent vs. adult) and visit event (baseline, 72 hours, and 10 days post-sexual intercourse) using a linear mixed-effects model, including random intercepts for subject ID and site to account for potential clustering effects. At baseline, no significant differences in alpha diversity were observed between adolescents and adults (mean difference = -0.023, 95% CI [-0.16, 0.11], p = >0.305), indicating that microbiome diversity was comparable across these age groups, regardless of location (Figures 4.2b-c). In addition, alpha diversity did not significantly change over time within either age group (β = 0.53, 95% CI [-0.41, 1.5], p = >0.11), and there was no significant interaction between age class and time (Figures 4.2A-C). However, a marginal decrease in alpha diversity was observed 10 days post-sexual intercourse in adolescent compared to adult (p = 0.078), suggesting a temporal reduction in microbiome diversity at this time point (Figure 4.2C).

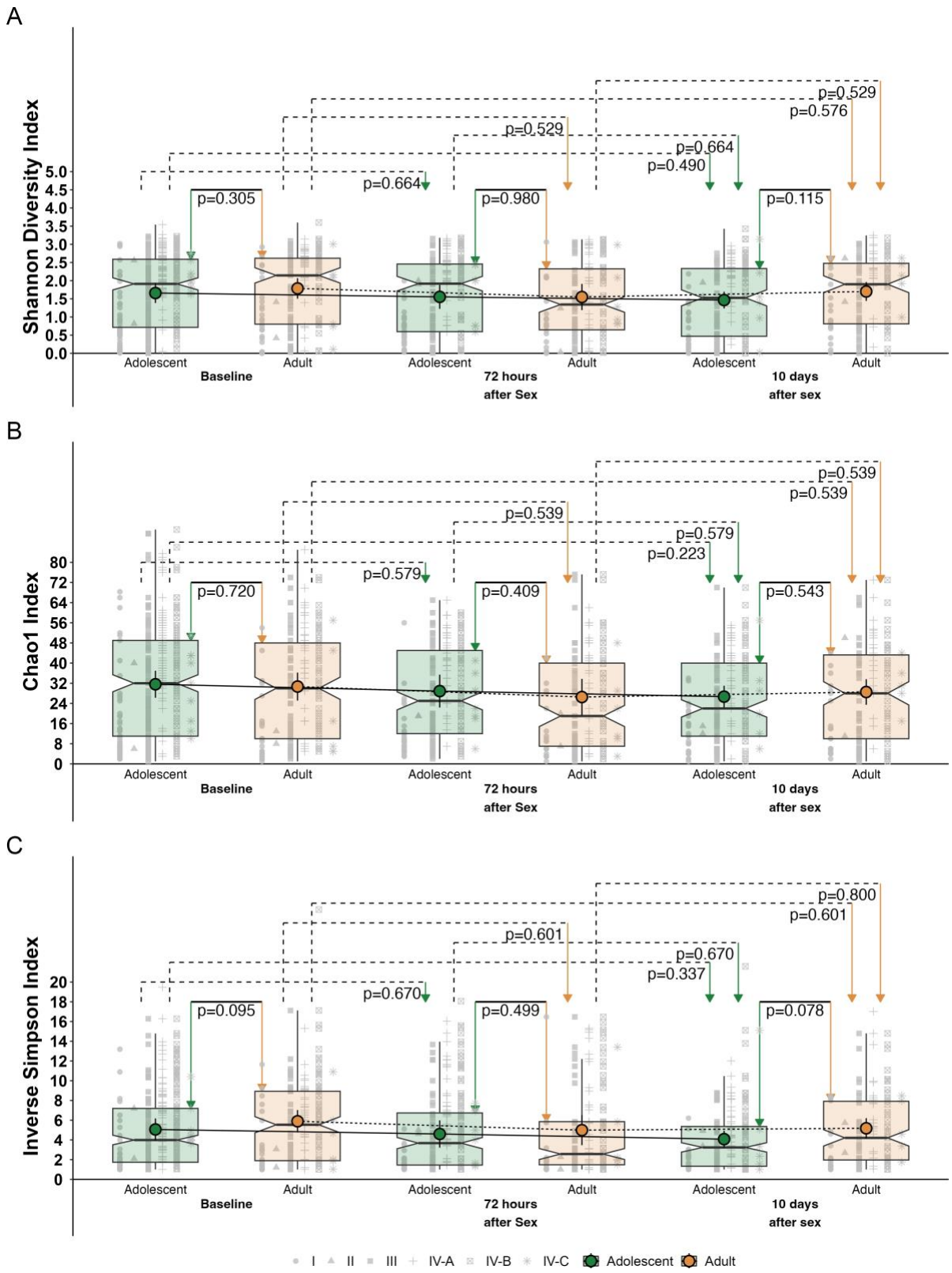


Figure 4.2: Alpha diversity indices of vaginal microbial communities across different age groups and time points relative to sexual activity. Microbial diversity was assessed among adolescent girls and adult women at baseline, 72 hours post-sex (B), and 10 days post-sex. Alpha diversity was quantified using three complementary indices: Shannon diversity, which accounts for both species richness and evenness; Chao1 richness, which estimates the total number of species including rare or potentially

undetected taxa; and Inverse Simpson diversity, which emphasizes species evenness and dominance. Higher values of Shannon and Inverse Simpson indicate greater diversity and more balanced communities, while higher Chao1 values reflect greater species richness. Statistical comparisons across groups were performed using the Kruskal–Wallis test with post-hoc Dunn's correction.

4.3.3. Post-Coital Changes in BVAB1 Dominance Among Adults

To assess the potential impacts of geography and sexual activity on vaginal microbial communities, we conducted Non-metric Multidimensional Scaling (NMDS) using Bray–Curtis distance metrics across all time points. Our analysis did not reveal any clustering patterns based on age, geography, or sexual activity (see Figure 4.3). To further explore the relative contribution of age to microbial diversity in relation to other unidentified factors, we performed a Permutational Multivariate Analysis of Variance (PERMANOVA) employing the Adonis method. The results showed that the effect of age class was not significant in the overall distances between samples ($F = 1.4814$, $R^2 = 0.0021$, $p = 0.182$), explaining only 0.21% of the variance in the community composition. In contrast, significant differences in bray curtis distances were observed between samples, due to differences in geography ($F = 8.8229$, $R^2 = 0.01261$, $p = 0.001$). The tests were based on 999 permutations.

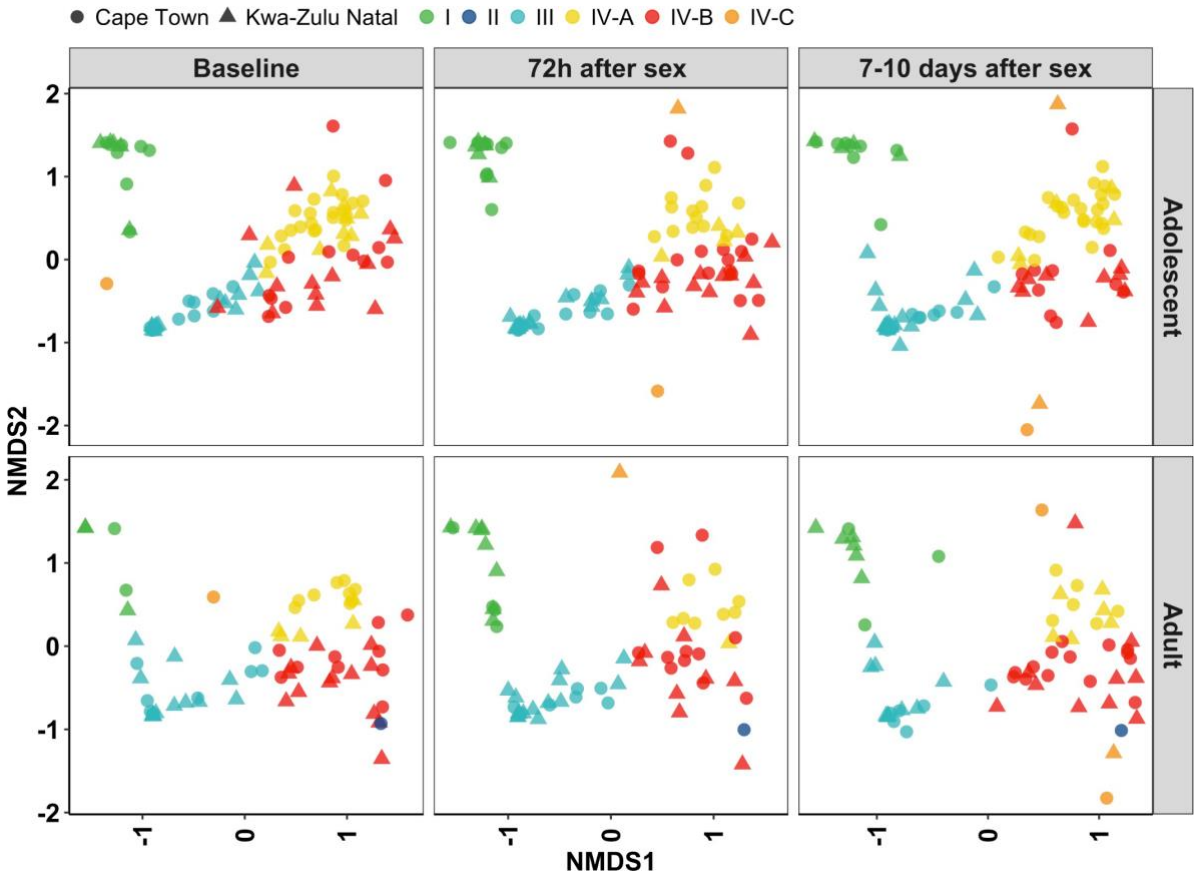


Figure 4.3: Non-metric Multidimensional Scaling (NMDS) of Bray-Curtis dissimilarity showing no distinct clustering of species-level relative abundances in adolescent girls and adult women at baseline, 72 hours, and 10 days post-sex. Samples are colour-coded by Community State Types (CSTs), with study sites represented by different shapes.

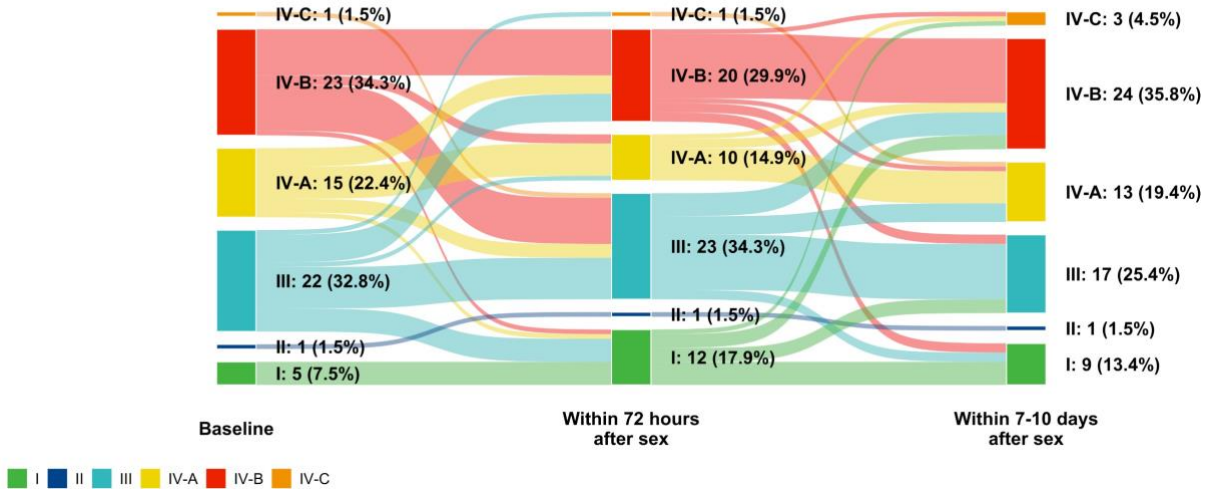
4.3.4. Transitions in CSTs Following Sexual Activity in Adult Women and Adolescent Girls

We investigated whether sexual activity induced transitions between CSTs in both adult women and adolescent girls, focusing on shifts toward BV-related CSTs. This analysis was performed using 164 individuals who had microbiome data at all three time points (baseline, within 72h after sex, and 7-10 days after sex). In adult women, 34% (23/67) maintained their baseline CSTs over time, including CST I (6%, 4/67), CST II (1.5% 1/67) CST III (6%, 4/67),CST IV-A (9%, 6/67) and CST IV-B (12%, 8/67). Moderate shifts in CST distribution were observed between baseline and 7-10 days post-coitus. Specifically, women initially classified as CST IV-B at baseline predominantly transitioned to CST III (24%,10/41). Women with CST I showed stability, as no transitions were observed between baseline and 72 hours post-coitus. In contrast, over half of the women with CST III transitioned to either CST I (23%, 5/22) or CST IV-B (27%, 6/22).Once women transitioned to CST IV-B, their microbiota remained relatively stable between 72 hours and 7-10 days post-coitus, with few transitions to other CSTs: CST I (10 %, 2/20), CST III (10%, 2/20), CST IV-A (5%, 1/20), or CST IV-C (5%, 1/20, Figure 4.4).

Although a limited number of women in CST III (41%, 9/22) remained in their baseline CST after 72 hours post-coitus, a significant proportion transitioned to CST IV-A, (5%, 1/22), CST IV-B (27%, 6/22) and CST IV-C (5%, 1/22). Some women transitioned from CST III to CST I (23%, 5/22). Within 7-10 days after sex, women in CST III mostly transitioned towards CST IV-A (17%, 4/23) and CST IV-B (22%, 5/23), and only 9% (2/23) transitioned to CST I, highlighting the relative difficulty of returning to a more stable and lactobacillus-dominant microbiota after dysbiosis. Similar trends were observed in adolescent girls, although there were some key differences. In adolescents, CST III was the most prevalent and stable CST throughout the study.Some girls maintained their CST III status (14%,14/97) throughout all visits. Girls showed a tendency to transition to CST III with increasing percentages of CST III prevalence over time (Baseline :29.9%, Within 72h after sex: 35.1% and 7-10 days after sex: 36.1%). Within 72 hours after sex, minimal transitions were observed towards CST I (10%, 3/29) , IV-A (7%, 2/29) and IV-B (10%,3/29). Similarly, within 7-10 days after sex, transitions from CST III to CST I (3%, 1/34), CST IV-A(12%, 4/34), and CST IV-B (12%, 4/34) were observed. In adolescents, CST IV-B showed an increase in prevalence within 72 hours after sex, followed by a decline within 7–10 days after sex. This may highlight instability in this cervicotype or a faster restoration of other CSTs after temporary shifts (Figure 4.4).

Transitions were mostly observed from CST IV-B towards CST III within 72h of sex (20%, 5/25) which is greater than the 10% transition observed in adults at this timepoint. Within 7-10 days a moderate shift from CST IV-B to III was also observed (17%,5/30). Some CST IV-B transitions were towards to CST IV-A (1/25), CST IV-C (1/25) and CST I (1/25) within 72 hours of sex. Within 7-10 days after sex, other CST IV-B were towards CST IV-A (37%, 11/30) and CST I (7%, 2/30). Furthermore, adolescent girls showed flactuations in CST IV-A prevalence over time (Baseline (29.9%) 72 hours post sex (16.55 %) and 7-10 days post sex (28.9%)) Similarly, the same decrease in CST IV-A pattern of prevalence was noted in adults (Baseline (22.4%),72 hours post sex (14.9%) and 7-10 days post sex (19.4%)). This may indicate sensitivity of this community group towards external factors. At baseline, adolescent girls with CST I were more likely to transition to CST III (31%, 4/13) than to CST IV (7%, 1/13).While adult women exhibited greater stability after transitioning to CST IV-B, adolescent girls demonstrated a more dynamic pattern of shifts between CSTs, particularly from CST IV-B to CST III.

Adults



Adolescents

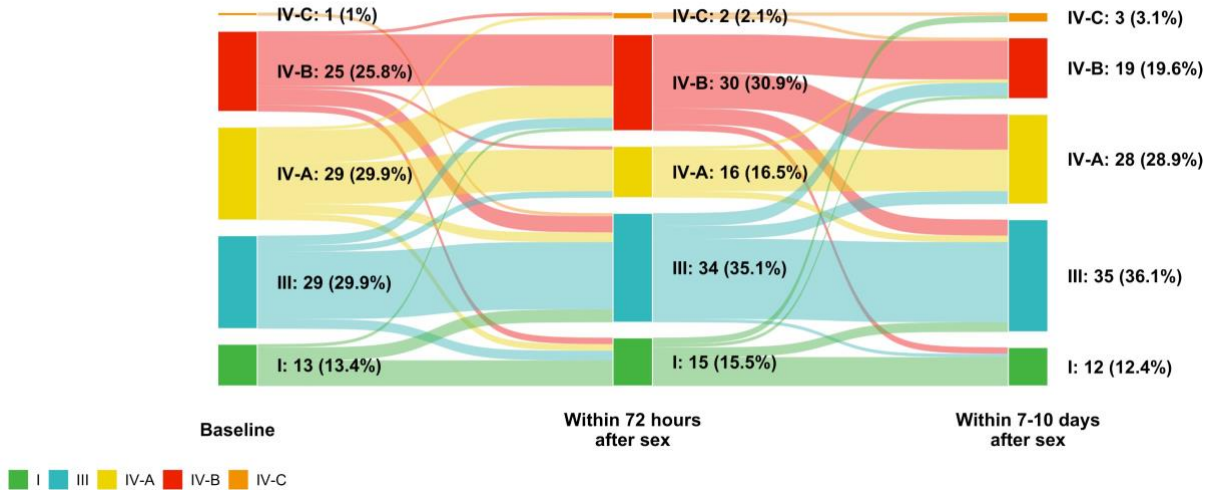


Figure 4.4: Sankey diagram illustrating transitions between Community State Types (CSTs) over time in (A) Adult women and (B) Adolescent girls. CST I (green) represents *L. crispatus* dominance, CST III (teal) represents *L. iners* dominance, CST IV-A (yellow) includes diverse bacterial communities characterised by moderate to high abundances of BVAB1, CST IV-B is characterized by moderate to high abundances of *G. vaginalis* and *Atopobium vaginae*, and CST IV-C represents a mixed BV-related community.

4.3.5. Impact of VIP Use on Vaginal Microbiota

Since no significant differences in species diversity or microbial community clustering were observed between KZN adolescent girls and adult women, these age groups were combined to evaluate the impact of VIP use on the vaginal microbiota over time. At baseline, no significant differences were observed in the relative abundances of bacterial species between women using VIP and non-VIP users. Over time, VIP users demonstrated significantly higher abundances of *Sneathia* and *Lactobacillus iners* compared to non-VIP users (Figure 4.5). In contrast, the abundances of *Megasphaera lornae*, *Gardnerella vaginalis*, *Eggerthella T1*, and *Atopobium vaginae* were significantly reduced in VIP users relative to non-VIP users throughout the study period.

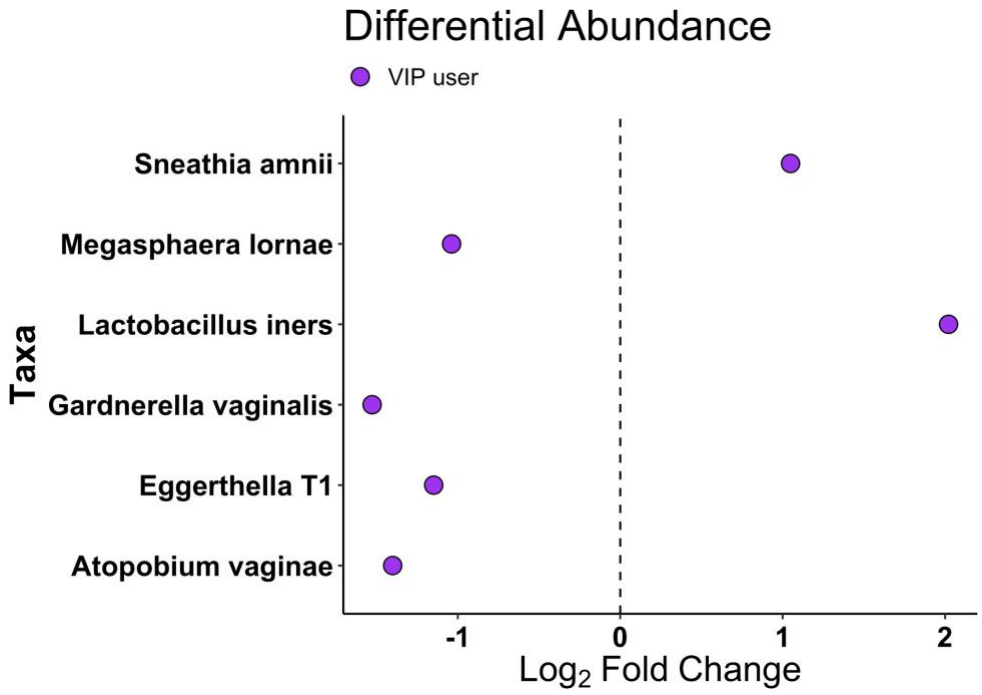


Figure 4.5: Differential abundance of the 20 most abundant bacterial species found in women using any VIP and non-VIP users. Log2 fold change of significant differentially abundant species between any VIP and non-VIP users at baseline.

4.3.6. Elevated MIP-3α concentrations in adult women compared to adolescent Girls, irrespective of the vaginal microbiota status

We investigated how the composition of vaginal microbiota (*Lactobacillus*-dominant vs. non-dominant) influences cytokine expression and immune cell activation in adult women compared to adolescent girls, and to examine the role of sexual activity timing in modulating these immune responses. Overall, the mean concentrations of MIP-3α were significantly higher by 127% in adult women with a *Lactobacillus*-dominant vaginal microbiota (geometric mean = 2.27; CI: (1.13,4.54) p = 0.021) in comparison to adolescent girls with a *Lactobacillus*-dominant vaginal microbiota. Similarly, although not statistically significant, the adult women with a non-*Lactobacillus*-dominant microbiota exhibited mean concentrations of MIP-3α which was higher by 93% (GM = 1.93; CI: (0.96,3.90) p = 0.065) compared to adolescent girls with a non-dominant vaginal microbiota (Figure 4.6). Futhermore, the non-*Lactobacillus*-dominate adult women had significantly higher MIP-3α by 162% (GM = 2.63 CI: (1.04,6.65), p = 0.041) compared to adolescent girls 72 hours post-coitus (Figure 4.7). Regardless of the vaginal microbiota status, adults had consistently higher MIP-3α (compared to any of the cytokines) concentrations at each visit than adolescents, notwithstanding the statistical significance. Once more, regardless of the vaginal microbiota status, but in contrast to MIP-3α, IL-1β was shown to be lower in adults than in adolescents (ranging from -17% to -61%) at any visit, with the exception of *Lactobacillus*-dominant women 0–3 days post-sex.

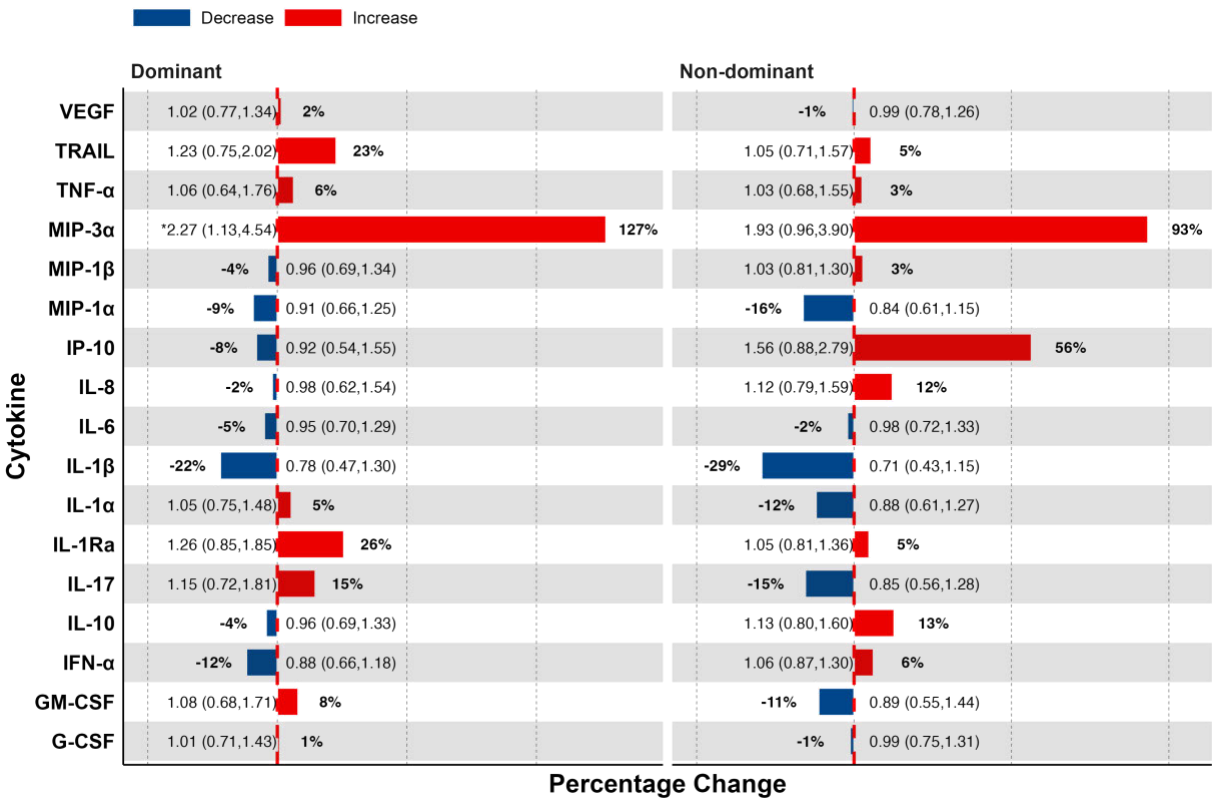
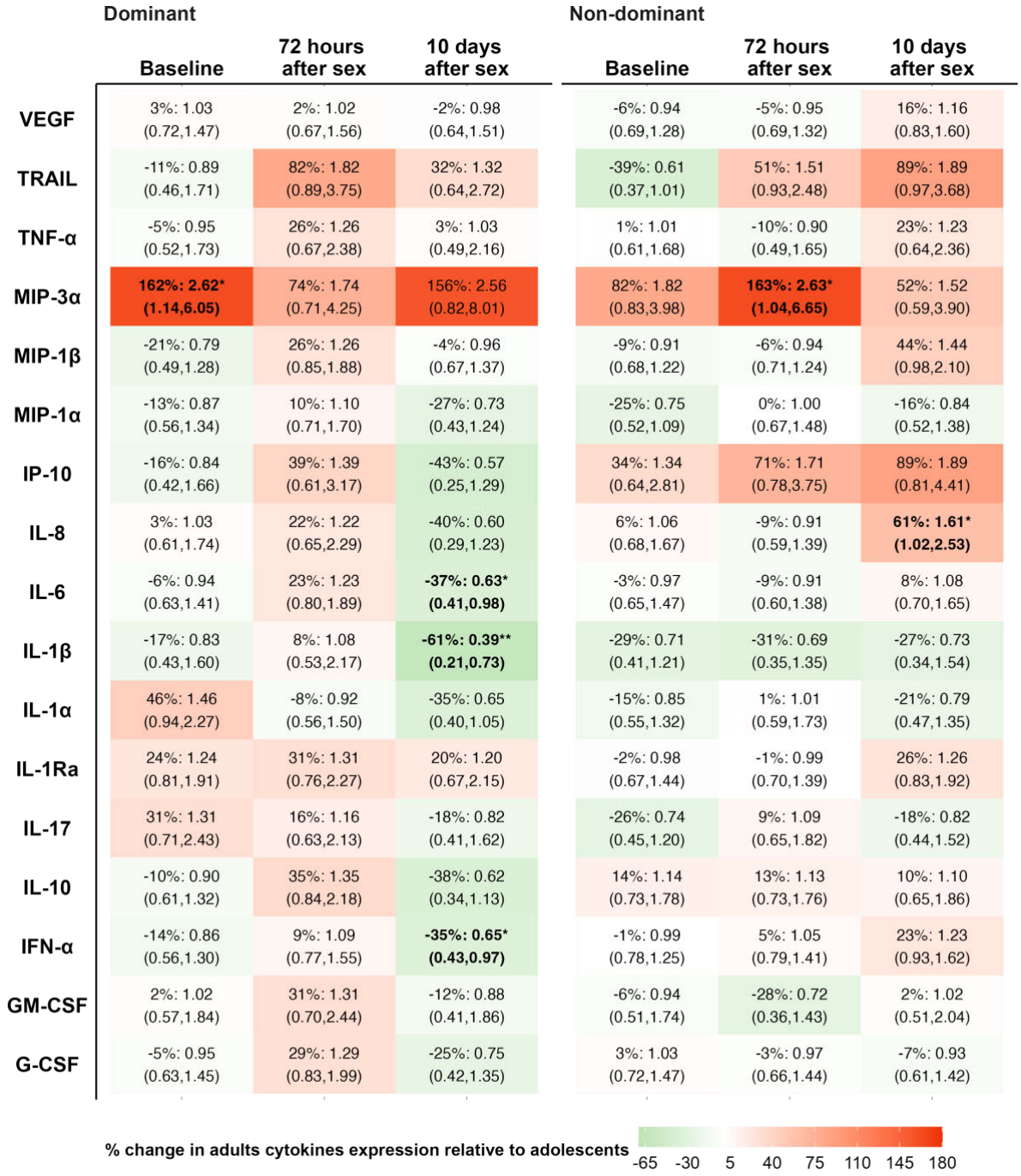


Figure 4.6: Effect of vaginal microbiota composition on relative abundance changes of dominant and non-dominant bacterial taxa over the study period. The left panel represents dominant taxa, and the right panel represents non-dominant taxa. Blue bars indicate relative changes in bacterial abundance in the reference group, while red bars indicate relative changes in the comparison group. The numeric values inside or adjacent to each bar represent the relative abundance change (as a percentage) and the corresponding 95% confidence intervals (shown in parentheses). The vertical red line at 0% represents no change in relative abundance. Bars extending to the right of the line indicate an increase in relative abundance, whereas bars extending to the left indicate a decrease. Statistical analyses were performed to assess differences between groups; odds ratios and 95% confidence intervals are displayed for each taxa. Only taxa with measurable relative abundance in ≥1% of samples are shown. This figure highlights taxa-specific shifts in microbial composition, illustrating differences in dynamics between dominant and non-dominant vaginal bacteria.

Despite the significance, adults in the *Lactobacillus* dominant group continuously had greater cytokine concentrations than adolescents at 0–3 days post-sex (Figure 4.7). In contrast, the majority of the cytokines in adult women exhibited reduced amounts compared to adolescents in the same microbial community group (*Lactobacillus* dominant) 7-10 days after intercourse.

The cytokine concentrations in adult women throughout all visits, however, did not show any age-specific patterns in the non-dominant group (Figure 4.7).



*The p-value significance: * < 0.05; ** < 0.01; *** < 0.001.*

Figure 4.7. Effect sizes (Odds Ratios [OR] and 95% Confidence Intervals [CI]) for muscle activity during various tasks, separated by dominant and non-dominant limbs. This heatmap presents the odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for muscle activity across various tasks and muscle groups for both dominant and non-dominant limbs. Each cell shows the percentage difference in muscle activation (top value), followed by the OR and CI (in parentheses). Cells are color-coded based on the magnitude and direction of the effect size: green shades indicate reductions and red shades indicate increases in muscle activity. Darker shades reflect greater deviations from the null effect

(OR = 1). Bold text indicates statistically significant findings ($p < 0.05$), with double asterisks (**) denoting more robust significance. Muscle groups are listed along the vertical axis, while different task conditions or comparisons are represented along the horizontal axis. The left panel shows data for the dominant limb, and the right panel shows data for the non-dominant limb. Color bar at the bottom denotes the scale of ORs, from 0.3 (green) to 3.0 (red), with white representing neutral or no effect (OR ≈ 1.0).

We further assessed whether similar effects could be observed in other immune markers. Adult women demonstrated higher odds of having high CD8+HLA-DR frequency compared to adolescent girls, regardless of *Lactobacillus* status (*Lactobacillus* dominant: OR= 1.55, CI: (0.99, 2.44), p=0.057, Non-*Lactobacillus* dominant: OR=2.88, CI: (1.54, 5.38), p = 0.001) (Table 4.2**)**. Similarly, adult women had significantly higher odds of having high CD4+α4β7 frequency relative to adolescent girls, regardless of microbiota status (*Lactobacillus* dominant: OR=2.94, CI: (1.08, 8.03), p=0.036, Non-dominant: OR=4.58, CI: (1.05,19.93), p=0.043). Although not significant, other cells which showed increased odds were CD4+HLA-DR and CD8+α4β7. Lower odds of having high cell frequency in adults relative to adolescents were observed in CD4+CCR5, irrespective of *Lactobacillus* status (*Lactobacillus* dominant: OR: 0.62, CI: (0.39,0.97), p=0.039, Non-dominant: OR=0.61, CI: (0.31, 1.17), p=0.133). Similar trends were observed in other cells such as CD4+CCR10, CD4+ CCR6, CD4+ CD38, CD8+ CCR5 and CD8+ CD38 in both dominant and non-dominant microbial communities. Interestingly, under *Lactobacillus* dominant conditions, higher odds of increased frequencies of CD8+ CCR10 (OR=2.37, CI: (0.97, 5.75), p=0.055), and CD8+CCR6 (OR=1.61, CI: (0.90, 2.86), p=0.104) in adults relative to adolescents. Whereas lower odds of increased frequencies in adults compared to adolescents were observed under Non-dominant conditions.

Four types of immune cells were found at significantly higher frequencies in adults at specific time points. At baseline, adult women had significantly more CD4+α4β7 (OR = 8.87, CI: (3.68,21.41), p<0.001) and CD8+α4β7 (OR = 6.03, CI: (1.43,25.40), p=0.015) cells, essential for mucosal immunity, compared to adolescents. This translates to adult women being approximately 9 times and 6 times more likely to have higher frequencies of these cells, respectively. A few days after sex (72 hours), adults continued to show significantly higher frequencies of CD8+CCR10 (OR = 4.18, CI: (1.19,14.72), p=0.027), involved in tissue-resident memory, and CD8+α4β7 (OR=37.33, CI: (6.08, 229.06), p<0.001). These elevated odds ratios suggest a strong likelihood of these cells being more abundant in adult women at

these specific times. Conversely, three immune cell types were significantly lower in *Lactobacillus*-dominant 72 hours after sex: CD4+CD38 (OR=0.42 CI: (0.23,0.77), p=0.006), CD8+CCR5 (0.22 CI: (0.12,0.42), p<0.001), and CD8+CD38 (OR=0.33 CI: (0.17,0.63), p=0.001). This indicates that adolescent girls are more likely to have higher frequencies of these cells at this time point.

Interestingly, at least 7-10 days after sex, a different set of immune cells showed significant differences in the non-*Lactobacillus* dominant group. Adults had higher frequencies of CD4+α4β7 (11.07 CI: (1.25,97.89), p=0.031), CD8+HLA-DR (5.24 CI: (1.88,14.62), p=0.002), and CD4+CCR5 (21.90 CI: (1.61,297.47), p=0.021). Be that as may, the resulting estimates had wide confidence intervals, particularly for some post-sex findings, indicating the need for further research with a larger sample size to obtain more precise estimates.

4.3.7. Impact of non-Lactobacillus-Dominated Microbiome on cytokine concentrations in Women Using and Not Using VIPs

Given the absence of strong, age-specific differences in immune responses related to microbiota composition and sexual activity in our analysis, we concluded that combining adolescent girls and adult women in this study may be a more appropriate approach for examining the influence of microbiota composition, VIP use and sexual activity on immune responses. To explore this further, we investigated the impact of a non-Lactobacillus-dominated microbiome on the levels of chemokines, growth factors, and inflammatory cytokines in women not using VIPs, comparing these to levels in women with Lactobacillus-dominated microbiota. Among the 17 markers assessed, 8 (%) exhibited significantly higher mean concentrations in women with a non-Lactobacillus-dominated microbiome who were not using VIPs, but not in users, after adjusting for age and timing post-sexual activity (Figure 4.8). These elevated markers included several chemokines MIP-1α at 27%: (GM=1.27, p=0.013), MIP-1β at 23%: (GM=1.23, p=0.035), hematopoietic growth factors G-CSF at 24%(GM=1.24, p = 0.044), the innate cytokine IFNα at 27%(GM=1.27, p=0.008), and the pro-inflammatory cytokines TRAIL at 226%: (GM= 3.26, p<0.001), IL-6 at 27%: (GM=1.27, p= 0.021), IL-1α at 90%: (GM=1.90, p<0.001), and IL-1β at165% (GM=2.65,p= <0.001). TRAIL, IL-1β and IL-1α showed a percentage increase of approximately 50% and more in both users and non-users (Figure 4.8). A non-Lactobacillus-dominated microbiome was associated with significantly lower mean concentrations of GM-CSF by -52% (GM=0.48, p<0.001), IL-1Rα by 29% (GM=0.71, p=0.008), and IP-10 by -73% (GM=0.27, p<0.001) (Figure 4.8). In all these markers, there was a decrease in the users, although not statistically significant. In VIP users, VEGF was significantly higher in those with a non-Lactobacillus-dominated microbiome (122%, p =0.015), but no such association was found in non-users.

Table 4.2: Odds Ratios for Immune Cell Frequencies within groups of Women's *Lactobacillus*-vaginal microbiota status by age group and time since intercourse

			Visit-specific estimates		
Cells	**Sub-group**	**Overall**	**Baseline**	**72 hours after sex**	**10 days after sex**
CD4+CCR10	Dominant	0.96 (0.51,1.81) p=0.901	0.79 (0.28,2.24) p=0.660	1.26 (0.54,2.96) p=0.594	0.89 (0.34,2.34) p=0.811
	Non-dominant	0.77 (0.39,1.49) p=0.427	1.25 (0.54,2.90) p=0.604	0.68 (0.25,1.87) p=0.446	0.58 (0.18,1.92) p=0.367
CD4+CCR5	Dominant	**0.62 (0.39,0.97) p=0.039**	0.69 (0.33,1.43) p=0.309	0.59 (0.30,1.20) p=0.143	0.58 (0.32,1.02) p=0.060
	Non-dominant	0.61 (0.31,1.17) p=0.133	0.86 (0.33,2.27) p=0.760	0.75 (0.30,1.92) p=0.545	**0.39 (0.16,0.97) p=0.042**
CD4+CCR6	Dominant	0.93 (0.52,1.65) p=0.798	1.21 (0.49,2.96) p=0.674	0.52 (0.23,1.17) p=0.114	1.43 (0.49,4.16) p=0.504
	Non-dominant	0.53 (0.25,1.11) p=0.090	0.81 (0.30,2.14) p=0.661	0.38 (0.12,1.18) p=0.092	0.51 (0.21,1.22) p=0.127
CD4+CD38	Dominant	0.93 (0.59,1.45) p=0.734	1.63 (0.98,2.71) p=0.057	**0.42 (0.23,0.77) p=0.006**	1.35 (0.65,2.80) p=0.418
	Non-dominant	0.67 (0.41,1.10) p=0.112	0.67 (0.29,1.58) p=0.359	0.82 (0.40,1.68) p=0.589	0.56 (0.28,1.10) p=0.090
CD4+HLA-DR	Dominant	1.34 (0.76,2.37) p=0.304	1.40 (0.62,3.14) p=0.412	1.49 (0.62,3.56) p=0.367	1.14 (0.53,2.47) p=0.731
	Non-dominant	1.54 (0.86,2.75) p=0.143	0.95 (0.36,2.55) p=0.921	2.07 (0.82,5.21) p=0.120	1.78 (0.64,4.90) p=0.261
CD4+α4β7	Dominant	**2.94 (1.08,8.03) p=0.036**	**8.87 (3.68,21.41) p<0.001§**	2.66 (0.38,18.72) p=0.321	0.56 (0.11,2.94) p=0.489
	Non-dominant	**4.58 (1.05,19.93) p=0.043**	3.61 (0.64,20.47) p=0.144	0.45 (0.06,3.27) p=0.426	**11.07 (1.25,97.89) p=0.031§**
CD8+CCR10	Dominant	2.37 (0.98,5.75) p=0.055	1.97 (0.37,10.60) p=0.425	**4.18 (1.19,14.72) p=0.027**	1.76 (0.47,6.63) p=0.397
	Non-dominant	0.97 (0.28,3.37) p=0.958	2.53 (0.31,20.44) p=0.379	0.14 (0.02,1.08) p=0.059	2.90 (0.38,21.88) p=0.296
CD8+CCR5	Dominant	**0.53 (0.30,0.93) p=0.027**	0.75 (0.30,1.88) p=0.541	**0.22 (0.12,0.42) p<0.001**	0.89 (0.41,1.93) p=0.758
	Non-dominant	0.86 (0.46,1.63) p=0.645	1.10 (0.48,2.52) p=0.819	0.97 (0.35,2.70) p=0.952	0.59 (0.24,1.48) p=0.258
CD8+CCR6	Dominant	1.61 (0.90,2.86) p=0.104	2.21 (0.80,6.14) p=0.125	0.72 (0.27,1.92) p=0.511	2.92 (0.84,10.16) p=0.091
	Non-dominant	0.88 (0.34,2.32) p=0.800	1.13 (0.31,4.13) p=0.848	0.62 (0.10,3.84) p=0.599	0.91 (0.27,3.11) p=0.884
CD8+CD38	Dominant	0.67 (0.40,1.10) p=0.113	1.07 (0.37,3.12) p=0.902	**0.33 (0.17,0.63) p=0.001**	0.90 (0.46,1.77) p=0.760
	Non-dominant	**0.54 (0.32,0.90) p=0.018**	0.53 (0.24,1.18) p=0.119	0.54 (0.22,1.32) p=0.171	0.54 (0.22,1.36) p=0.190
CD8+HLA-DR	Dominant	1.55 (0.99,2.44) p=0.057	1.17 (0.48,2.88) p=0.726	1.71 (0.73,4.00) p=0.212	1.99 (0.91,4.36) p=0.082
	Non-dominant	**2.88 (1.54,5.38) p=0.001**	2.28 (0.79,6.58) p=0.126	2.67 (0.91,7.85) p=0.074	**5.24 (1.88,14.62) p=0.002§**
CD8+α4β7	Dominant	3.83 (1.08,13.55) p=0.038	**6.03 (1.43,25.40) p=0.015§**	**37.33 (6.08,229.06) p<0.001§**	0.32 (0.03,3.13) p=0.322
	Non-dominant	4.35 (0.72,26.34) p=0.107	3.96 (0.26,59.86) p=0.314	0.01 (0.00,6.10) p=0.143	**21.90 (1.61,297.47) p=0.021§**

§ - indicating extremely wide confidence interval; Estimates presented as GM (95% CI)

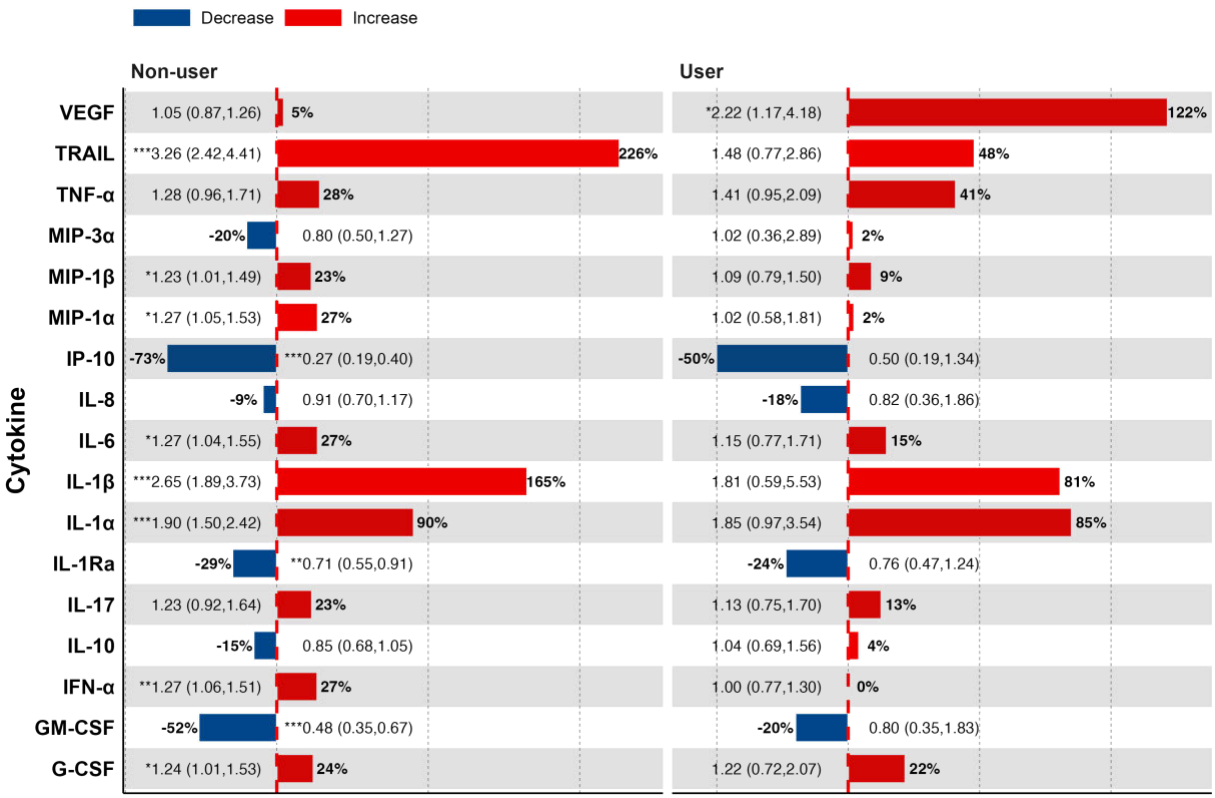


Figure 4.8: *Comparison of odds ratios (ORs) and percentage differences in outcome measures between users and non-users.* This figure presents odds ratios (ORs) with 95% confidence intervals (CIs) and percentage changes in a series of outcomes, stratified by user status: non-users (left panel, blue bars) and users (right panel, red bars). Each row corresponds to a specific outcome or condition. OR values are displayed within parentheses beside the respective bars, with asterisks (*) denoting statistically significant findings ($p < 0.05$). Percentage values to the right of each bar indicate the relative difference from baseline. Blue bars represent negative effects (reduction), while red bars indicate positive effects (increase). The length of the bars visually reflects the magnitude of the percentage change. Statistically significant ORs are marked with asterisks, and values with confidence intervals that do not cross 1.00 are emphasized. This visualization highlights key differences in the relationship between the outcome variables and user status, with particularly notable differences in measures such as those marked with high percentage changes (e.g., 90%, 85%) and statistically significant ORs.

4.3.8. Interactions Between VIP Use, Cytokine Profiles, and Sexual Activity Over Time

Here, we seek to identify whether the timing of sex (72 hours vs. 10 days post-sex) modulates the immune response, and whether this effect differs between individuals with a non-*Lactobacillus*-dominant microbiome compared to those with other microbiome profiles. Within non-VIP users (Figure 4.9A), TRAIL (72 hours: GM=4.01~301%, 10 days: GM=1.75~75%), IL-1β (72 hours: GM=2.69~169%, 10 days: GM=2.02~102%) and IL-1α (72 hours: GM=1.89~89%, 10 days GM=1.54~54%) were significantly increased in the non-*Lactobacillus* dominant women relative to *Lactobacillus* dominant women, regardless of

timing of sex.The oppositre effect was observed in IP-10 (72 hours: GM=0.27~ -75%, 10 days: GM=0.21~ -79%), and GM-CSF (72 hours: GM=0.34~ -66%, 10 days: GM=0.45~ -55%) as they were significantly reduced. The timing of sexual activity influenced the relationship between *Lactobacillus* status and immune markers, as significant changes were observed at 72 hours but not at 10 days post-sex in non-VIP users. MIP-1β (72 hours: GM=1.47~47%, 10 days GM=0.86~ -14%) and MIP-1α (72 hours: GM=1.38~38%, 10 days GM=1.95~95%) at 72 hours after sex, however, it reduced after 10 days post sex showing no significant difference.IL-1R (72 hours: GM=0.63~ -37%, 10 days: GM=0.77~ -33%) was reduced only at 72 hours. In Vip-users (Figure 4.9B), there were no markers that were consistently increased or reduced throughout the visits. However, two immune markers showed significant changes at 72 hours but not at 10 hours post sex; TRAIL (72 hours: GM=3.22~222%, 10 days: GM=1.40~ 40%) and MIP-3α (72 hours: GM=13.92~1292%, 10 days: GM=0.34~ 66%).

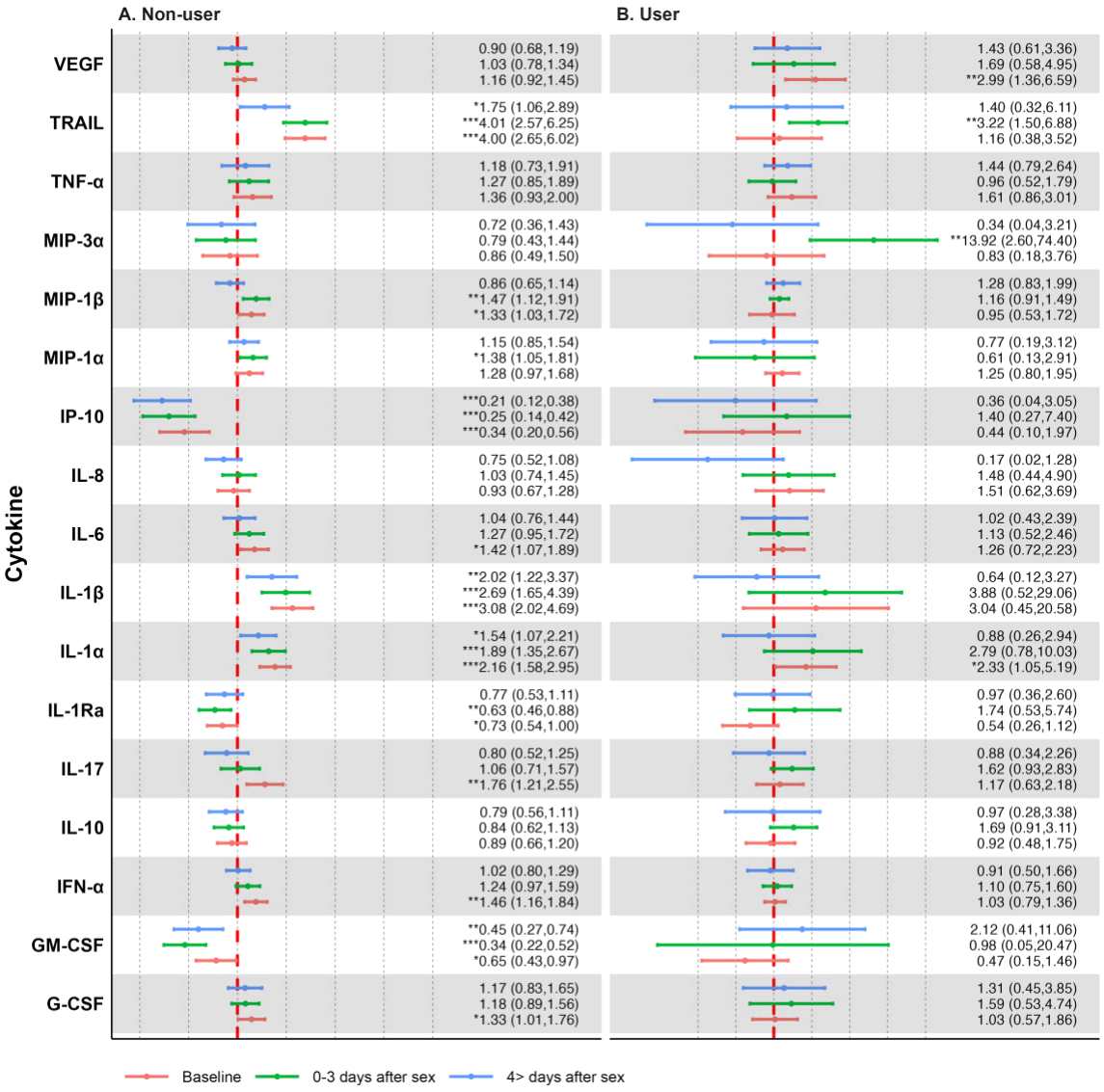


Figure 4.9: Geometric mean with 95% CI of the effect of non-*Lactobacillus*-dominated relative to *Lactobacillus*-dominated on cytokine expressions given visits (A. VIP non-users and B. VIP users). This forest plot presents odds ratios (ORs) with 95% confidence intervals (CIs) for various outcomes, stratified by user status: non-users (Panel A, left) and users (Panel B, right). Each row represents a distinct outcome, with ORs shown for three different conditions, color-coded as red, blue, and green. The vertical dashed red line represents the null value (OR = 1.0), with values to the left indicating decreased odds and those to the right indicating increased odds. For each condition, horizontal lines denote the 95% confidence interval, and asterisks indicate statistical significance ($p < 0.05$, $p < 0.01$, $p < 0.001$). The figure reveals several significant reductions in odds among non-users, particularly for outcomes in the middle section of the panel, whereas users show a broader range of effects, including some markedly increased odds for certain outcomes. This visual summary facilitates direct comparison of the impact of each condition between user groups.

4.3.9. Impact of *Lactobacillus* status on Immune Cell Expression and the Influence of Timing After Sex in VIP Users and non-users

Here, we investigated the impact of *Lactobacillus* status on immune cell frequency, highlighting the across and within visit effect for non-VIP and VIP users, while controlling for age and sexual activity as well as the number of days of last sexual intercourse. Women with a non-*Lactobacillus*-dominant microbiome exhibited significantly lower expression of CD4+CD38 (OR=0.70 CI: (0.51,0.96), p=0.026), CD8+HLA-DR (OR=0.54 CI: (0.36,0.80), p=0.002) in non-VIP users, with no such effects observed in VIP users. Whereas, the non-*Lactobacillus*-dominant microbiome was associated with significantly a higher CD4+HLA-DR (OR=2.13 CI: (1.06,4.27), p=0.035) frequency in VIP users, but had no significant impact in non-users (Table 4.3). In vip-users, two immune markers [CD4+CD38 (72 hours: OR=0.51, p=0.047; 10 days: OR=3.60, p=0.001) and CD8+CCR6 (72 hours: OR=0.05, p=0.001; 10 days: OR=5.89, p=0.012)] showed a significant increase in non-*Lactobacillus* dominant participants at 72 hours relative to *Lactobacillus* dominant participants, whereas, at 10 days, a reduction was observed. Contrary to the effect observed at 10 days after sex, non-*lactobacillus* dominant participants showed a significant increased cell frequency for markers; CD4+HLA-DR (OR= 3.62, p=0.038), CD8+CD38 (OR=6.36, p=0.002), and CD8+HLA-DR (OR=5.26, p=0.001). This same effect was observed at 72 hours after sex but it was not statistically significant. (Table 4.3)

Table 4.3: The impact of lactobacillus status on immune cell frequency in non-VIP and VIP users

Cells	Sub-group	Overall	Baseline	72 hours after sex	10 days after sex
CD4+CCR10	Non-user	1.02 (0.68,1.52) p=0.921	0.86 (0.46,1.62) p=0.634	1.27 (0.66,2.44) p=0.480	0.97 (0.41,2.29) p=0.949
	User	0.77 (0.34,1.75) p=0.517	0.44 (0.15,1.34) p=0.140	2.47 (0.63,9.63) p=0.181	0.50 (0.12,2.09) p=0.325
CD4+CCR5	Non-user	0.89 (0.68,1.16) p=0.389	0.89 (0.55,1.44) p=0.636	0.80 (0.48,1.34) p=0.389	1.00 (0.64,1.57) p=1.000
	User	0.69 (0.39,1.23) p=0.196	0.41 (0.14,1.15) p=0.086	0.71 (0.30,1.69) p=0.418	1.15 (0.49,2.68) p=0.738
CD4+CCR6	Non-user	0.92 (0.65,1.30) p=0.633	0.70 (0.39,1.24) p=0.221	0.77 (0.40,1.48) p=0.431	1.48 (0.72,3.02) p=0.282
	User	0.74 (0.41,1.35) p=0.312	0.58 (0.21,1.60) p=0.280	**0.38 (0.23,0.63) p=0.001**	1.85 (0.80,4.28) p=0.144
CD4+CD38	Non-user	**0.70 (0.51,0.96) p=0.026**	0.91 (0.54,1.53) p=0.708	**0.52 (0.30,0.91) p=0.021**	0.72 (0.45,1.16) p=0.180
	User	0.95 (0.58,1.54) p=0.818	0.53 (0.26,1.06) p=0.071	**0.51 (0.26,0.99) p=0.047**	**3.60 (1.78,7.32) p=0.001**
CD4+HLA-DR	Non-user	1.00 (0.72,1.40) p=0.985	0.97 (0.56,1.67) p=0.901	1.16 (0.63,2.13) p=0.633	0.89 (0.49,1.63) p=0.706
	User	**2.13 (1.06,4.27) p=0.035**	1.83 (0.54,6.20) p=0.312	1.72 (0.88,3.35) p=0.107	**3.62 (1.08,12.09) p=0.038**
CD4+α4β7	Non-user	1.04 (0.42,2.58) p=0.924	0.70 (0.31,1.58) p=0.384	1.48 (0.43,5.12) p=0.536	1.86 (0.28,12.29) p=0.514
	User	—	—	—	—
CD8+CCR10	Non-user	0.79 (0.29,2.14) p=0.644	0.93 (0.21,4.16) p=0.926	1.35 (0.30,6.14) p=0.694	0.30 (0.09,1.01) p=0.052
	User	1.80 (0.44,7.34) p=0.393	3.73 (0.45,31.00) p=0.209	0.98 (0.16,6.00) p=0.981	0.39 (0.06,2.39) p=0.294
CD8+CCR5	Non-user	0.98 (0.63,1.51) p=0.920	0.94 (0.51,1.73) p=0.840	0.92 (0.48,1.77) p=0.801	1.09 (0.55,2.16) p=0.794
	User	1.61 (0.66,3.93) p=0.282	2.02 (0.66,6.22) p=0.206	0.86 (0.19,3.81) p=0.832	1.96 (0.59,6.51) p=0.258
CD8+CCR6	Non-user	0.84 (0.49,1.43) p=0.516	0.83 (0.35,1.99) p=0.680	0.83 (0.33,2.10) p=0.691	0.85 (0.33,2.24) p=0.747
	User	1.33 (0.48,3.69) p=0.564	1.25 (0.32,4.88) p=0.734	**0.05 (0.01,0.25) p=0.001**	**5.89 (1.54,22.61) p=0.012**
CD8+CD38	Non-user	0.92 (0.61,1.40) p=0.705	1.13 (0.56,2.32) p=0.727	0.70 (0.38,1.29) p=0.249	1.06 (0.56,2.00) p=0.863
	User	0.86 (0.48,1.54) p=0.590	**0.33 (0.14,0.73) p=0.009**	1.19 (0.73,1.96) p=0.470	**6.36 (2.20,18.34) p=0.002**
CD8+HLA-DR	Non-user	**0.54 (0.36,0.80) p=0.002**	**0.46 (0.22,0.99) p=0.048**	0.79 (0.36,1.73) p=0.558	0.42 (0.17,1.02) p=0.054
	User	1.60 (0.53,4.78) p=0.384	2.06 (0.44,9.54) p=0.337	1.40 (0.58,3.36) p=0.432	**5.26 (2.15,12.88) p=0.001**
CD8+α4β7	Non-user	2.06 (0.42,10.02) p=0.367	2.70 (0.55,13.29) p=0.219	0.15 (0.02,1.10) p=0.062	3.26 (0.30,35.65) p=0.329
	User	—	—	—	—

Estimates presented as GM (95% CI), cell presented as — indicates that the model failed to converge

4.4. DISCUSSION

The use of VIPs is common among women in Southern Africa, with many seeking to achieve a dry, tight vaginal environment during sexual intercourse (3-10). Although these practices are culturally ingrained, research suggests that certain VIPs can alter the *Lactobacillus*-dominant vaginal microbiota, shifting it towards a more diverse, anaerobic bacterial community associated with BV (17, 18). This study sought to longitudinally characterise the vaginal microbiome composition in South African adolescent girls and adult women, with a specific focus on the influence of sexual activity and intravaginal practices on microbial dynamics and cytokine and cellular markers of genital inflammation. This study found that, at the WC site, both adolescent girls and adult women reported using only soap and water for washing their FGTs, primarily for hygiene purposes. This practice highlights a more basic and minimalistic approach to FGT hygiene in this population. In contrast, at the KZN site, participants reported using a variety of VIPs for FGT hygiene, including those intended for both hygiene and sexual enhancement (7-10). The use of VIPs at the KZN site aligns with broader trends documented in other African studies, which report widespread use of intravaginal practices in regions with high HIV prevalence (3-10), often aimed at enhancing sexual experiences, maintaining vaginal dryness and tightness, preventing unintended pregnancies, and addressing STIs and vaginal discharge (5, 7-14). In many African cultures, the use of natural products to promote "dry sex" is a socially ingrained norm, with vaginal wetness often being associated with unfaithfulness, promiscuity, and perceived sexual inadequacy (41, 42). Despite these regional and cultural differences in hygiene practices, the study found significant differences in STI prevalence between the age groups, with adolescent girls exhibiting a higher prevalence of *Chlamydia trachomatis* compared to adult women at both sites. This finding is consistent with existing literature that highlights the increased susceptibility of adolescent women to STIs, particularly *Chlamydia* (43-45), due to a combination of biological factors (such as lower production of cervical mucous and increased cervical ectopy) (46-48) and sexual behaviours (such as higher rates of unprotected sex and multiple partners) (49, 50). These results underscore the need for targeted health interventions addressing both the regional cultural practices, the widespread use of vaginal products in high HIV prevalence areas, and the increased STI vulnerability of adolescent girls.

Although several studies have shown that the vaginal microbiota of adolescents resembles those of reproductive-age women of non-African descent (51, 52), our understanding of the microbial composition differences between adolescent girls and adult women of African

descent remains limited, primarily due to the lack of targeted research addressing these age-related and ethnogeographic variations. In this study, we found that microbial diversity, both alpha and beta diversity, did not significantly differ based on age. The vaginal microbiome of adolescent girls in our cohort was predominantly dominated by *Lactobacillus iners* and BV-associated communities, mirroring the microbiota profiles observed in adult women. Furthermore, both adolescent girls and adult women exhibited transitions toward BV-related CSTs. However, adolescent girls demonstrated more dynamic and frequent shifts between CSTs, particularly between CST IV-B and CST III, whereas adult women exhibited more stability, particularly after transitioning to CST IV-B. These findings suggest that while the vaginal microbiome of adolescent girls may resemble that of adult women, there is greater microbial flux and instability in younger women. This may reflect developmental factors, hormonal fluctuations, or other contextual influences (53).

Several studies have suggested that intravaginal practices may be associated with an increased prevalence of bacterial vaginosis and other disruptions in the vaginal microbiome, including shifts from a beneficial *Lactobacillus*-dominant flora to more diverse microbial communities (1, 3, 9, 19-23). We observed that, at baseline, there were no significant differences in the relative abundances of bacterial species between women using VIPs and non-VIP users. However, over the study period, VIP users exhibited significantly higher abundances of *Sneathia* and *Lactobacillus iners* compared to non-users. These findings are consistent with previous research, which has shown that VIP use is associated with a shift from a *Lactobacillus*-dominant vaginal microbiota to a less protective microbial community, characterized by a broader diversity of primarily anaerobic bacteria (1, 9, 19-23). In contrast, the abundances of several bacterial species commonly linked to BV, including *Megasphaera lornae*, *Gardnerella vaginalis*, *Eggerthella T1*, and *Atopobium vaginae*, were significantly reduced in VIP users relative to non-users throughout the study period. These findings suggest that while VIP use may initially lead to microbial shifts towards species typically associated with dysbiosis, over time it appears to be linked with a reduction in the abundance of key BV-associated pathogens. Additionally, these findings suggest a complex, dynamic interaction between VIP use and the vaginal microbiome, highlighting the need for further research to elucidate the long-term implications for vaginal health and the clinical significance of VIP use in microbial modulation.

The study examined the relationship between vaginal microbiota composition and immune markers of genital inflammation in both VIP and non-VIP users. The results align with previous studies (25, 54) linking non-*Lactobacillus*-dominated microbiota to elevated inflammatory cytokine levels, which are associated with increased susceptibility to HIV infection due to immune activation and facilitation of viral entry (55). Specifically, women with non-*Lactobacillus*-dominated microbiota who did not use VIPs had significantly higher concentrations of several cytokines (MIP-1β, MIP-1α, IFNα, IL-6, TRAIL, IL-1β, and IL-1α and G-CSF). Conversely, MIP-3α, an important chemokine for immune cell recruitment (56, 57), was─lower in non-VIP users, suggesting impaired immune recruitment despite inflammation. Both VIP and non-VIP users with non-*Lactobacillus* microbiomes exhibited higher levels (approximately >50%) of IL-1α, IL-1β,─and TRAIL, indicating ongoing inflammation and immune cell recruitment(58-60). However, both groups showed moderately lower levels of IP-10 and GM-CSF, which are involved in lymphocyte trafficking (61, 62) and immune cell activation (63), respectively. These reduced levels of IP-10 are consistent with previous findings in women with BV, suggesting a dysregulated immune response in the context of vaginal dysbiosis (61, 64, 65). The lower expression of immune markers such as CD4+CD38 and CD8+HLA-DR further supports this, suggesting a suppressed immune response despite the presence of inflammation. Overall, these results highlight that while non-*Lactobacillus*-dominated microbiota is associated with immune activation, VIP use may have a less pronounced impact on immune responses, indicating that other biological factors may play a role in modulating how VIPs interact with the vaginal immune environment.

Next, we assessed how the timing of sexual activity (72 hours vs. 10 days post-coitus) influences immune responses in women with different vaginal microbiota compositions, and whether these effects are modulated by the use of VIPs. At 10 days post-sex, there was a significant decrease in several cytokines IP-10 and GM-CSF, indicating a resolution of inflammation and a return to immune homeostasis (66-68). This decrease aligned with increased CD4+HLA-DR expression, suggesting that the immune system is attenuating the initial inflammatory response over time. However, in VIP users, there were notable increases in IFN-α and G-CSF at the same time points, implying that VIP use may prolong or enhance certain immune activation markers, potentially affecting the resolution of inflammation. Overall, these findings highlight how vaginal microbiota composition, sexual activity timing,

and VIP use influence immune responses, with implications for infection risk and vaginal health.

Several limitations should be considered when interpreting the findings of this study. First, while the longitudinal design allows for the examination of temporal changes in vaginal microbiota composition and immune markers, the duration of follow-up may not have been long enough to fully capture the long-term impacts of VIP use on microbiota stability and immune response. Second, the study's sample size and focus on a specific population (adolescent girls and adult women from South Africa) may limit the generalizability of the findings to other regions or populations with differing microbiome profiles, sexual behaviors, and hygiene practices. Additionally, self-reported data on the use of VIPs and sexual behaviors may be subject to recall bias or underreporting, which could affect the accuracy of these measurements, particularly when addressing sensitive topics such as sexual activity and intravaginal product use. Finally, while the findings suggest a complex interaction between microbiota composition, sexual activity, and VIP use, other unmeasured factors (e.g., STIs, presence of semen exposure, hormonal fluctuations, diet, or genetic predispositions) may also influence the results, warranting further investigation to disentangle these variables.

In conclusion, this study demonstrated that the vaginal microbiota of adolescent girls may closely resemble that of adult women, though with more dynamic microbial flux and instability in younger women. Additionally, non-dominated microbiota were found to modulate immune responses, while the use of VIPs did not exhibit the same effect, suggesting that other biological factors may contribute to immune modulation. Moreover, the timing of sexual activity significantly influenced immune responses, with changes observed 10 days post-coitus, while VIP use appeared to sustain immune activation. These findings highlight the complex interplay between microbial composition, sexual activity, and VIP use in shaping vaginal health and immune function, underscoring the need for future studies with larger cohorts, longer follow-up periods, and comprehensive clinical data.

Declarations:

Ethics approval:

The protocol for this study was approved by the Ethics Review Committee of the University of KwaZulu-Natal (BREC number: BE603/18). Written informed consent was obtained from all CAPRISA 090 participants.

Consent for publication:

Not applicable

Availability of data and materials:

The raw 16S rRNA gene sequence, along with all the associated experimental and procedural metadata such as unrarefied OTU tables, and R scripts used in this study will be deposited in the Sequence Read Archive (SRA) at NCBI and GitHub (https://github.com).

Conflicts of Interest: The authors declare no conflicts of interest.

Authors Contributions: NMM and SN conceived and designed the analysis, and NMM, SN, OAK and LM performed the analyses. LGB, JSP, LM and HBJ were the principal investigators of the CAPRISA 090 study. NMM, SN, OAK, LM, SN, PR, PG, CML, NR, AGA, SS, RH, MTM, BM, HA, DP, WH, LJPL, DA, AS, QAK, LGB, JSP, HH, LM, HBJ and SN contributed to the interpretation and discussion of the results and writing of the manuscript.

References:

1. Hilber AM, Chersich MF, van de Wijgert JHHM, Rees H, Temmerman M. 2007. Vaginal practices, microbicides and HIV: what do we need to know? *Sexually Transmitted Infections* 83: 505-8
2. Alcaide ML, Rodriguez VJ, Fischl MA, Jones DL, Weiss SM. 2017. Addressing intravaginal practices in women with HIV and at-risk for HIV infection, a mixed methods pilot study. *Int J Womens Health* 9: 123-32
3. Myer L, Kuhn L, Stein ZA, Wright TC, Jr., Denny L. 2005. Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. *The Lancet Infectious Diseases* 5: 786-94
4. Beksinska ME, Rees HV, Kleinschmidt I, McIntyre J. 1999. The practice and prevalence of dry sex among men and women in South Africa: a risk factor for sexually transmitted infections? *Sex Transm Infect* 75: 178-80
5. Dallabetta GA, Miotti PG, Chiphangwi JD, Liomba G, Canner JK, Saah AJ. 1995. Traditional vaginal agents: use and association with HIV infection in Malawian women. *Aids* 9: 293-7
6. Runganga A, Pitts M, McMaster J. 1992. The use of herbal and other agents to enhance sexual experience. *Soc Sci Med* 35: 1037-42
7. Morar NS, Ramjee G, Abdool Karim SS. 1998. Vaginal insertion and douching practices among sex workers at truck stops in KwaZulu-Natal. *S Afr Med J* 88: 470
8. Scorgie F, Kunene B, Smit JA, Manzini N, Chersich MF, Preston-Whyte EM. 2009. In search of sexual pleasure and fidelity: vaginal practices in KwaZulu-Natal, South Africa. *Cult Health Sex* 11: 267-83
9. van De Wijgert JH, Mason PR, Gwanzura L, Mbizvo MT, Chirenje ZM, Iliff V, Shiboski S, Padian NS. 2000. Intravaginal practices, vaginal flora disturbances, and acquisition of sexually transmitted diseases in Zimbabwean women. *J Infect Dis* 181: 587-94
10. Sandala L, Lurie P, Sunkutu MR, Chani EM, Hudes ES, Hearst N. 1995. 'Dry sex' and HIV infection among women attending a sexually transmitted diseases clinic in Lusaka, Zambia. *Aids* 9 Suppl 1: S61-8
11. Bui TC, Scheurer ME, Pham VT, Tran LT, Hor LB, Vidrine DJ, Ross MW, Markham CM. 2018. Intravaginal practices and genital human papillomavirus infection among female sex workers in Cambodia. *J Med Virol* 90: 1765-74

12. Baleta A. 1998. Concern voiced over "dry sex" practices in South Africa. *Lancet* 352: 1292
13. Brown JE, Brown RC. 2000. Traditional intravaginal practices and the heterosexual transmission of disease: a review. *Sex Transm Dis* 27: 183-7
14. Rosenberg J. 2001. Douching is indirectly linked to HIV infection in female sex workers. *International Family Planning Perspectives* 27: 215-
15. Brown RC, Brown JE, Ayowa OB. 1993. The use and physical effects of intravaginal substances in Zairian women. *Sex Transm Dis* 20: 96-9
16. Shattock RJ, Moore JP. 2003. Inhibiting sexual transmission of HIV-1 infection. *Nature reviews. Microbiology* 1: 25-34
17. Lokken EM, Manguro GO, Abdallah A, Ngacha C, Shafi J, Kiarie J, Jaoko W, Srinivasan S, Fiedler TL, Munch MM, Fredricks DN, McClelland RS, Balkus JE. 2019. Association between vaginal washing and detection of Lactobacillus by culture and quantitative PCR in HIV-seronegative Kenyan women: a cross-sectional analysis. *Sex Transm Infect* 95: 455-61
18. Ness RB, Hillier SL, Richter HE, Soper DE, Stamm C, McGregor J, Bass DC, Sweet RL, Rice P. 2002. Douching in relation to bacterial vaginosis, lactobacilli, and facultative bacteria in the vagina. *Obstet Gynecol* 100: 765
19. Shipitsyna E, Roos A, Datcu R, Hallen A, Fredlund H, Jensen JS, Engstrand L, Unemo M. 2013. Composition of the vaginal microbiota in women of reproductive age--sensitive and specific molecular diagnosis of bacterial vaginosis is possible? *PLoS One* 8: e60670
20. Hawes SE, Hillier SL, Benedetti J, Stevens CE, Koutsky LA, Wølner-Hanssen P, Holmes KK. 1996. Hydrogen Peroxide—Producing Lactobacilli and Acquisition of Vaginal Infections. *The Journal of Infectious Diseases* 174: 1058-63
21. van de Wijgert JHHM. 2017. The vaginal microbiome and sexually transmitted infections are interlinked: Consequences for treatment and prevention. *PLOS Medicine* 14: e1002478
22. Myer L, Kuhn L, Stein ZA, Wright TC, Jr., Denny L. 2005. Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. *Lancet Infect Dis* 5: 786-94
23. McClelland RS, Lavreys L, Hassan WM, Mandaliya K, Ndinya-Achola JO, Baeten JM. 2006. Vaginal washing and increased risk of HIV-1 acquisition among African women: a 10-year prospective study. *Aids* 20: 269-73

24. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, Padavattan N, Ismail N, Moodley A, Sabatini ME, Ghebremichael MS, Nusbaum C, Huttenhower C, Virgin HW, Ndung'u T, Dong KL, Walker BD, Fichorova RN, Kwon DS. 2015. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* 42: 965-76
25. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, Padavattan N, Desai C, Droit L, Moodley A, Dong M, Chen Y, Ismail N, Ndung'u T, Ghebremichael MS, Wesemann DR, Mitchell C, Dong KL, Huttenhower C, Walker BD, Virgin HW, Kwon DS. 2017. Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. *Immunity* 46: 29-37
26. Lennard K, Dabee S, Barnabas SL, Havyarimana E, Blakney A, Jaumdally SZ, Botha G, Mkhize NN, Bekker LG, Lewis DA, Gray G, Mulder N, Passmore JS, Jaspan HB. 2018. Microbial Composition Predicts Genital Tract Inflammation and Persistent Bacterial Vaginosis in South African Adolescent Females. *Infect Immun* 86
27. Cohen CR, Moscicki AB, Scott ME, Ma Y, Shiboski S, Bukusi E, Daud I, Rebbapragada A, Brown J, Kaul R. 2010. Increased levels of immune activation in the genital tract of healthy young women from sub-Saharan Africa. *Aids* 24: 2069-74
28. Boyle AK, Rinaldi SF, Norman JE, Stock SJ. 2017. Preterm birth: Inflammation, fetal injury and treatment strategies. *J Reprod Immunol* 119: 62-6
29. Clark P, Kurtzer T, Duff P. 1994. Role of bacterial vaginosis in peripartum infections. *Infect Dis Obstet Gynecol* 2: 179-83
30. Haggerty CL, Totten PA, Tang G, Astete SG, Ferris MJ, Norori J, Bass DC, Martin DH, Taylor BD, Ness RB. 2016. Identification of novel microbes associated with pelvic inflammatory disease and infertility. *Sex Transm Infect* 92: 441-6
31. Nelson DB, Hanlon A, Nachamkin I, Haggerty C, Mastrogiannis DS, Liu C, Fredricks DN. 2014. Early pregnancy changes in bacterial vaginosis-associated bacteria and preterm delivery. *Paediatr Perinat Epidemiol* 28: 88-96
32. Masese L, McClelland RS, Gitau R, Wanje G, Shafi J, Kashonga F, Ndinya-Achola JO, Lester R, Richardson BA, Kurth A. 2013. A pilot study of the feasibility of a vaginal washing cessation intervention among Kenyan female sex workers. *Sexually Transmitted Infections* 89: 217-22

33. Esber A, Rao N, Norris A, Carr Reese P, Kandodo J, Nampandeni P, Jumbe E, Norris Turner A. 2016. Intravaginal Practices and Prevalence of Sexual and Reproductive Tract Infections Among Women in Rural Malawi. *Sex Transm Dis* 43: 750-5
34. Perfetto SP, Chattopadhyay PK, Roederer M. 2004. Seventeen-colour flow cytometry: unravelling the immune system. *Nature Reviews Immunology* 4: 648-55
35. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 13: 581-3
36. McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One* 8: e61217
37. France MT, Ma B, Gajer P, Brown S, Humphrys MS, Holm JB, Waetjen LE, Brotman RM, Ravel J. 2020. VALENCIA: a nearest centroid classification method for vaginal microbial communities based on composition. *Microbiome* 8: 166
38. McKinney W. 2010. *Data structures for statistical computing in Python*. Presented at SciPy
39. Yue JC, Clayton MK. 2005. A Similarity Measure Based on Species Proportions. *Communications in Statistics - Theory and Methods* 34: 2123-31
40. Mkhize P, Mehou-Loko C, Maphumulo N, Radzey N, Abrahams AG, Sibeko S, Harryparsad R, Manhanzva M, Meyer B, Radebe P. 2024. Differences in HIV risk factors between South African adolescents and adult women and their association with sexually transmitted infections. *Sexually Transmitted Infections*
41. Francis SC, Baisley K, Lees SS, Andrew B, Zalwango F, Seeley J, Vandepitte J, Ao TT, van de Wijgert J, Watson-Jones D, Kapiga S, Grosskurth H, Hayes RJ. 2013. Vaginal practices among women at high risk of HIV infection in Uganda and Tanzania: recorded behaviour from a daily pictorial diary. *PLoS One* 8: e59085
42. Nsereko E, Moreland PJ, Dunlop AL, Nzayirambaho M, Corwin EJ. 2021. Consideration of Cultural Practices When Characterizing the Vaginal Microbiota Among African and African American Women. *Biol Res Nurs* 23: 91-9
43. Balle C, Lennard K, Dabee S, Barnabas SL, Jaumdally SZ, Gasper MA, Maseko V, Mbulawa ZZA, Williamson AL, Bekker LG, Lewis DA, Passmore JS, Jaspan HB. 2018. Endocervical and vaginal microbiota in South African adolescents with asymptomatic Chlamydia trachomatis infection. *Sci Rep* 8: 11109
44. Barnabas SL, Dabee S, Passmore J-AS, Jaspan HB, Lewis DA, Jaumdally SZ, Gamieldien H, Masson L, Muller E, Maseko VD. 2018. Converging epidemics of

sexually transmitted infections and bacterial vaginosis in southern African female adolescents at risk of HIV. *International journal of STD & AIDS* 29: 531-9

45. Jarolimova J, Chidumwa G, Chimbindi N, Okesola N, Dreyer J, Smit T, Seeley J, Harling G, Copas A, Baisley K, Shahmanesh M, Group tIR, Herbst, McGrath N, Zuma T, Khoza T, Behuhuma N, Bassett IV, Sherr L. 2023. Prevalence of Curable Sexually Transmitted Infections in a Population-Representative Sample of Young Adults in a High HIV Incidence Area in South Africa. *Sexually Transmitted Diseases* 50: 796-803
46. Control CfD, Prevention. 2008. Sexually transmitted disease surveillance. *http://www. cdc. gov/std/stats08/syphilis. htm*
47. Burchell AN, Winer RL, de Sanjosé S, Franco EL. 2006. Chapter 6: Epidemiology and transmission dynamics of genital HPV infection. *Vaccine* 24 Suppl 3: S3/52-61
48. Shannon CL, Klausner JD. 2018. The growing epidemic of sexually transmitted infections in adolescents: a neglected population. *Curr Opin Pediatr* 30: 137-43
49. Lara LAS, Abdo CHN. 2016. Age at Time of Initial Sexual Intercourse and Health of Adolescent Girls. *J Pediatr Adolesc Gynecol* 29: 417-23
50. Monteiro IP, Azzi CFG, Bilibio JP, Monteiro PS, Braga GC, Nitz N. 2023. Prevalence of sexually transmissible infections in adolescents treated in a family planning outpatient clinic for adolescents in the western Amazon. *PLoS One* 18: e0287633
51. Hickey RJ, Zhou X, Settles ML, Erb J, Malone K, Hansmann MA, Shew ML, Van Der Pol B, Fortenberry JD, Forney LJ. 2015. Vaginal microbiota of adolescent girls prior to the onset of menarche resemble those of reproductive-age women. *mBio* 6
52. Thoma ME, Gray RH, Kiwanuka N, Aluma S, Wang MC, Sewankambo N, Wawer MJ. 2011. Longitudinal changes in vaginal microbiota composition assessed by gram stain among never sexually active pre- and postmenarcheal adolescents in Rakai, Uganda. *J Pediatr Adolesc Gynecol* 24: 42-7
53. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. 2011. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 108 Suppl 1: 4680-7
54. Mtshali A, San JE, Osman F, Garrett N, Balle C, Giandhari J, Onywera H, Mngomezulu K, Mzobe G, de Oliveira T, Rompalo A, Mindel A, Abdool Karim SS, Ravel J, Passmore J-AS, Abdool Karim Q, Jaspan HB, Liebenberg LJP, Ngcapu S. 2021. Temporal Changes in Vaginal Microbiota and Genital Tract Cytokines Among South African Women Treated for Bacterial Vaginosis. *Frontiers in Immunology* 12

55. Masson L, Passmore JA, Liebenberg LJ, Werner L, Baxter C, Arnold KB, Williamson C, Little F, Mansoor LE, Naranbhai V, Lauffenburger DA, Ronacher K, Walzl G, Garrett NJ, Williams BL, Couto-Rodriguez M, Hornig M, Lipkin WI, Grobler A, Abdool Karim Q, Abdool Karim SS. 2015. Genital inflammation and the risk of HIV acquisition in women. *Clin Infect Dis* 61: 260-9

56. Dieu-Nosjean MC, Massacrier C, Homey B, Vanbervliet B, Pin JJ, Vicari A, Lebecque S, Dezutter-Dambuyant C, Schmitt D, Zlotnik A, Caux C. 2000. Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J Exp Med* 192: 705-18

57. Ngo VN, Tang HL, Cyster JG. 1998. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *J Exp Med* 188: 181-91

58. Voronov E, Dotan S, Krelin Y, Song X, Elkabets M, Carmi Y, Rider P, Idan C, Romzova M, Kaplanov I, Apte RN. 2013. Unique Versus Redundant Functions of IL-1α and IL-1β in the Tumor Microenvironment. *Front Immunol* 4: 177

59. Ribatti D, Crivellato E. 2009. Immune cells and angiogenesis. *J Cell Mol Med* 13: 2822-33

60. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. 2008. Growth factors and cytokines in wound healing. *Wound Repair Regen* 16: 585-601

61. Hoermannsperger G, Clavel T, Hoffmann M, Reiff C, Kelly D, Loh G, Blaut M, Hölzlwimmer G, Laschinger M, Haller D. 2009. Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation. *PLoS One* 4: e4365

62. Jauregui CE, Wang Q, Wright CJ, Takeuchi H, Uriarte SM, Lamont RJ. 2013. Suppression of T-cell chemokines by Porphyromonas gingivalis. *Infect Immun* 81: 2288-95

63. Gomez-Cambronero J, Horn J, Paul CC, Baumann MA. 2003. Granulocyte-macrophage colony-stimulating factor is a chemoattractant cytokine for human neutrophils: involvement of the ribosomal p70 S6 kinase signaling pathway. *J Immunol* 171: 6846-55

64. Masson L, Mlisana K, Little F, Werner L, Mkhize NN, Ronacher K, Gamieldien H, Williamson C, Mckinnon LR, Walzl G. 2014. Defining genital tract cytokine signatures

of sexually transmitted infections and bacterial vaginosis in women at high risk of HIV infection: a cross-sectional study. *Sex Transm Infect*: sextrans-2014-051601

65. Kyongo JK, Crucitti T, Menten J, Hardy L, Cools P, Michiels J, Delany-Moretlwe S, Mwaura M, Ndayisaba G, Joseph S, Fichorova R, van de Wijgert J, Vanham G, Arien KK, Jespers V. 2015. Cross-Sectional Analysis of Selected Genital Tract Immunological Markers and Molecular Vaginal Microbiota in Sub-Saharan African Women, with Relevance to HIV Risk and Prevention. *Clin Vaccine Immunol* 22: 526-38
66. Oppenheim JJ, Tewary P, de la Rosa G, Yang D. 2007. Alarmins initiate host defense. *Adv Exp Med Biol* 601: 185-94
67. Atanasova M, Whitty A. 2012. Understanding cytokine and growth factor receptor activation mechanisms. *Crit Rev Biochem Mol Biol* 47: 502-30
68. Broughton SE, Hercus TR, Lopez AF, Parker MW. 2012. Cytokine receptor activation at the cell surface. *Curr Opin Struct Biol* 22: 350-9

4.5. Supplementary Tables and Figures

Table S4.1: Descriptive statistics of cytokine expression by age class based on *Lactobacillus* status

	Non *Lactobacillus*-dominant, N = 167		***Lactobacillus* Dominant**, N = 158	
Characteristic	**Adult**, N = 72	**Adolescent**, N = 95	**Adult**, N = 63	**Adolescent**, N = 95
MIP-1β				
Mean (SD)	1,763 (1,107)	1,976 (1,514)	1,533 (1,167)	1,518 (1,299)
Median (IQR)	1,506 (1,209, 2,025)	1,721 (1,134, 2,409)	1,378 (1,027, 1,949)	1,357 (916, 1,729)
IP-10				
Mean (SD)	3,824 (8,097)	4,518 (9,456)	6,355 (9,386)	10,872 (13,340)
Median (IQR)	1,500 (371, 3,373)	994 (203, 4,125)	3,498 (913, 7,776)	5,875 (2,100, 12,635)
GM-CSF				
Mean (SD)	214 (310)	228 (283)	300 (265)	328 (284)
Median (IQR)	65 (16, 333)	68 (16, 353)	299 (16, 491)	289 (53, 506)
IL-1α				
Mean (SD)	6,428 (8,002)	8,274 (9,706)	3,050 (3,762)	3,303 (3,759)
Median (IQR)	3,802 (1,295, 9,375)	5,485 (1,793, 11,880)	1,437 (723, 3,827)	1,676 (801, 4,437)
IL-1Ra				
Mean (SD)	167,036 (436,459)	127,078 (334,154)	314,650 (630,059)	143,875 (291,234)
Median (IQR)	55,567 (41,286, 80,513)	52,461 (39,941, 83,542)	72,312 (51,836, 104,682)	77,578 (57,186, 106,295)
IL-8				
Mean (SD)	14,008 (18,781)	12,990 (10,944)	17,798 (26,229)	17,013 (24,648)
Median (IQR)	9,679 (4,487, 16,207)	10,630 (3,549, 17,229)	9,018 (2,893, 17,075)	10,863 (5,614, 17,324)
IL-17				
Mean (SD)	243 (203)	352 (428)	209 (201)	330 (686)
Median (IQR)	203 (81, 383)	237 (95, 448)	150 (70, 292)	195 (44, 338)
TRAIL				
Mean (SD)	443 (623)	489 (622)	210 (321)	191 (324)
Median (IQR)	302 (106, 531)	297 (95, 754)	100 (10, 274)	57 (10, 232)
MIP-1α				
Mean (SD)	114 (184)	150 (482)	92 (160)	79 (105)
Median (IQR)	68 (42, 99)	74 (48, 116)	55 (32, 97)	65 (40, 91)
MIP-3α				
Mean (SD)	2,692 (5,990)	1,298 (1,557)	4,411 (17,493)	1,823 (2,253)
Median (IQR)	1,036 (104, 4,656)	740 (100, 1,751)	1,060 (333, 4,304)	937 (160, 2,619)
G-CSF				
Mean (SD)	3,549 (3,264)	4,366 (4,794)	3,009 (3,215)	3,672 (4,887)
Median (IQR)	2,645 (1,024, 4,838)	2,746 (1,547, 4,991)	1,866 (694, 4,347)	1,956 (956, 3,794)
IFN-α				
Mean (SD)	183 (100)	186 (99)	149 (86)	163 (95)
Median (IQR)	166 (110, 237)	175 (101, 265)	147 (83, 201)	150 (92, 228)
IL-1β				
Mean (SD)	3,177 (5,034)	5,395 (13,191)	1,290 (3,324)	1,574 (3,042)
Median (IQR)	1,099 (234, 3,927)	2,477 (536, 5,036)	382 (70, 1,178)	456 (180, 1,232)
IL-6				
Mean (SD)	2,073 (3,336)	2,252 (2,896)	1,246 (1,312)	1,649 (2,559)
Median (IQR)	1,037 (449, 1,898)	1,130 (682, 3,202)	812 (337, 1,625)	938 (465, 1,707)
IL-10				
Mean (SD)	1,983 (2,030)	2,670 (6,595)	1,968 (1,442)	2,348 (3,233)
Median (IQR)	1,345 (690, 2,650)	1,559 (611, 2,783)	1,839 (645, 2,967)	1,918 (1,127, 2,902)
TNF-α				
Mean (SD)	175 (211)	196 (199)	137 (163)	132 (214)
Median (IQR)	105 (51, 219)	151 (49, 272)	81 (33, 186)	81 (42, 156)
VEGF				
Mean (SD)	12,248 (11,681)	13,283 (12,025)	10,492 (9,709)	12,919 (14,579)
Median (IQR)	9,130 (4,745, 14,404)	9,909 (5,393, 17,104)	8,968 (3,636, 15,174)	8,620 (5,268, 14,361)
Range	337, 56,669	542, 70,809	122, 56,620	147, 89,644
IFN-β	21 (29%)	15 (16%)	11 (17%)	16 (17%)
RANTES	53 (77%)	75 (84%)	47 (80%)	69 (79%)

Table S4.2: Descriptive statistics of cell frequencies by age class based *on Lactobacillus* status.

	Non-*Lactobacillus* dominant, N = 36		***Lactobacillus* Dominant**, N = 43	
Characteristic	**Adult**, N = 19	**Adolescent**, N = 17	**Adult**, N = 24	**Adolescent**, N = 19
CD4+α4β7				
Mean (SD)	12 (17)	8 (21)	12 (13)	4 (9)
Median (IQR)	2 (0, 20)	2 (0, 6)	10 (3, 14)	1 (0, 2)
CD4+CCR5				
Mean (SD)	22 (17)	31 (12)	27 (14)	36 (16)
Median (IQR)	18 (9, 30)	30 (20, 42)	24 (17, 35)	32 (26, 50)
CD4+CCR6				
Mean (SD)	43 (23)	56 (25)	51 (24)	49 (26)
Median (IQR)	45 (32, 57)	60 (46, 73)	59 (33, 68)	57 (30, 64)
CD4+CCR10				
Mean (SD)	38 (24)	39 (21)	34 (26)	35 (24)
Median (IQR)	32 (20, 50)	37 (25, 50)	31 (14, 43)	27 (18, 50)
CD4+CD38				
Mean (SD)	39 (22)	36 (12)	44 (15)	42 (19)
Median (IQR)	36 (28, 51)	37 (25, 45)	46 (33, 55)	37 (32, 60)
CD4+HLA-DR				
Mean (SD)	59 (23)	55 (19)	54 (24)	48 (21)
Median (IQR)	63 (41, 75)	57 (40, 68)	54 (39, 70)	43 (34, 64)
CD8+α4β7				
Mean (SD)	5 (8)	6 (19)	5.8 (11.1)	1.3 (3.9)
Median (IQR)	1 (0, 5)	0 (0, 1)	0.9 (0.0, 4.7)	0.0 (0.0, 0.9)
CD8+CCR5				
Mean (SD)	58 (20)	59 (23)	51 (25)	66 (22)
Median (IQR)	61 (45, 74)	61 (36, 80)	55 (31, 74)	68 (48, 81)
CD8+CCR6				
Mean (SD)	18 (20)	24 (27)	25 (22)	17 (19)
Median (IQR)	11 (3, 23)	11 (6, 33)	18 (7, 45)	13 (2, 22)
CD8+CCR10				
Mean (SD)	7 (13)	13 (27)	12 (22)	5 (8)
Median (IQR)	2 (0, 6)	2 (1, 3)	5 (0, 12)	2 (1, 5)
CD8+CD38				
Mean (SD)	65 (21)	64 (21)	64 (19)	71 (18)
Median (IQR)	65 (51, 78)	71 (48, 78)	66 (53, 80)	70 (66, 86)
CD8+HLA-DR				
Mean (SD)	34 (24)	27 (25)	33 (19)	29 (18)
Median (IQR)	29 (19, 50)	20 (12, 26)	34 (18, 49)	34 (14, 41)

Table S4.3: Descriptive statistics of cytokine expression by age class based on *Lactobacillus* status

Characteristic	Non-*Lactobacillus* dominant, N = 167		*Lactobacillus* Dominant, N = 158	
	Adult, N = 72	**Adolescent**, N = 95	**Adult**, N = 63	**Adolescent**, N = 95
MIP-1β				
Mean (SD)	1,763 (1,107)	1,976 (1,514)	1,533 (1,167)	1,518 (1,299)
Median (IQR)	1,506 (1,209, 2,025)	1,721 (1,134, 2,409)	1,378 (1,027, 1,949)	1,357 (916, 1,729)
IP-10				
Mean (SD)	3,824 (8,097)	4,518 (9,456)	6,355 (9,386)	10,872 (13,340)
Median (IQR)	1,500 (371, 3,373)	994 (203, 4,125)	3,498 (913, 7,776)	5,875 (2,100, 12,635)
GM-CSF				
Mean (SD)	214 (310)	228 (283)	300 (265)	328 (284)
Median (IQR)	65 (16, 333)	68 (16, 353)	299 (16, 491)	289 (53, 506)
IL-1α				
Mean (SD)	6,428 (8,002)	8,274 (9,706)	3,050 (3,762)	3,303 (3,759)
Median (IQR)	3,802 (1,295, 9,375)	5,485 (1,793, 11,880)	1,437 (723, 3,827)	1,676 (801, 4,437)
IL-1Ra				
Mean (SD)	167,036 (436,459)	127,078 (334,154)	314,650 (630,059)	143,875 (291,234)
Median (IQR)	55,567 (41,286, 80,513)	52,461 (39,941, 83,542)	72,312 (51,836, 104,682)	77,578 (57,186, 106,295)
IL-8				
Mean (SD)	14,008 (18,781)	12,990 (10,944)	17,798 (26,229)	17,013 (24,648)
Median (IQR)	9,679 (4,487, 16,207)	10,630 (3,549, 17,229)	9,018 (2,893, 17,075)	10,863 (5,614, 17,324)
IL-17				
Mean (SD)	243 (203)	352 (428)	209 (201)	330 (686)
Median (IQR)	203 (81, 383)	237 (95, 448)	150 (70, 292)	195 (44, 338)
TRAIL				
Mean (SD)	443 (623)	489 (622)	210 (321)	191 (324)
Median (IQR)	302 (106, 531)	297 (95, 754)	100 (10, 274)	57 (10, 232)
MIP-1α				
N	72	95	63	95
Mean (SD)	114 (184)	150 (482)	92 (160)	79 (105)
Median (IQR)	68 (42, 99)	74 (48, 116)	55 (32, 97)	65 (40, 91)
MIP-3α				
Mean (SD)	2,692 (5,990)	1,298 (1,557)	4,411 (17,493)	1,823 (2,253)
Median (IQR)	1,036 (104, 4,656)	740 (100, 1,751)	1,060 (333, 4,304)	937 (160, 2,619)
G-CSF				
Mean (SD)	3,549 (3,264)	4,366 (4,794)	3,009 (3,215)	3,672 (4,887)
Median (IQR)	2,645 (1,024, 4,838)	2,746 (1,547, 4,991)	1,866 (694, 4,347)	1,956 (956, 3,794)
IFN-α				
Mean (SD)	183 (100)	186 (99)	149 (86)	163 (95)
Median (IQR)	166 (110, 237)	175 (101, 265)	147 (83, 201)	150 (92, 228)
IL-1β				
Mean (SD)	3,177 (5,034)	5,395 (13,191)	1,290 (3,324)	1,574 (3,042)
Median (IQR)	1,099 (234, 3,927)	2,477 (536, 5,036)	382 (70, 1,178)	456 (180, 1,232)
IL-6				
Mean (SD)	2,073 (3,336)	2,252 (2,896)	1,246 (1,312)	1,649 (2,559)
Median (IQR)	1,037 (449, 1,898)	1,130 (682, 3,202)	812 (337, 1,625)	938 (465, 1,707)
IL-10				
Mean (SD)	1,983 (2,030)	2,670 (6,595)	1,968 (1,442)	2,348 (3,233)
Median (IQR)	1,345 (690, 2,650)	1,559 (611, 2,783)	1,839 (645, 2,967)	1,918 (1,127, 2,902)
TNF-α				
Mean (SD)	175 (211)	196 (199)	137 (163)	132 (214)
Median (IQR)	105 (51, 219)	151 (49, 272)	81 (33, 186)	81 (42, 156)
VEGF				
Mean (SD)	12,248 (11,681)	13,283 (12,025)	10,492 (9,709)	12,919 (14,579)
Median (IQR)	9,130 (4,745, 14,404)	9,909 (5,393, 17,104)	8,968 (3,636, 15,174)	8,620 (5,268, 14,361)
IFN-β	21 (29%)	15 (16%)	11 (17%)	16 (17%)
RANTES	53 (77%)	75 (84%)	47 (80%)	69 (79%)

Table S4.4: Descriptive statistics of cell frequencies by age class based on *Lactobacillus* status

	Non-*Lactobacillus* dominant, N = 36		***Lactobacillus* Dominant**, N = 43	
Characteristic	**Adult**, N = 19	**Adolescent**, N = 17	**Adult**, N = 24	**Adolescent**, N = 19
CD4+α4β7				
Mean (SD)	12 (17)	8 (21)	12 (13)	4 (9)
Median (IQR)	2 (0, 20)	2 (0, 6)	10 (3, 14)	1 (0, 2)
CD4+CCR5				
Mean (SD)	22 (17)	31 (12)	27 (14)	36 (16)
Median (IQR)	18 (9, 30)	30 (20, 42)	24 (17, 35)	32 (26, 50)
CD4+CCR6				
Mean (SD)	43 (23)	56 (25)	51 (24)	49 (26)
Median (IQR)	45 (32, 57)	60 (46, 73)	59 (33, 68)	57 (30, 64)
CD4+CCR10				
Mean (SD)	38 (24)	39 (21)	34 (26)	35 (24)
Median (IQR)	32 (20, 50)	37 (25, 50)	31 (14, 43)	27 (18, 50)
CD4+CD38				
Mean (SD)	39 (22)	36 (12)	44 (15)	42 (19)
Median (IQR)	36 (28, 51)	37 (25, 45)	46 (33, 55)	37 (32, 60)
CD4+HLA-DR				
Mean (SD)	59 (23)	55 (19)	54 (24)	48 (21)
Median (IQR)	63 (41, 75)	57 (40, 68)	54 (39, 70)	43 (34, 64)
CD8+α4β7				
Mean (SD)	5 (8)	6 (19)	5.8 (11.1)	1.3 (3.9)
Median (IQR)	1 (0, 5)	0 (0, 1)	0.9 (0.0, 4.7)	0.0 (0.0, 0.9)
CD8+CCR5				
Mean (SD)	58 (20)	59 (23)	51 (25)	66 (22)
Median (IQR)	61 (45, 74)	61 (36, 80)	55 (31, 74)	68 (48, 81)
CD8+CCR6				
Mean (SD)	18 (20)	24 (27)	25 (22)	17 (19)
Median (IQR)	11 (3, 23)	11 (6, 33)	18 (7, 45)	13 (2, 22)
CD8+CCR10				
Mean (SD)	7 (13)	13 (27)	12 (22)	5 (8)
Median (IQR)	2 (0, 6)	2 (1, 3)	5 (0, 12)	2 (1, 5)
CD8+CD38				
Mean (SD)	65 (21)	64 (21)	64 (19)	71 (18)
Median (IQR)	65 (51, 78)	71 (48, 78)	66 (53, 80)	70 (66, 86)
CD8+HLA-DR				
Mean (SD)	34 (24)	27 (25)	33 (19)	29 (18)
Median (IQR)	29 (19, 50)	20 (12, 26)	34 (18, 49)	34 (14, 41)

CHAPTER 5

5.1 SUMMARY AND DISCUSSION OF THE MAIN FINDING

The vaginal ecosystem is a dynamic and complex microbial environment that plays a critical role in women's reproductive and overall health. An optimal ("healthy") vaginal microbiome is typically dominated by *Lactobacillus* species, which contribute to vaginal health by producing lactic acid, maintaining a low pH, and inhibiting the growth of pathogenic organisms (1, 2). However, shifts in the microbiome away from *Lactobacillus* dominance, often towards a diverse community of polymicrobial anaerobes, are associated with increased susceptibility to sexually transmitted infections (STIs), including HIV (3-7). These shifts are also implicated in reduced efficacy of HIV PrEP, likely due to increased inflammation, reduced epithelial barrier integrity, and altered drug pharmacokinetics in the vaginal environment (8-11). Various factors influence the vaginal microbiota, including hormonal fluctuations, sexual behaviour, menstrual hygiene practices, and the use of vaginal insertion products (12, 13). These products, which are common among women in Southern Africa, have been linked to an increased prevalence of bacterial vaginosis (BV), a condition characterised by dysbiotic microbiota and associated inflammation (14-17).

This dissertation systematically investigates the impact of oral and topical PrEP on the vaginal microbiome and explores additional factors such as sexual activity and intravaginal practices that may contribute to microbial dynamics and genital inflammation. The research is divided into four distinct but interrelated components: (i) Introduction and Literature Review (CHAPTER 1): This chapter highlights the significance of the study, focusing on the high HIV incidence among adolescent girls and young women (AGYW) in South Africa, with rates as high as 7.8%−8.6% in high-burden areas. It reviews the interplay between the vaginal microbiome, genital inflammation, and HIV susceptibility, emphasizing the role of *Lactobacillus*-dominant microbiota in reducing STI risks and enhancing PrEP efficacy. The chapter also outlines the study's design and methodologies. (ii) Impact of Oral TDF/FTC PrEP on Vaginal Microbiota: A longitudinal analysis evaluates the effects of daily oral PrEP on the vaginal microbiome composition in healthy South African women (CHAPTER 2). A longitudinal analysis evaluates whether daily oral TDF/FTC PrEP alters the composition of the vaginal microbiota in healthy South African women. The findings indicate that oral PrEP does not significantly disrupt microbial community structure or *Lactobacillus* dominance, reassuring its compatibility with microbiome health in high-risk populations. (iii) Influence of Vaginal Microbiota on Cervicovaginal Tenofovir Levels (CHAPTER 3): This chapter examines

how microbiome composition affects local tenofovir pharmacokinetics. It explores the potential interactions between vaginal microbial communities, drug metabolism, and tissue drug penetration, which may impact PrEP efficacy. (iv) Effects of Intravaginal Practices and Sexual Activity on vaginal microbiota, cytokine and immune markers of genital inflammation (CHAPTER 4): This component explored the role of cultural and behavioural practices, including intravaginal cleansing, the use of insertion products, and sexual activity, on the composition of the vaginal microbiota in adolescent girls and young women. It also examines the relationship between these practices, cytokine and immune markers of genital inflammation, which are critical for understanding the biological mechanisms underlying increased STI susceptibility.

A. Exploring the Impact of Oral PrEP on the Vaginal Microbiome

While PrEP has been shown to be highly effective in preventing HIV acquisition in men who have sex with men (MSM), studies in women have yielded suboptimal outcomes. Previous research suggests that the vaginal microbiome can significantly influence the efficacy of topical tenofovir-based PrEP (8, 18), but this effect has not been observed with oral tenofovir-based PrEP (19). Oral tenofovir disoproxil fumarate/emtricitabine (TDF/FTC) PrEP was found to be equally effective among women with normal and abnormal vaginal microbiota. However, these studies relied on Gram stain and qPCR testing to characterise vaginal dysbiosis, which are limited in their ability to detect the full range of microbial species.

To address this gap, we investigated the longitudinal effects of oral TDF/FTC PrEP on the vaginal microbiome of healthy South African women, using 16S rRNA gene amplicon sequencing for molecular characterisation. This study represents the first to assess the impact of oral PrEP on the composition of the vaginal microbiota with this level of precision. Our data revealed that fewer women in both the PrEP and non-PrEP groups had increased relative abundance of *Lactobacillus crispatus*. Instead, the cervicovaginal microbiota was predominantly dominated by *Lactobacillus iners* and *Gardnerella vaginalis* communities, consistent with prior findings showing a lack of *L. crispatus* dominance in the vaginal microbiota of young Black South African women aged 18–23 years (1, 5). We further explored the temporal dynamics of microbial communities over time. No significant differences in microbial community transitions were observed between PrEP and non-PrEP groups. However, communities dominated by *L. iners* were less likely to transition to other states, and when

transitions occurred, they often shifted toward bacterial vaginosis (BV)-associated communities associated with an increased risk of HIV. This aligns with recent findings indicating that transitions from *L. crispatus* dominance to *L. iners* communities are less likely to revert to *L. crispatus* but more likely to progress to BV-associated states (20). The role of *L. iners* in vaginal ecology remains unclear. It is uncertain whether *L. iners* merely reflects transitional changes or actively contributes to BV risk and impacts the mucosal immune environment. Understanding the resilience and ecological role of *L. iners* is critical for addressing vaginal dysbiosis and improving interventions. Additionally, BV treatments have been shown to induce temporal changes in the vaginal microbiota (21-23). Our findings revealed that after antibiotic treatment for BV, *G. vaginalis*-dominant communities often transitioned to *L. iners*. This highlights the need for supplementary treatments, such as probiotics, aimed at fostering a transition from *L. iners* to *L. crispatus*, a state associated with vaginal health. Effective probiotic therapies could potentially enhance BV treatment outcomes and reduce the risk of HIV acquisition by promoting the restoration of a healthy vaginal microbiome.

B. Impact of Vaginal Microbiota on Topical Tenofovir Gel Efficacy

Recent studies have highlighted that vaginal microbiota may influence the efficacy of topical tenofovir (TFV) gel through several potential mechanisms, including the proteolytic degradation of TFV by microbial enzymes (8, 18). These enzymes, produced by BV-associated bacteria such as *Gardnerella vaginalis* and *Prevotella spp.*, may alter the stability and bioavailability of TFV, potentially reducing its efficacy in preventing HIV infection (8, 11, 18). Proteomic analyses have identified bacterial-associated proteins involved in the degradation process; however, these analyses are often limited in their sensitivity to low-abundance species, particularly when using mass spectrometry-based approaches (8). While in vitro studies have demonstrated TFV biodegradation in the presence of specific BV-associated bacteria, these findings may not fully capture the complexity of in vivo conditions. The vaginal environment is influenced by factors such as pH, host immune responses, and the presence of mucus, which can modulate microbial activity and drug bioavailability (24-26). Furthermore, the interactions between bacterial communities and TFV, including potential competitive or synergistic effects among microbial species, remain poorly understood.

To address these gaps, we conducted a comprehensive investigation into how vaginal microbiota composition affects TFV pharmacokinetics (PK) in women participating in the CAPRISA 008 1% tenofovir gel study (Chapter 3). This study utilized culture-independent bacterial 16S rRNA gene sequencing and community profiling to characterize the cervicovaginal microbiota. These advanced molecular methods enabled us to capture the diversity and relative abundance of bacterial taxa without the limitations of traditional culture techniques. Our analysis revealed that most participants exhibited vaginal microbiomes dominated by *Lactobacillus iners* (CST III), a community type associated with a less stable and more dynamic microbial environment compared to *Lactobacillus crispatus*-dominated microbiota. *L. iners* is known for its ability to adapt to a wide range of vaginal conditions, but it is also more prone to transitioning into diverse microbial states linked to bacterial vaginosis (BV). This instability could potentially influence TFV gel efficacy by altering the vaginal environment, including pH and mucosal barrier integrity. Interestingly, the prevalence of *L. crispatus*, a species associated with more stable and protective vaginal conditions, was low among participants. This observation aligns with findings from other sub-Saharan African cohorts (5-7, 27-29), where *L. iners* and BV-associated communities are more common. These microbiota profiles are thought to reflect a combination of genetic, environmental, and behavioural factors prevalent in these regions.

The highlight of our findings is that the composition of the vaginal microbiota, including the presence of BV-associated bacteria and *Lactobacillus*-dominated communities, does not significantly influence tenofovir (TFV) gel pharmacokinetics or its concentration levels in the vaginal environment. Specifically, we observed no correlation between the relative abundance of bacterial species such as *Gardnerella vaginalis*, *Prevotella spp.*, or *Lactobacillus spp.* (including *L. iners*, *L. crispatus*, and *L. jensenii*) and TFV concentrations. Additionally, microbial diversity (both alpha and beta) and community state types (CSTs) were not associated with differences in TFV levels. Most participants exhibited CST III microbiota dominated by *L. iners*, a less stable microbial community prone to transitioning to BV-associated states. Despite this, TFV concentrations remained unaffected, suggesting that TFV gel efficacy is robust against variations in vaginal microbiota composition. This is particularly significant for populations in high HIV-prevalence regions where BV is common, as our findings support the use of TFV-based PrEP regardless of vaginal microbial profiles.

C. Lack of Correlation Between Microbiota and TFV Levels

Our results showed no correlation between the relative abundance of BV-associated bacteria (*G. vaginalis*, *Prevotella spp.*) and *Lactobacillus spp.* (*L. iners*, *L. crispatus*, *L. jensenii*) with TFV concentrations. In contrast, Hillier et al. and Klatt et al. demonstrated significantly lower TFV concentrations in cervical-vaginal aspirates from users of TFV 1% gel and film who exhibited CST IV microbiota (8, 30). Similarly, other studies have found that participants with diverse anaerobic CST IVA/B microbiota exhibited higher *in vivo* TFV release compared to those with *Lactobacillus*-dominated microbiota. While a sub-analysis of CAPRISA 004 samples suggested reduced TFV gel efficacy in women with non–*Lactobacillus*-dominated microbiomes (31), our findings do not support this role. Additionally, no significant differences in microbial diversity or community state types were observed between women with high and low TFV concentrations. These findings align with a recent study of TFV-based rings, which observed no changes in mucosal TFV concentrations in the presence of BV-associated microbiota (32-34). Overall, our results do not indicate significant interactions between vaginal microbiota composition and *in vivo* TFV concentrations.

D. Implications of Vaginal Microbiota on Tenofovir Gel Efficacy and the Importance of PrEP Accessibility for BV-Positive Women

The key takeaway from our study (Chapter 2 and 3) is that vaginal microbiota composition, including BV-associated bacteria and *Lactobacillus*-dominated communities, does not significantly modulate TFV pharmacokinetics or gel efficacy. This underscores that TFV gel remains a robust and effective PrEP strategy, even in the presence of BV-associated or diverse microbiota. Continuing PrEP use in BV-positive women is essential to ensuring equitable and effective HIV prevention, particularly in high-burden regions like sub-Saharan Africa, where BV is highly prevalent. BV disrupts the protective vaginal microbiota, increasing HIV susceptibility due to inflammation, immune activation, and compromised epithelial barriers (7, 35, 36). Providing PrEP to BV-positive women addresses this heightened vulnerability, as studies, including our findings, demonstrate that TFV-based PrEP remains effective regardless of vaginal microbiota composition. Excluding BV-positive women from PrEP access would disproportionately impact a large segment of women in these regions, exacerbating health disparities. Furthermore, BV is often linked to sociocultural and behavioural practices, such as intravaginal practices, making it critical for prevention strategies to accommodate real-world conditions (37, 38). By including BV-positive women in PrEP programs, public health efforts

can significantly reduce HIV incidence at the population level, ultimately contributing to epidemic control and reinforcing the importance of equitable healthcare access.

E. Age-Related Differences in STI Prevalence and Microbiota

Our study also reveals significant age-related differences in STI prevalence and vaginal microbiome dynamics, highlighting the unique vulnerabilities of adolescent girls compared to adult women. Adolescent girls exhibited a higher prevalence of *Chlamydia trachomatis*, attributed to biological factors such as an immature cervical epithelium that is more susceptible to infection, and behavioural risks like inconsistent condom use and limited access to STI prevention services (39-46). While microbial diversity (both alpha and beta) was comparable across age groups, adolescent girls displayed greater microbial instability and flux, characterized by frequent shifts in the vaginal microbiome. Their microbiomes were predominantly dominated by *L. iners* and bacterial vaginosis-associated communities, patterns that reflect developmental and hormonal changes during puberty. The reduced resilience associated with *L. iners* and the increased presence of BV-associated bacteria suggest a higher risk for microbial dysbiosis and STI susceptibility. These patterns likely reflect the influence of developmental, hormonal, and contextual factors (47). These findings underscore the critical role of developmental, hormonal, and contextual factors in shaping adolescent vaginal health and emphasise the need for targeted public health interventions to address their heightened vulnerability.

F. Impact of Vaginal Practices on Microbiota Composition, Immune Profiles, and Health Risks in High HIV-Prevalence Regions

Microbial composition and dynamics in the vaginal environment are influenced by a range of factors, including VIPs, sexual activity, and hygiene practices (48-50). These factors can alter the balance of vaginal microbiota, which plays a crucial role in maintaining vaginal health and modulating immune responses. Intravaginal practices, including the use of VIPs, have been shown to be widespread in regions with a high prevalence of HIV, particularly in South Africa, where such practices are influenced by cultural, social, and economic factors (16, 17, 49-54). In Chapter 3, we conducted a longitudinal evaluation of the effects of VIP use and sexual activity on vaginal microbiota composition and stability in South African adolescent girls and adult women. Our findings revealed that participants frequently used a variety of VIPs, often

for purposes such as hygiene or sexual enhancement. Notably, the prevalence of VIP use was higher at the KwaZulu-Natal (KZN) site, consistent with trends reported in other African studies (16, 17, 49-54). These practices are important to investigate given their potential to disrupt vaginal microbiota and impact reproductive and sexual health. Analysis of microbial composition demonstrated that VIP users had increased abundances of *Sneathia* and *L. iners*, a pattern previously linked to shifts from Lactobacillus-dominated microbiota, which are protective, to less protective and more diverse anaerobic communities (49, 55-60). Such shifts are often associated with bacterial vaginosis (BV) and other dysbiotic states that can increase the risk of sexually transmitted infections (STIs), including HIV.

In addition to microbial shifts, our study identified distinct immune profiles associated with VIP use and non-*Lactobacillus*-dominated microbiota. Both VIP users and non-users with disrupted microbiota exhibited elevated levels of inflammatory markers, including IL-1α, IL-1β, VEGF, and TRAIL (61-63). These markers are indicative of an activated immune state, which may reflect an inflammatory response to microbial imbalance or external irritants. Concurrently, there were reductions in key immune markers, such as IP-10 and GM-CSF, suggesting an impaired ability to mount a robust immune defence (64, 65). The combination of heightened inflammation and diminished immune signalling creates an environment that may increase susceptibility to infections.

Further supporting this, reduced expression of immune activation markers, such as CD4+CCR10, CD4+HLA-DR, and CD8+HLA-DR, was observed. This finding indicates a paradoxical dampening of adaptive immune responses, even in the context of inflammation (66). Such immune suppression could compromise the body's ability to effectively counteract pathogens, potentially exacerbating vulnerability to STIs, including HIV. Interestingly, while non-*Lactobacillus*-dominated microbiota was clearly associated with immune activation, the impact of VIP use on immune responses appeared more nuanced. VIP-related disruptions to the microbiota were not uniformly associated with severe immune consequences, suggesting that other biological factors may influence the interplay between VIP use, microbial composition, and immune activity. These factors could include the specific types of VIPs used, frequency and duration of use, individual genetic and hormonal variations, and co-existing health conditions.

Overall, our findings highlight the complex relationships between intravaginal practices, microbial dynamics, and immune function. While VIP use is associated with shifts in microbial composition and immune responses, the full extent of its impact likely depends on a combination of individual behaviours, environmental influences, and biological factors. Further research is needed to disentangle these interactions and to develop interventions that promote vaginal health while reducing risks associated with intravaginal practices.

G. E%ects of Intravaginal Practices and Sexual Activity on vaginal microbiota, cytokine and immune markers of genital inflammation

The timing of sexual activity in relation to immune responses provides critical insights into the interplay between inflammation and immune regulation within the female reproductive tract. This study identified distinct temporal variations in cytokine levels and immune markers, shaped by the timing of coitus and the composition of vaginal microbiota. At 10 days post-coitus, a significant reduction in IP-10 and GM-CSF levels was observed, indicative of the resolution of the early inflammatory response. This reduction aligns with prior studies (67-69) which associate declines in these cytokines with the re-establishment of immune homeostasis (64, 65). IP-10, a chemokine that facilitates T-cell recruitment, and GM-CSF, a cytokine essential for dendritic cell and macrophage activation, are key players in the inflammatory cascade (64, 68). Their downregulation at the later time point reflects a well-regulated transition from immune activation to a homeostatic state, critical for preserving reproductive health. Additionally, the observed increase in CD4+HLA-DR expression supports the hypothesis of immune modulation over time. HLA-DR expression on CD4+ T cells serves as an indicator of antigen presentation and immune activation (70). Its elevation at the 10-day mark suggests an adaptive regulatory phase of the immune response, potentially balancing inflammation to maintain tissue integrity and prevent excessive immune activation.

Although the vaginal microbiota did not exhibit any clustering based on sexual activity, its role in immune dynamics remains a crucial avenue for further investigation. Differences in immune responses may be influenced by the dominance of lactobacilli compared to dysbiotic microbial communities, which could modulate the inflammatory environment and its resolution. Furthermore, the impact of VIPs on these immune trajectories highlights the need for focused studies to understand their influence on inflammatory resolution. This research underscores the

significance of temporal dynamics in immune responses following sexual activity and highlights the intricate interactions between host immunity, microbial composition, and external modulators. These insights are fundamental for developing targeted interventions to enhance reproductive health and mitigate risks associated with unresolved inflammation.

5.2. STUDY LIMITATIONS

This thesis revealed several limitations that may impact the reliability and applicability of its findings. Firstly, PrEP adherence data relied on pill count and self-reporting, and systemic or genital PrEP levels were measured only in a sub-set of women. Secondly, The CAPRISA 082 trial lacked longitudinal data on STIs and BV, while CAPRISA 084 had missing BV treatment, this hindered a detailed analysis of the effects of PrEP and BV and BV treatment on the vaginal microbiota. Additionally, the study was conducted with a coitally dependent, unscheduled application of tenofovir gel, which may not align with clinic visits when pharmacokinetic (PK) samples were collected. Due to the nature of the trial, we could not assess the longitudinal impact of vaginal microbiota on TFV concentration even though some participants had more than one visit, as the vaginal microbiome can change over time. Participants self-reported the timing and frequency of gel use, which could not be fully reliable, as self-reports may not accurately reflect actual usage patterns. Previous studies have suggested that genital inflammation is associated with an increased risk of HIV (27, 35, 71) and also undermines PrEP efficacy (9), however, we did not include genital cellular and cytokines data, and further studies are required to determine the relationship between TFV levels, microbiome composition alterations, genital cytokines and the frequency of HIV target cells. Chapter 4; While the longitudinal design allows for the examination of temporal changes in vaginal microbiota composition and immune markers, the duration of follow-up may not have been long enough to fully capture the long-term impacts of VIP use on microbiota stability and immune response. Additionally, self-reported data on the use of VIPs and sexual behaviours may be subject to recall bias or underreporting, which could affect the accuracy of these measurements, particularly when addressing sensitive topics such as sexual activity and intravaginal product use. Overall, we did not investigate the impact of other potential co-factors such as STIs, human papillomavirus, herpes simplex virus, hormonal contraceptives, diets, hormonal status, semen exposure, genetic predispositions, and other vaginal disorders (e.g., aerobic vaginitis), these factors may have different biological effects on microbial

communities, ultimately contributing to more refined and comprehensive knowledge in this field.

5.3 STRENGTHS

Pre-Exposure Prophylaxis (PrEP) has proven to be a highly effective HIV prevention strategy, particularly among women with high adherence. However, questions remain about the potential influence of abnormal vaginal microbiota on PrEP efficacy. Previous studies using Nugent scoring to assess bacterial vaginosis (BV) concluded that BV does not compromise the effectiveness of oral PrEP. Building on this, our study focused on understanding how PrEP impacts vaginal microbial communities longitudinally, particularly in South African women who face a disproportionately high burden of BV and HIV. A major advancement of this study is the use of high-throughput 16S rRNA gene sequencing, which provides a more accurate and comprehensive characterisation of the vaginal microbiome compared to traditional methods like Nugent scoring, Gram staining, or qPCR. By capturing bacterial species and genera with greater precision, this approach allowed us to evaluate temporal dynamics of the vaginal microbiota before and after initiating PrEP. Additionally, the inclusion of participants who were not taking PrEP facilitated a comparative analysis, enhancing the robustness of our findings.

Our thesis also explored the impact of vaginal microbiota on the effectiveness of 1% TFV gel. Unlike previous studies that relied on proteomic analyses, which are limited in detecting low-abundance bacterial species, we utilized 16S rRNA gene sequencing to provide a more detailed understanding of microbial composition. Furthermore, by quantifying TFV levels, we were able to stratify participants based on these levels and identify bacterial species and genera associated with TFV concentration. These insights significantly advance our knowledge of how vaginal microbiota may influence microbicide efficacy, forming a foundation for developing localized HIV prevention methods. South Africa, as a region with high rates of BV and HIV among young women, offered a critical context for this research. Sociocultural practices such as VIP are prevalent in this region and are known to promote a diverse, anaerobic bacterial community associated with BV. Leveraging this context, our study longitudinally evaluated the impact of VIP on vaginal microbiota using 16S rRNA sequencing. Additionally, by recruiting participants from two provinces, we accounted for geographic variations in microbiota composition. The inclusion of adolescent girls alongside young and older women further allowed us to investigate age-related differences in vaginal microbiota profiles.

This thesis's longitudinal design stands out as a key strength, enabling us to capture the temporal dynamics of vaginal microbiota and their interplay with immune markers. By integrating microbial and immune analyses, we gained valuable insights into the complex interactions between vaginal microbiota and immune responses, contributing to a more comprehensive understanding of reproductive health. Importantly, our findings demonstrate that oral PrEP remains effective across diverse vaginal microbiota profiles, reinforcing its potential as a robust HIV prevention strategy for high-risk populations. These results not only confirm the efficacy of PrEP but also provide critical data for refining HIV prevention strategies tailored to the unique microbial and sociocultural contexts of vulnerable populations.

5.4. ENHANCING VAGINAL HEALTH AND HIV PREVENTION: RECOMMENDATIONS FOR FUTURE RESEARCH ON MICROBIOME DYNAMICS AND PREP EFFICACY

To address these gaps and advance our understanding, several recommendations for future research are proposed. First, longitudinal studies are needed to clarify the role of *Lactobacillus iners* in vaginal ecology, particularly its association with BV and susceptibility to HIV. These studies should explore whether *L. iners* actively promotes dysbiosis or serves as an indicator of transitional microbial states. Experimental models and metagenomic analyses could provide valuable insights into its metabolic pathways and ecological interactions. Second, future research should prioritise integrating microbiome studies with immunological assessments to better understand how vaginal microbiota influence the mucosal immune environment and the efficacy of tenofovir-based and other novel PrEP strategies. Advanced molecular techniques, such as transcriptomics and proteomics, can illuminate functional relationships between microbial communities, host immune responses, and drug bioavailability. Third, studies should account for individual-level factors, such as hormonal contraceptive use, diet, genetic predispositions, co-infections with STIs, sexual activity and VIP use. These variables may play a critical role in shaping the vaginal microbiome and influencing PrEP outcomes. Stratified analyses and multi-variable models could help disentangle these complex interactions. Additionally, more objective measures of adherence to PrEP and other intravaginal products are necessary to overcome the limitations of self-reported data. Wearable technologies, drug-level monitoring, and digital adherence tools may provide more accurate insights into user

behaviours and product effectiveness. Finally, intervention-based studies should assess whether tailored approaches, such as probiotics, prebiotics, or microbiome-targeted therapies, can stabilise vaginal microbiota and improve PrEP efficacy. These studies should prioritise women in high HIV-prevalence regions, with an emphasis on community engagement and culturally sensitive approaches to ensure relevance and acceptance. By addressing these research priorities, future studies can provide a more comprehensive understanding of vaginal microbiome dynamics, refine PrEP interventions, and improve reproductive health outcomes for women worldwide.

5.5. CONCLUSIONS

Our findings indicate that the initiation of PrEP does not result in significant changes to vaginal microbial communities in HIV-negative women from sub-Saharan Africa, suggesting that PrEP use does not disrupt vaginal microbiota. Additionally, vaginal microbiota composition did not significantly affect tenofovir levels, though adherence to PrEP and timing of application remain critical for effective HIV prevention. Minor variations in tenofovir levels were observed across groups defined by BV status, Lactobacillus dominance, and STI presence; however, these differences were not statistically significant. These results support the feasibility of PrEP implementation in women with both optimal and suboptimal vaginal microbiota. Nevertheless, routine STI and BV screening during PrEP care is recommended, particularly in high HIV-prevalence regions. The thesis also revealed that while the vaginal microbiota of adolescent girls resembles that of adult women, younger individuals exhibit greater microbial flux and instability. Furthermore, non-Lactobacillus-dominated microbiota was associated with modulation of immune responses, an effect not observed with the use of vaginal insertion products (VIPs), suggesting other biological factors may drive immune modulation. Timing of sexual activity was found to influence immune responses, with notable changes occurring 10 days post-coitus, whereas VIP use appeared to sustain immune activation.

These findings emphasize the intricate relationships between vaginal microbiota composition, sexual behaviour, and VIP use in shaping genital health and immune responses. They highlight the need for further research involving larger cohorts, extended follow-up periods, and comprehensive clinical data. This research contributes to our understanding of how vaginal microbiota, PrEP use, and vaginal insertion products impact genital inflammatory profiles,

microbiome dynamics, and HIV susceptibility, providing a foundation for optimizing HIV prevention strategies. Our findings indicate that the initiation of PrEP does not result in significant changes to vaginal microbial communities in HIV-negative women from sub-Saharan Africa, suggesting that PrEP use does not disrupt vaginal microbiota. Additionally, vaginal microbiota composition did not significantly affect tenofovir levels, though adherence to PrEP and timing of application remain critical for effective HIV prevention. Minor variations in tenofovir levels were observed across groups defined by BV status, Lactobacillus dominance, and STI presence; however, these differences were not statistically significant. These results support the feasibility of PrEP implementation in women with both optimal and suboptimal vaginal microbiota. Nevertheless, routine STI and BV screening during PrEP care is recommended, particularly in high HIV-prevalence regions. The study also revealed that while the vaginal microbiota of adolescent girls resembles that of adult women, younger individuals exhibit greater microbial flux and instability. Furthermore, non-Lactobacillus-dominated microbiota was associated with modulation of immune responses, an effect not observed with the use of vaginal insertion products (VIPs), suggesting other biological factors may drive immune modulation. Timing of sexual activity was found to influence immune responses, with notable changes occurring 10 days post-coitus, whereas VIP use appeared to sustain immune activation.

These findings highlight the intricate relationships between vaginal microbiota composition, sexual behaviour, and VIP use in modulating genital health and immune responses. They underscore the need for additional studies with larger sample sizes, extended follow-up durations, and more comprehensive clinical data to further elucidate these interactions. This research contributes to a deeper understanding of how vaginal microbiota, PrEP use, and VIP use influence genital inflammatory profiles, microbiome dynamics, and susceptibility to HIV, providing a basis for optimising HIV prevention strategies and therapeutic interventions.

Reference list

1. Anahtar MN, Gootenberg DB, Mitchell CM, Kwon DS. 2018. Cervicovaginal Microbiota and Reproductive Health: The Virtue of Simplicity. *Cell Host Microbe* 23: 159-68
2. Cohen CR, Lingappa JR, Baeten JM, Ngayo MO, Spiegel CA, Hong T, Donnell D, Celum C, Kapiga S, Delany S. 2012. Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: a prospective cohort analysis among African couples. *PLoS medicine* 9: e1001251
3. Petrova MI, van den Broek M, Balzarini J, Vanderleyden J, Lebeer S. 2013. Vaginal microbiota and its role in HIV transmission and infection. *FEMS microbiology reviews* 37: 762-92
4. Nagot N, Ouedraogo A, Defer M-C, Vallo R, Mayaud P, Van de Perre P. 2007. Association between bacterial vaginosis and Herpes simplex virus type-2 infection: implications for HIV acquisition studies. *Sexually transmitted infections* 83: 365-8
5. Gosmann C, Anahtar MN, Handley SA, Farcasanu M, Abu-Ali G, Bowman BA, Padavattan N, Desai C, Droit L, Moodley A, Dong M, Chen Y, Ismail N, Ndung'u T, Ghebremichael MS, Wesemann DR, Mitchell C, Dong KL, Huttenhower C, Walker BD, Virgin HW, Kwon DS. 2017. Lactobacillus-Deficient Cervicovaginal Bacterial Communities Are Associated with Increased HIV Acquisition in Young South African Women. *Immunity* 46: 29-37
6. Borgdorff H, Tsivtsivadze E, Verhelst R, Marzorati M, Jurriaans S, Ndayisaba GF, Schuren FH, Van De Wijgert JH. 2014. Lactobacillus-dominated cervicovaginal microbiota associated with reduced HIV/STI prevalence and genital HIV viral load in African women. *The ISME journal* 8: 1781-93
7. Lennard K, Dabee S, Barnabas SL, Havyarimana E, Blakney A, Jaumdally SZ, Botha G, Mkhize NN, Bekker L-G, Lewis DA. 2018. Microbial composition predicts genital tract inflammation and persistent bacterial vaginosis in South African adolescent females. *Infection and immunity* 86: 10.1128/iai. 00410-17
8. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noël-Romas L, Grobler A, Westmacott G, Xie IY, Butler J. 2017. Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356: 938-45
9. McKinnon LR, Liebenberg LJ, Yende-Zuma N, Archary D, Ngcapu S, Sivro A, Nagelkerke N, Garcia Lerma JG, Kashuba AD, Masson L. 2018. Genital inflammation undermines the effectiveness of tenofovir gel in preventing HIV acquisition in women. *Nature medicine* 24: 491-6
10. Arnold KB, Burgener A, Birse K, Romas L, Dunphy LJ, Shahabi K, Abou M, Westmacott GR, McCorrister S, Kwatampora J. 2016. Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells. *Mucosal immunology* 9: 194
11. Cheu RK, Gustin AT, Lee C, Schifanella L, Miller CJ, Ha A, Kim C, Rodriguez VJ, Fischl M, Burgener AD. 2020. Impact of vaginal microbiome communities on HIV

antiretroviral-based pre-exposure prophylaxis (PrEP) drug metabolism. *PLoS pathogens* 16: e1009024

12. Lokken EM, Manguro GO, Abdallah A, Ngacha C, Shafi J, Kiarie J, Jaoko W, Srinivasan S, Fiedler TL, Munch MM, Fredricks DN, McClelland RS, Balkus JE. 2019. Association between vaginal washing and detection of Lactobacillus by culture and quantitative PCR in HIV-seronegative Kenyan women: a cross-sectional analysis. *Sex Transm Infect* 95: 455-61
13. Ness RB, Hillier SL, Richter HE, Soper DE, Stamm C, McGregor J, Bass DC, Sweet RL, Rice P. 2002. Douching in relation to bacterial vaginosis, lactobacilli, and facultative bacteria in the vagina. *Obstet Gynecol* 100: 765
14. Myer L, Denny L, Telerant R, de Souza M, Wright Jr TC, Kuhn L. 2005. Bacterial vaginosis and susceptibility to HIV infection in South African women: a nested case-control study. *The Journal of infectious diseases* 192: 1372-80
15. Myer L, Denny L, De Souza M, Wright Jr TC, Kuhn L. 2006. Distinguishing the temporal association between women's intravaginal practices and risk of human immunodeficiency virus infection: a prospective study of South African women. *American Journal of Epidemiology* 163: 552-60
16. Morar NS, Ramjee G, Abdool Karim SS. 1998. Vaginal insertion and douching practices among sex workers at truck stops in KwaZulu-Natal. *S Afr Med J* 88: 470
17. Scorgie F, Kunene B, Smit JA, Manzini N, Chersich MF, Preston-Whyte EM. 2009. In search of sexual pleasure and fidelity: vaginal practices in KwaZulu-Natal, South Africa. *Cult Health Sex* 11: 267-83
18. Taneva E, Sinclair S, Mesquita PM, Weinrick B, Cameron SA, Cheshenko N, Reagle K, Frank B, Srinivasan S, Fredricks D. 2018. Vaginal microbiome modulates topical antiretroviral drug pharmacokinetics. *JCI insight* 3
19. Heffron R, McClelland RS, Balkus JE, Celum C, Cohen CR, Mugo N, Bukusi E, Donnell D, Lingappa J, Kiarie J, Fiedler T, Munch M, Fredricks DN, Baeten JM, Partners Pr EPST. 2017. Efficacy of oral pre-exposure prophylaxis (PrEP) for HIV among women with abnormal vaginal microbiota: a post-hoc analysis of the randomised, placebo-controlled Partners PrEP Study. *Lancet HIV*
20. Munoz A, Hayward MR, Bloom SM, Rocafort M, Ngcapu S, Mafunda NA, Xu J, Xulu N, Dong M, Dong KL, Ismail N, Ndung'u T, Ghebremichael MS, Kwon DS. 2021. Modeling the temporal dynamics of cervicovaginal microbiota identifies targets that may promote reproductive health. *Microbiome* 9: 163
21. Mtshali A, San JE, Osman F, Garrett N, Balle C, Giandhari J, Onywera H, Mngomezulu K, Mzobe G, de Oliveira T. 2021. Temporal changes in vaginal microbiota and genital tract cytokines among South African women treated for bacterial vaginosis. *Frontiers in immunology* 12: 730986
22. Ahrens P, Andersen LO, Lilje B, Johannesen TB, Dahl EG, Baig S, Jensen JS, Falk L. 2020. Changes in the vaginal microbiota following antibiotic treatment for Mycoplasma genitalium, Chlamydia trachomatis and bacterial vaginosis. *PLoS One* 15: e0236036
23. Joag V, Obila O, Gajer P, Scott MC, Dizzell S, Humphrys M, Shahabi K, Huibner S, Shannon B, Tharao W, Mureithi M, Oyugi J, Kimani J, Kaushic C, Ravel J, Anzala O,

Kaul R. 2019. Impact of Standard Bacterial Vaginosis Treatment on the Genital Microbiota, Immune Milieu, and Ex Vivo Human Immunodeficiency Virus Susceptibility. *Clin Infect Dis* 68: 1675-83

24. Biswas M, Nurunnabi M, Khatun Z. 2024. Understanding Mucosal Physiology and Rationale of Formulation Design for Improved Mucosal Immunity. *ACS Applied Bio Materials*
25. Dong M, Dong Y, Bai J, Li H, Ma X, Li B, Wang C, Li H, Qi W, Wang Y. 2023. Interactions between microbiota and cervical epithelial, immune, and mucus barrier. *Frontiers in cellular and infection microbiology* 13: 1124591
26. Armstrong E, Kaul R, Cohen CR. 2023. Optimizing the vaginal microbiome as a potential strategy to reduce heterosexual HIV transmission. *Journal of Internal Medicine* 293: 433-44
27. Anahtar MN, Byrne EH, Doherty KE, Bowman BA, Yamamoto HS, Soumillon M, Padavattan N, Ismail N, Moodley A, Sabatini ME, Ghebremichael MS, Nusbaum C, Huttenhower C, Virgin HW, Ndung'u T, Dong KL, Walker BD, Fichorova RN, Kwon DS. 2015. Cervicovaginal bacteria are a major modulator of host inflammatory responses in the female genital tract. *Immunity* 42: 965-76
28. Mtshali A, San JE, Osman F, Garrett N, Balle C, Giandhari J, Onywera H, Mngomezulu K, Mzobe G, de Oliveira T, Rompalo A, Mindel A, Abdool Karim SS, Ravel J, Passmore J-AS, Abdool Karim Q, Jaspan HB, Liebenberg LJP, Ngcapu S. 2021. Temporal Changes in Vaginal Microbiota and Genital Tract Cytokines Among South African Women Treated for Bacterial Vaginosis. *Frontiers in Immunology* 12
29. Mazibuko-Motau N, Sobia P, Xu J, Elsherbini JA, San JE, Lewis L, Mtshali A, Mzobe G, Ntuli L, Abdool Karim SS. 2022. Vaginal microbial shifts are unaffected by oral pre-exposure prophylaxis in South African women. *Scientific Reports* 12: 16187
30. Hillier SL, Meyn LA, Bunge A, Austin M, Moncla BJ, Dezzutti CS, Schwartz J, Marzinke MA, Hendrix C, Rohan LC. 2017. *IMPACT OF VAGINAL MICROBIOTA ON GENITAL TISSUE AND PLASMA CONCENTRATIONS OF TENOFOVIR*. Presented at Conference on Retroviruses and Opportunistic Infections, Seattle, WA
31. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noel-Romas L, Grobler A, Westmacott G, Xie IY, Butler J, Mansoor L, McKinnon LR, Passmore JS, Abdool Karim Q, Abdool Karim SS, Burgener AD. 2017. Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women. *Science* 356: 938-45
32. Thurman AR, Schwartz JL, Ravel J, Gajer P, Marzinke MA, Yousefieh N, Anderson SM, Doncel GF. 2019. Vaginal microbiota and mucosal pharmacokinetics of tenofovir in healthy women using tenofovir and tenofovir/levonorgestrel vaginal rings. *PLoS One* 14: e0217229
33. Thurman AR, Ravel J, Gajer P, Marzinke MA, Ouattara LA, Jacot T, Peet MM, Clark MR, Doncel GF. 2022. Vaginal microbiota and mucosal pharmacokinetics of tenofovir in healthy women using a 90-day tenofovir/levonorgestrel vaginal ring. *Frontiers in Cellular and Infection Microbiology* 12: 799501
34. Dabee S, Mugo N, Mudhune V, McLellan-Lemal E, Peacock S, O'Connor S, Njoroge B, Nyagol B, Thurman AR, Ouma E. 2022. Genital microbiota of women using a 90

day tenofovir or tenofovir and levonorgestrel intravaginal ring in a placebo controlled randomized safety trial in Kenya. *Scientific Reports* 12: 12040

35. Masson L, Passmore J-AS, Liebenberg LJ, Werner L, Baxter C, Arnold KB, Williamson C, Little F, Mansoor LE, Naranbhai V. 2015. Genital inflammation and the risk of HIV acquisition in women. *Clinical Infectious Diseases* 61: 260-9
36. Selhorst P, Masson L, Ismail SD, Samsunder N, Garrett N, Mansoor LE, Abdool Karim Q, Abdool Karim SS, Passmore J-AS, Williamson C. 2017. Cervicovaginal inflammation facilitates acquisition of less infectious HIV variants. *Clinical Infectious Diseases* 64: 79-82
37. Low N, Chersich MF, Schmidlin K, Egger M, Francis SC, HHM van de Wijgert J, Hayes RJ, Baeten JM, Brown J, Delany-Moretlwe S. 2011. Intravaginal practices, bacterial vaginosis, and HIV infection in women: individual participant data meta-analysis. *PLoS medicine* 8: e1000416
38. Simbayi LC, Chauveau J, Shisana O. 2004. Behavioural responses of South African youth to the HIV/AIDS epidemic: a nationwide survey. *AIDS care* 16: 605-18
39. Balle C, Lennard K, Dabee S, Barnabas SL, Jaumdally SZ, Gasper MA, Maseko V, Mbulawa ZZA, Williamson AL, Bekker LG, Lewis DA, Passmore JS, Jaspan HB. 2018. Endocervical and vaginal microbiota in South African adolescents with asymptomatic Chlamydia trachomatis infection. *Sci Rep* 8: 11109
40. Barnabas SL, Dabee S, Passmore J-AS, Jaspan HB, Lewis DA, Jaumdally SZ, Gamieldien H, Masson L, Muller E, Maseko VD. 2018. Converging epidemics of sexually transmitted infections and bacterial vaginosis in southern African female adolescents at risk of HIV. *International journal of STD & AIDS* 29: 531-9
41. Jarolimova J, Chidumwa G, Chimbindi N, Okesola N, Dreyer J, Smit T, Seeley J, Harling G, Copas A, Baisley K, Shahmanesh M, Group tIR, Herbst, McGrath N, Zuma T, Khoza T, Behuhuma N, Bassett IV, Sherr L. 2023. Prevalence of Curable Sexually Transmitted Infections in a Population-Representative Sample of Young Adults in a High HIV Incidence Area in South Africa. *Sexually Transmitted Diseases* 50: 796-803
42. Control CfD, Prevention. 2008. Sexually transmitted disease surveillance. *http://www. cdc. gov/std/stats08/syphilis. htm*
43. Burchell AN, Winer RL, de Sanjosé S, Franco EL. 2006. Chapter 6: Epidemiology and transmission dynamics of genital HPV infection. *Vaccine* 24 Suppl 3: S3/52-61
44. Shannon CL, Klausner JD. 2018. The growing epidemic of sexually transmitted infections in adolescents: a neglected population. *Curr Opin Pediatr* 30: 137-43
45. Lara LAS, Abdo CHN. 2016. Age at Time of Initial Sexual Intercourse and Health of Adolescent Girls. *J Pediatr Adolesc Gynecol* 29: 417-23
46. Monteiro IP, Azzi CFG, Bilibio JP, Monteiro PS, Braga GC, Nitz N. 2023. Prevalence of sexually transmissible infections in adolescents treated in a family planning outpatient clinic for adolescents in the western Amazon. *PLoS One* 18: e0287633
47. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. 2011. Vaginal microbiome of reproductive-age women. *Proc Natl Acad Sci U S A* 108 Suppl 1: 4680-7

48. Alcaide ML, Rodriguez VJ, Brown MR, Pallikkuth S, Arheart K, Martinez O, Roach M, Fichorova RN, Jones DL, Pahwa S. 2017. High levels of inflammatory cytokines in the reproductive tract of women with BV and engaging in intravaginal douching: a cross-sectional study of participants in the women interagency HIV study. *AIDS research and human retroviruses* 33: 309-17
49. van De Wijgert JH, Mason PR, Gwanzura L, Mbizvo MT, Chirenje ZM, Iliff V, Shiboski S, Padian NS. 2000. Intravaginal practices, vaginal flora disturbances, and acquisition of sexually transmitted diseases in Zimbabwean women. *J Infect Dis* 181: 587-94
50. Sandala L, Lurie P, Sunkutu MR, Chani EM, Hudes ES, Hearst N. 1995. 'Dry sex' and HIV infection among women attending a sexually transmitted diseases clinic in Lusaka, Zambia. *Aids* 9 Suppl 1: S61-8
51. Myer L, Kuhn L, Stein ZA, Wright TC, Jr., Denny L. 2005. Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. *The Lancet Infectious Diseases* 5: 786-94
52. Beksinska ME, Rees HV, Kleinschmidt I, McIntyre J. 1999. The practice and prevalence of dry sex among men and women in South Africa: a risk factor for sexually transmitted infections? *Sex Transm Infect* 75: 178-80
53. Dallabetta GA, Miotti PG, Chiphangwi JD, Liomba G, Canner JK, Saah AJ. 1995. Traditional vaginal agents: use and association with HIV infection in Malawian women. *Aids* 9: 293-7
54. Runganga A, Pitts M, McMaster J. 1992. The use of herbal and other agents to enhance sexual experience. *Soc Sci Med* 35: 1037-42
55. Shipitsyna E, Roos A, Datcu R, Hallen A, Fredlund H, Jensen JS, Engstrand L, Unemo M. 2013. Composition of the vaginal microbiota in women of reproductive age--sensitive and specific molecular diagnosis of bacterial vaginosis is possible? *PLoS One* 8: e60670
56. Hawes SE, Hillier SL, Benedetti J, Stevens CE, Koutsky LA, Wølner-Hanssen P, Holmes KK. 1996. Hydrogen Peroxide—Producing Lactobacilli and Acquisition of Vaginal Infections. *The Journal of Infectious Diseases* 174: 1058-63
57. van de Wijgert JHHM. 2017. The vaginal microbiome and sexually transmitted infections are interlinked: Consequences for treatment and prevention. *PLOS Medicine* 14: e1002478
58. Hilber AM, Chersich MF, van de Wijgert JHHM, Rees H, Temmerman M. 2007. Vaginal practices, microbicides and HIV: what do we need to know? *Sexually Transmitted Infections* 83: 505-8
59. Myer L, Kuhn L, Stein ZA, Wright TC, Jr., Denny L. 2005. Intravaginal practices, bacterial vaginosis, and women's susceptibility to HIV infection: epidemiological evidence and biological mechanisms. *Lancet Infect Dis* 5: 786-94
60. McClelland RS, Lavreys L, Hassan WM, Mandaliya K, Ndinya-Achola JO, Baeten JM. 2006. Vaginal washing and increased risk of HIV-1 acquisition among African women: a 10-year prospective study. *Aids* 20: 269-73

61. Voronov E, Dotan S, Krelin Y, Song X, Elkabets M, Carmi Y, Rider P, Idan C, Romzova M, Kaplanov I, Apte RN. 2013. Unique Versus Redundant Functions of IL-1α and IL-1β in the Tumor Microenvironment. *Front Immunol* 4: 177
62. Ribatti D, Crivellato E. 2009. Immune cells and angiogenesis. *J Cell Mol Med* 13: 2822-33
63. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. 2008. Growth factors and cytokines in wound healing. *Wound Repair Regen* 16: 585-601
64. Hoermannsperger G, Clavel T, Hoffmann M, Reiff C, Kelly D, Loh G, Blaut M, Hölzlwimmer G, Laschinger M, Haller D. 2009. Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation. *PLoS One* 4: e4365
65. Jauregui CE, Wang Q, Wright CJ, Takeuchi H, Uriarte SM, Lamont RJ. 2013. Suppression of T-cell chemokines by Porphyromonas gingivalis. *Infect Immun* 81: 2288-95
66. Gomez-Cambronero J, Horn J, Paul CC, Baumann MA. 2003. Granulocyte-macrophage colony-stimulating factor is a chemoattractant cytokine for human neutrophils: involvement of the ribosomal p70 S6 kinase signaling pathway. *J Immunol* 171: 6846-55
67. Oppenheim JJ, Tewary P, de la Rosa G, Yang D. 2007. Alarmins initiate host defense. *Adv Exp Med Biol* 601: 185-94
68. Atanasova M, Whitty A. 2012. Understanding cytokine and growth factor receptor activation mechanisms. *Crit Rev Biochem Mol Biol* 47: 502-30
69. Broughton SE, Hercus TR, Lopez AF, Parker MW. 2012. Cytokine receptor activation at the cell surface. *Curr Opin Struct Biol* 22: 350-9
70. Revenfeld ALS, Steffensen R, Pugholm LH, Jørgensen MM, Stensballe A, Varming K. 2016. Presence of HLA-Dr Molecules and HLA-DRB 1 Mrna in Circulating CD 4+ T Cells. *Scandinavian journal of immunology* 84: 211-21
71. Passmore J-AS, Jaspan HB, Masson L. 2016. Genital inflammation, immune activation and risk of sexual HIV acquisition. *Current opinion in HIV and AIDS* 11: 156-62

APPENDICES

Appendix A: Detailed methodologies

6.1. Processing of genital specimens

A. *Processing of genital SoftCup specimens*

Cervicovaginal fluid for genital tract tenofovir detection was collected using SoftCup menstrual cups (SoftCup, San Diego, CA). SoftCup specimens were collected in 50 ml conical tubes and processed upon arrival at the laboratory. The tubes were first centrifuged at 2000 rpm for 10 minutes at 22°C to separate the CVL content, after which the SoftCup was discarded with forceps. A second centrifugation was performed under the same conditions. If supernatant separation occurred, 50 µl of the supernatant was aspirated into a cryovial, and 200 µl of PBS was added. The sample was then vortexed and divided into four cryovials, with the pellet transferred into a fifth cryovial. Specimens were stored at -80°C.

B. *Processing of vaginal swab specimens*

Dry vaginal swabs from -80°C were thawed at room temperature for 10 minutes and resuspended into the 1ml PBS and the e swabs were thawed on ice prior to Total nucleic Acid (TNA) extraction.

6.2. Genital Microbiome Characterisation by 16S rRNA sequencing

A. Total nucleic acid (TNA) extraction

We used both dry and wet swabs. The e-swab was thawed on ice and the dry vaginal swab was thawed, resuspended into 1 ml PBS, and allowed to sit on ice for 30 minutes. The samples (e-swab and dry swab) were vortexed for 15 seconds at high speed and 500 µl was transferred into a sarstedt 2 ml DNase-free tube containing beads. 500 µl buffer A, 210 µl of 20% sodium dodecyl sulfate (SDS) and 500 µl of phenol-chloroform*: isoamyl alcohol (IAA- pH 7.9, Ambion)* were added, and samples were homogenised using a bead beater for 2 min, and

centrifuged at 8000 rpm for 3 minutes at 4 °C. After centrifugation, the top layer was transferred into a new DNase free tube containing 600 µl phenol-chloroform, mixed briefly by vertexing and span down at 13,000 rpm (16,000 rcf) for 5 minutes at 4 °C. The top layer was transferred into a new 2 ml tube and 600 µl of isopropanol and 60 µl of 3MSodium Acetate (NaOAc, pH 5.5) were added. The mixture was briefly mixed and incubated at -20 °C for overnight precipitation (12-24 hrs). On the second day of the experiment, tubes were centrifuged at the maximum speed (16,000 rpm) for 30 minutes, and once the spin was complete, the supernatant was discarded, leaving the pellet in the tube. The pellet was washed with 500 µl of 100% ethanol (EtOH) and spun down at 16,000 rpm for 15 minutes, followed by discarding the ethanol and allowing the pellet to dry for 15 minutes. The pellet was resuspended by 20 µl of TE buffer and stored at -80 °C until used.

Buffer A was prepared as follows:

	Initial Concentration	**Final Concentration**	**100 mL**	**500 mL**
NaCl	5 M	200 mM	4 mL	20 mL
Tris-HCl	1 M	200 mM	20 mL	100 mL
EDTA	0.5 M	20 mM	4 mL	20 mL
UltraPure H2O			72 mL	360 mL

B. DNA quantification: Qubit

DNA quantification was performed using the Qubit 2.0 Fluorometer (Invitrogen, USA). To prepare the Qubit working solution, 1 µl of Qubit reagent was added to 199 µl of Qubit buffer. Standards 1 and 2 were prepared by adding 10 µl of each standard to 190 µl of the working solution. For each sample, 2 µl of DNA was added to 198 µl of the Qubit working solution. Both samples and standards were briefly vortexed and incubated for 2 minutes at room temperature. The standards and double-stranded DNA (dsDNA) high-sensitivity concentrations (ng/ml) were then measured using the fluorometer.

C. 16S rRNA gene V4 region Amplification

DNA samples were removed from the freezer and allowed to thaw at room temperature. 2 µl of DNA was plated in a 96-well plate. The mastermix was prepared for the appropriate number

of samples by adding 16.25 µl PCR clean water, 5ul 5X Q5 reaction buffer, 0.5 µl dNTP (10mM), 0.5µl 515Forward prime (10 µM), 0.25 µl Q5 polymerase. 22.5 µl of the Master mix was added into each reaction followed by 0.50 µl of 806Reverse primer. PCR was performed in a thermal cycler using the following program: 98°C for 30 seconds, 30 cycles of (98°C for 10 seconds, 60°C for 30 seconds, 72°C for 20 seconds), 72°C for 2 minutes and holding at 4°C. The amplicons were stored at 4°C.

Master Mix preparation:

Master Mix	**Per well (uL)**
PCR-Clean Water	16.25
5X Q5 Reaction buffer	5.00
10mM dNTPs (each at 10mM)	0.50
10uM forward primer	0.50
Q5 polymerase	0.25

D. Gel electrophoresis and Library pooling and preparation for sequencing

To confirm PCR amplification, we prepared 1.5 % agarose gel by adding 2 tablets into 50 ml of 1X TAE buffer, then microwaved the mixture to allow the tablet to dissolve. Once the was loaded the gel, we added the mixture of (1.5 µl of loading [6X 1ml Loading dye with 10 µl of gel red] and 5 µl of each sample) and (1.5 µl loading dye and 2µl DNA Ladder). The gel ran at 120V for 40 minutes, followed by visualisation under UV to confirm band size at 300-350bp. Samples amplified successfully were pooled into libraries using 7 µl for a faint band, 5 µl for a medium band and 3µl for a bright band. The libraries were cleaned up using the UltraClean PCR Clean-Up kit per the manufacturers protocol. Qubit™ dsDNA HS Assay Kit was used to determine the concentration of each library. Purified libraries consisting of 300 pooled samples were sequenced from both ends with the Illumina MiSeq platform

E. 16S data analysis

Divisive Amplicon Denoising Algorithm (DADA) 2 was used to filter for quality, trim, and identify inference of amplicon sequence variant (ASV). ASVs were taxonomically classified to genus or higher levels using a Naïve Bayes classification approach and SILVA ribosomal RNA database. The ASVs for *Lactobacillus, Prevotella, Sneathia* and *Mobiluncus* were further refined to the species level with speciateIT (version 1.0, http://ravel-lab.org/speciateIT). A

phyloseq object containing a phylogenetic tree, ASV table, taxonomic table, and sample metadata was created using the phyloseq R package. α-diversity including Richness (Chao1), evenness (Simpson's E) and phylogenetic diversity (Faith's PD) estimates were calculated using the *R vegan library*. β-diversity between samples was estimated by Bray–Curtis distances. Non-metric Multidimensional scaling (NMDS) was performed to assess differences in taxonomy profiles among samples based on the Bray–Curtis distance of ASV relative abundance using the *metaMDS function* in the *vegan R package*.

6.3. Detection of Tenofovir concentrations in CVL samples by Ion-pair (IP) hydrophilic interaction chromatography (HILIC) coupled to mass spectrometry

Tenofovir and deuterated labelled tenofovir (d6-TFV) was purchased from Clearsynth laboratories (Mumbai, India). Ion-pairing reagents; diethylamine (DEA) and hexafluoro-2-propanol (HFIP) were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, US). Acetonitrile, methanol, and formic acid was LC-MS grade purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained using the Millipore Direct-Q water purification system (Billerica, MA, USA).

A. Instrumentation

Sample analysis was performed using an Agilent high pressure liquid chromatography (HPLC) system coupled to an AB Sciex 5500, triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) TurboIonSpray source. Analyst software, version 1.6.2 was used for data acquisition and quantitative data analysis.

B. Sample preparation

Methanol was used as protein precipitation solvent to extract analytes from clinical samples. The CSs, QCs and CVL clinical samples were thawed, and vortex mixed prior to processing. A volume of 50 µl of CVL/CS/QC sample was mixed with 50 µl of ISTD solution and 200 µl of 70% MeOH was added to precipitate the proteins, the sample was then vortex mixed, sonicated (to facilitate analyte extraction) and centrifuged at 16000 rpm at 4°C for 10 minutes. A volume of 300 µl of the clear supernatant was transferred to a microcentrifuge tube and dried down in a SpeedVac at 35°C, overnight. The dried samples were reconstituted in 50 µl of 70% acetonitrile in 0.1% formic acid water, vortex mixed, sonicated and centrifuged at 16000 rpm at 4°C for 10 minutes then the entire volume was transferred to an amber vial and placed in auto-sampler rack and 2 µl of each sample was injected for subsequent LC-MS/MS analysis.

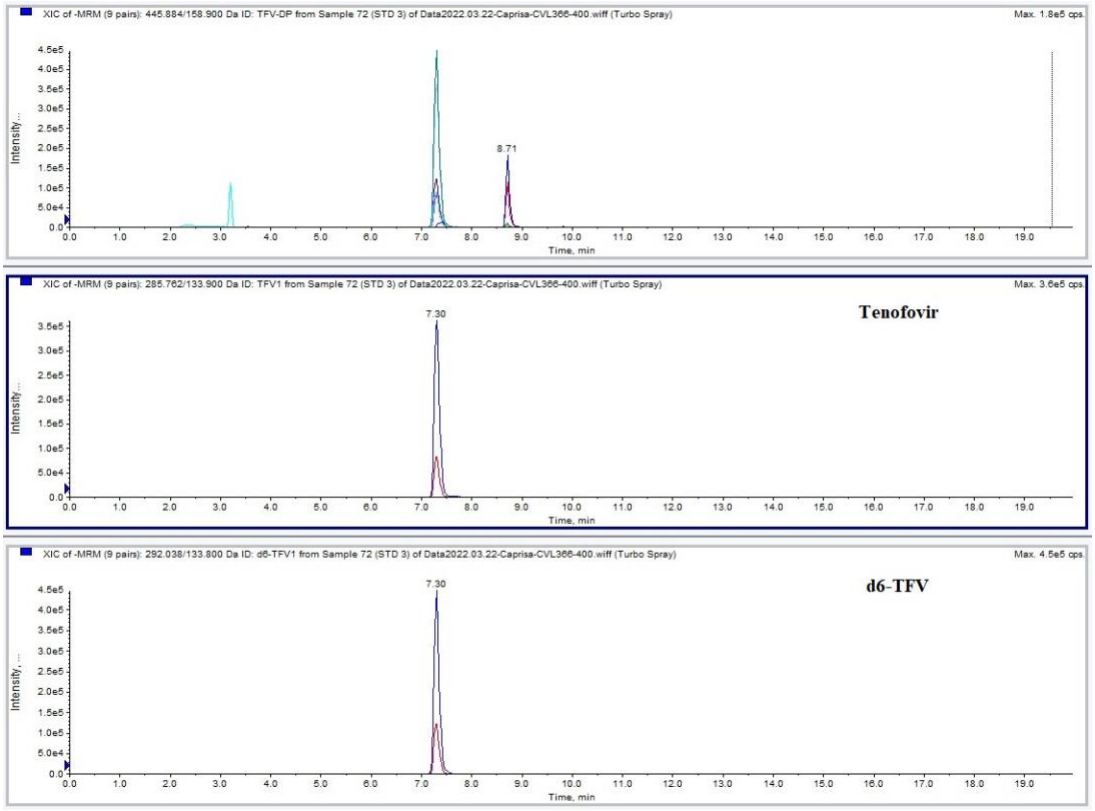
Each sample analysis batch included a 'blank' sample which was a processed drug-free or control CVL sample (no TFV) but included ISTD, and a 'double blank' sample, which was a processed drug-free or control CVL sample without any spiked TFV or ISTD. A processed blank CVL sample, spiked with TFV at a concentration of 500 ng/ml was used as a system performance verification sample (SPVS) for monitoring instrument stability throughout sample analytical runs.

C. Method validation

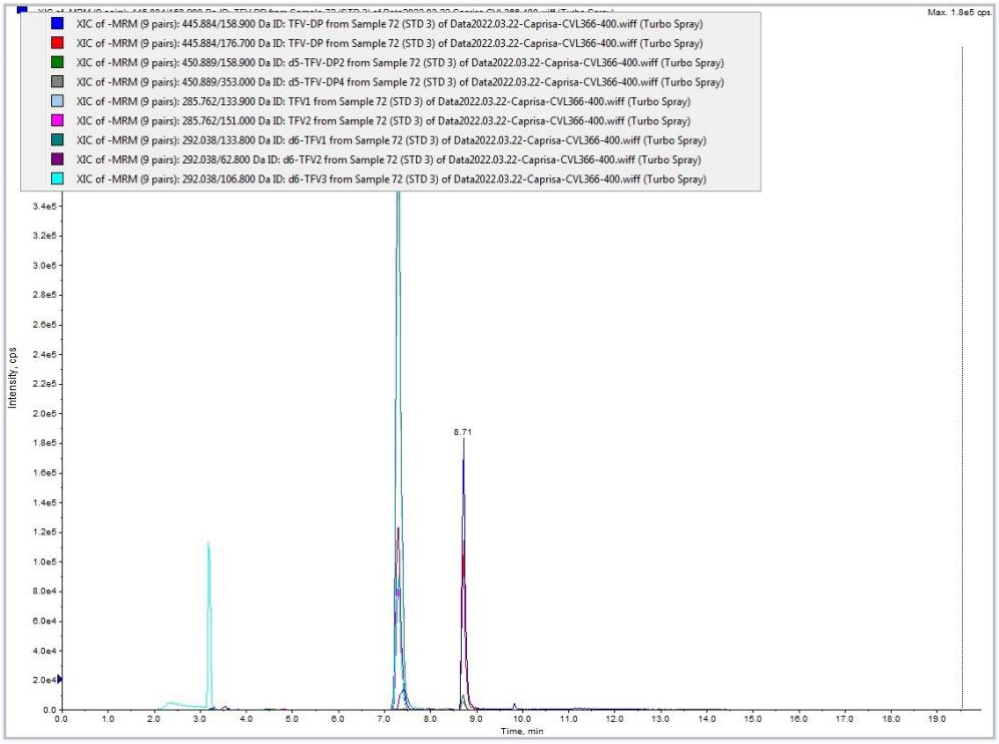
The method was validated for extraction efficiency, linearity, accuracy, precision and matrix effects using the USFDA guidelines for bioanalytical methods. Standard calibration curves were constructed using peak area ratio of the analyte to that of their corresponding ISTD peak area with a weighted (1/x) least-squares quadratic regression analysis. Precision (expressed as percent relative standard deviation, % RSD) and accuracy (expressed as percent error) were calculated for replicate CS and QC samples (in each batch), and a set limit of 15 % deviation from the nominal concentration was deemed acceptable for a successful analytical run.

D. Liquid chromatographic and mass spectrometric conditions

For the IP–HILIC–MS/MS method, chromatographic separations were performed at a flow rate of 0.2 mL/min on a Luna Amino (NH2) column (Phenomenex, Torrance, CA) 100 mm × 2.0 mm, packed with 3.0 µm particles. Mobile phase A consisted of 100 mM HFIP and 0.5% DEA (v/v) in water, and mobile phase B consisted of 0.1 M HFIP and 0.5% DEA (v/v) in acetonitrile. A sample volume of 5.0 µl was injected onto the HPLC column and the analytes were separated using a gradient elution. The column eluent was delivered into an AB Sciex 5500 mass spectrometer electrospray ionization spray (ESI) source. The autosampler syringe and the injection valve were washed with a water:acetonitrile (30:70, v/v) solution, post sample injection, to reduce carryover. The system was operated in negative-ion multiple reaction monitoring (MRM) mode set to detect parent $[M+H]^+ \rightarrow$ product ion transitions for TFV1 (*mz* 285.8 → *mz* 133.9), TFV2 (*mz* 285.8→ *mz* 151.0) and the internal standard; d6-TFV (*mz* 292.0→ *mz* 133.8). Optimized ESI source dependent parameters were set as follows; ion spray voltage (ISV): 5500V, temperature (TEM): 350°C, gas 1 (N_2) and gas 2 (N_2): 40 psi.



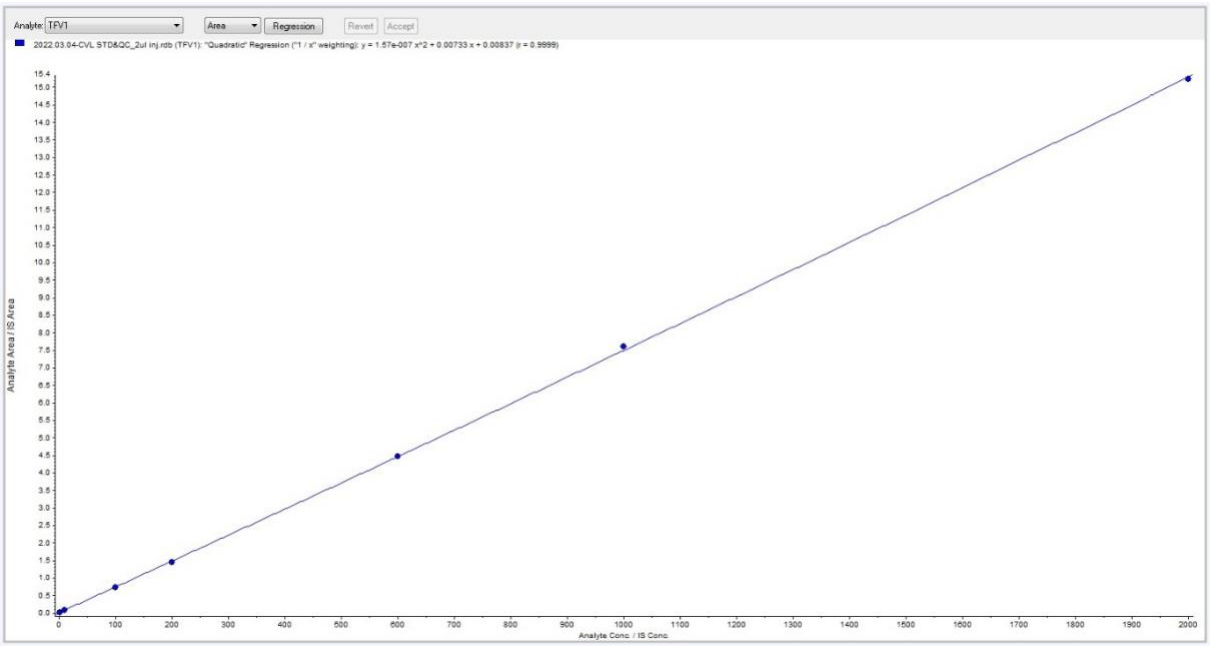
A representative chromatogram for tenofovir and deuterated internal standard; d6-TFV, with HPLC column retention time at 7.3 minutes.



A representative chromatogram showing all monitored and detected parent and precursor ions for each analyte and ISTD.

Preparation of standards and quality control (QC) samples

The main stock solutions of TFV and d6-TFV were prepared at a concentration level of 100 µg/ml in methanol: 0.1% formic acid-water (1:1, v/v) and stored at −80◦C until use. Further stock solutions of TFV, were prepared at 50, 10, 1 and 0.1 µg/ml. The calibration standards (CSs) were prepared by spiking the calculated amounts of the specific stock solution to 2 ml of methanol: 0.1% formic acid-water (1:1, v/v). The CSs covered a concentration range of 2 – 2000 ng/ml for TFV. The highest concentration quality control samples (HQC) were prepared at 800 ng/ml, medium quality controls (MQC) at 500 ng/ml and low-quality controls (LQC) at 5 ng/ml. The internal standard solution containing 5 ng/mL d6-TFV in water with 0.2% formic acid, was freshly prepared every day, prior to sample preparation.



A 7-point standard calibration concentration curve for tenofovir.

Appendix B: UKZN BREC approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

27 November 2018

Mrs Mazibuko-Motau (209502316)
School of Laboratory Medicine and Medical Sciences
College of Health Sciences
Mazibuko.noluthando@gmail.com

Dear Dr Mazibuko-Motau

Title: Longitudinal impact of BV treatment and oral PrEP use of vaginal microbiota in adolescent girls and young women at high risk of acquiring HIV in KwaZulu-Natal.
Degree: PhD
BREC REF NO: BE603/18

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received 27 September 2018.

The study was provisionally approved pending appropriate responses to queries raised. Your response received on 19 November 2018 to BREC correspondence dated 14 November 2018 has been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given **full ethics approval** and may begin as from 27 November 2018. Please ensure that site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is valid for one year from **27 November 2018.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

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

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

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

The sub-committee's decision will be **RATIFIED** by a full Committee at its next meeting taking place on **11 December 2018.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours since[redacted]

Professor V Rambiritch
Chair: Biomedical Research Ethics Committee

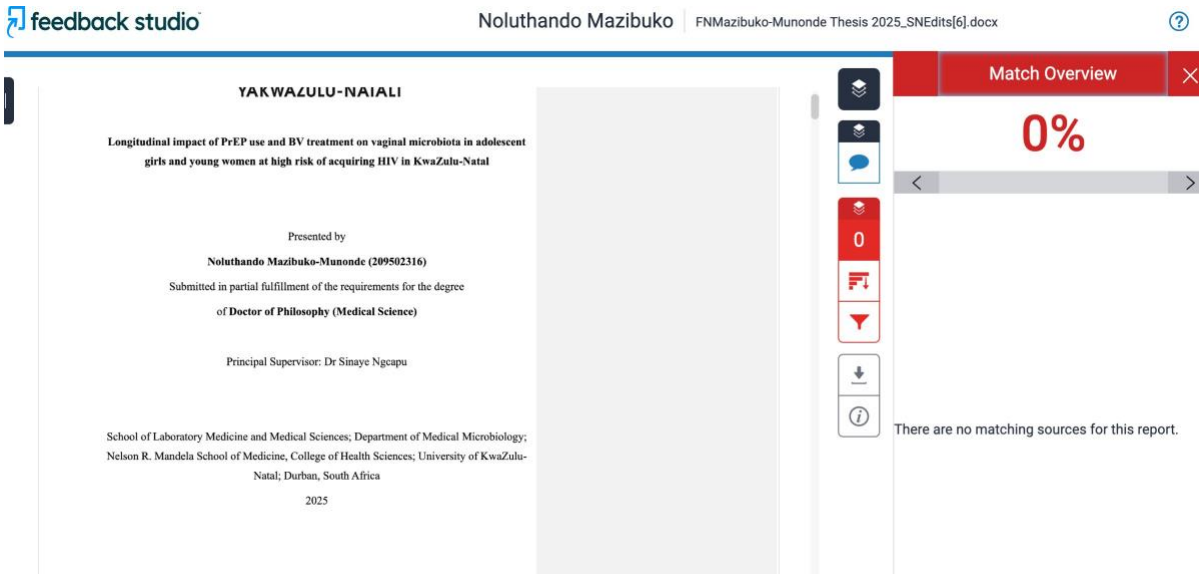
Supervisor: Ngcapu@ukzn.ac.za

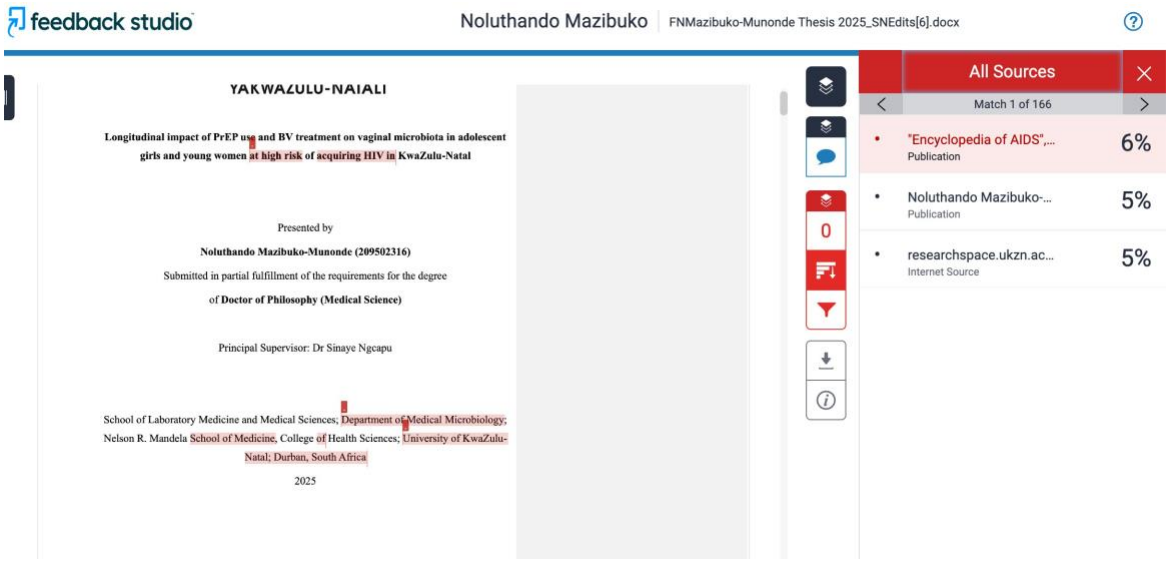
Biomedical Research Ethics Committee
Professor V Rambiritch (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2486 **Facsimile:** +27 (0) 31 260 4609 **Email:** brec@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

1910 - 2010
100 YEARS OF ACADEMIC EXCELLENCE

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

Plagiarism check





Appendix C: Statement of Contribution

To the dissertation committee,

In line with the examiner's request, I have provided a detailed account of my contributions to each publication and manuscript included in this thesis. These contributions have been reviewed and acknowledged by some of my co-authors and supervisor.

Chapter 2: Vaginal microbial shifts are unaffected by oral pre-exposure prophylaxis in South African women. *Published in Scientific Reports (2022), Article number: 16187. DOI: https://doi.org/10.1038/s41598-022-20486-z*

For this study, I (Noluthando Mazibuko-Motau) was centrally involved in the conception and design of the project, working jointly with Dr. Parveen Sobia, Dr. Derseree Archary, Prof. Douglas Kwon, and Dr. Sinaye Ngcapu. I was primarily responsible for conducting the laboratory experiments, including sample preparation and 16S rRNA sequencing workflows, with additional technical support from Andile Mtshali, Gugulethu Mzobe, and Lungelo Ntuli. In addition to the experimental work, I took a lead role in the analysis: I performed microbiome data processing, quality control, ecological diversity assessments, and community state type assignments in collaboration with Jiawu Xu, Ahmed Elsherbini, James San, and Dr. Sinaye Ngcapu. I also contributed extensively to the interpretation of results, drafted the initial versions of the manuscript, and worked iteratively with co-authors to refine the text. Statistical guidance was provided by Lara Lewis, and the principal investigators of the parent CAPRISA 082 and 084 studies (Dr. Leila Mansoor and Prof. Quarraisha Abdool Karim) provided oversight and access to study samples. Senior investigators (Prof. Douglas Kwon, Dr. Derseree Archary, and Dr. Sinaye Ngcapu) supervised and guided the overall study design and analysis framework, while I drove the day-to-day implementation of the work and took responsibility for preparing figures and integrating laboratory findings with the statistical results. In summary, my primary contributions to this publication included experimental execution, data analysis, figure preparation, and manuscript drafting, which together formed a substantial part of my PhD training.

Chapter 3: *Lactobacillus*-Dominant and Polymicrobial Vaginal Microbiomes not associated with High or Low levels of 1% Tenofovir Gel

For this study, I (Noluthando Mazibuko-Munonde) was centrally involved in conceptualizing and designing the project, in collaboration with Dr. Sinaye Ngcapu. I led the laboratory work, including DNA extraction, PCR amplification, and pooling of samples for 16S rRNA sequencing. I performed the data analysis and interpretation, integrating microbiome outputs with clinical metadata. My analyses were validated by our statistician, Marothi Peter Letsoalo, and our bioinformatician, Olona Asavela Kama, ensuring methodological rigor. I drafted the manuscript, which was subsequently reviewed and refined with input from all co-authors: Olona Asavela Kama, Jiawu Xu, Katya Govender, Andile Mtshali, Gugulethu Mzobe, Lungelo Ntuli, Leila Mansoor, Quarraisha Abdool Karim, Salim S. Abdool Karim, and Douglas S. Kwon. All co-authors contributed to the interpretation and discussion of the results and provided critical feedback on manuscript revisions. In summary, my contributions encompassed experimental execution, primary data analysis, interpretation, and manuscript drafting, forming a substantial and independent component of my PhD training, while the co-authors provided complementary support, oversight, and expert guidance throughout the study.

Chapter 4: Impact of Vaginal Insertion Products, Sexual Activity, and Vaginal Microbiome Dynamics on Genital Inflammation Markers Among South African Women. *Submitted to Microbiome;*

For this study, I (Noluthando Mazibuko-Munonde) was centrally involved in the conception and design of the analysis, working closely with Dr. Sinaye Ngcapu. I led and participated in the data analyses alongside Dr. Ngcapu, Dr. Olona Asavela Kama, and Dr. Leila Mansoor, performing microbiome data processing, statistical analyses, and integration with clinical metadata. The CAPRISA 090 study was overseen by principal investigators LGB, JSP, LM, and HBJ, who provided oversight and access to study samples. All other co-authors, including SN, PR, PG, CML, NR, AGA, SS, RH, MTM, BM, HA, DP, WH, LJPL, DA, AS, QAK, LGB, JSP, HH, LM, HBJ, and SN, contributed to the interpretation and discussion of results and provided critical input during manuscript preparation. I drafted the initial manuscript and coordinated revisions with all co-authors, incorporating feedback to finalise the publication. In summary, my primary contributions encompassed study conceptualisation, analysis, interpretation of findings, and manuscript drafting, forming a substantial and independent component of my PhD training, while co-authors provided complementary guidance, supervision, and critical review throughout the study.

Signed,

Noluthando Mazibuko-Munonde