Impact of the Vaginal Microbiome on HIV Transmission

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Abstract

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HIV is a global epidemic affecting almost 35 million people worldwide. Each year, almost 2 million new infections occur, with over 1 million new infections in women. The majority of these new infections take place in low income countries, primarily Sub-Saharan Africa. HIV transmission is highly understudied within women where each minute, two women are infected with HIV. Increased HIV transmission risk is characterized by three major mechanisms: barrier damage, inflammation, and an altered microbiome. The interplay between these mechanisms and their underlying causes have not been fully elucidated. The broad purpose of this thesis was to address several unanswered questions relating to mechanisms for an altered microbiome state within the female reproductive tract (FRT) and its impact on HIV transmission. We aimed to address three main objectives: 1) determining the impact of vaginal microbial communities on immune cells essential for pathogen protection and epithelial integrity; 2) identifying which specific bacterial species that are associated with HIV transmission and alter pre-exposure prophylaxis (PrEP) drug concentrations; and 3) characterizing the kinetics of PrEP uptake into target cells versus bacteria-mediated drug metabolism.

Within the FRT, an altered microbiome consists of the loss of a *Lactobacillus* dominant community replaced with a diverse, anaerobic community primarily consisting of *Gardnerella vaginalis, Atopobium vaginae, Prevotella* spp., and other bacterial vaginosis (BV)-associated bacteria. This shift in community is often referred to as vaginal

dysbiosis, or bacterial vaginosis (BV). BV is highly prevalent and associated with an 60% increase in HIV acquisition rate. The exact mechanisms by which BV impacts HIV acquisition rates are currently unknown and this thesis aims to address those questions.

The role of neutrophils in BV is a major theme of the studies described in chapter II. While neutrophils are important for pathogen containment, evidence has demonstrated their ability to contribute to tissue and barrier damage due to the release of reactive oxygen species and other potentially harmful effector molecules. Here, we demonstrate that BV associated bacteria increase neutrophil activation and increase lifespan, potentially contributing to accumulation in the FRT and reduced epithelial integrity. Healthy vaginal microbial communities, however, do not interfere with homeostatic apoptosis of neutrophils. The study described below provides potential mechanistic insights into how BV may lead to FRT inflammation and increased HIV transmission.

The success of pre-exposure prophylaxis (PrEP) drugs such as Truvada, have greatly decreased HIV transmission rates when adhered to. However, the efficacy seen in women highly varies and ranges from 75% to -49% in clinical trials. This variability is far more severe when compared to their male counterparts. While these studies have attributed these varying outcomes to adherence, recent evidence has highlighted the role biological factors, such as the vaginal microbiome, can play in impacting effectiveness of PrEP. In chapter III, through a secondary analysis, we demonstrate that vaginal microbial communities impact HIV incidence rates and that specific taxa, such as *G. vaginalis,* can directly impact tenofovir drug levels through a bacteria-mediated metabolic process. Indeed, in chapter IV, we show that this metabolism occurs at a faster rate than target cell conversion to the pharmacologically active drug and thus provide evidence linking vaginal bacteria to microbicide efficacy. When evaluating PrEP

in women, vaginal microbial communities must be considered and resolving dysbiosis may be critical for enhancing PrEP efficacy and prevention of HIV acquisition in women. Taken together, the data presented in this thesis improves our understanding of the mechanisms contributing to increased HIV transmission risk in women and the impact vaginal microbial communities can have. This thesis alludes to potential therapies that should be further investigated to improve overall health in women, a highly underserved community within HIV prevention studies.

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Dedication

For my future wife, Charlene Miller.

“audere est facere.”
-To dare is to do

Chapter I
Introduction

Adapted from: The role of the microbiome in pharmacokinetics and drug metabolism

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Global HIV Disease Burden

Human immunodeficiency syndrome (HIV) is one of the world's most serious health and developmental challenges[1,2]. Currently, close to 37 million people live with HIV worldwide and millions have died from acquired immunodeficiency syndrome (AIDS) since the beginning of this epidemic[1]. Despite attempts to identify 90% of people living with HIV knowing their status, 90% of those individuals on treatment, and 90% on treatment with suppressed viral loads, many people living with HIV or are at risk for HIV infection do not have access to prevention, treatment, and care. Additionally, there is no vaccine or cure available.

Despite a significant decline in new infections since the mid-1990s, there were still 1.8 million new infections in 2017[1]. The majority of these new infections occur in Sub-Saharan Africa, which includes nearly two-thirds of all people living with HIV[1]. Women represent almost half of all adults living with HIV and is currently the leading cause of death amongst reproductive age women[1]. Prevention strategies do exist to combat this HIV epidemic. They include behavioral awareness programs, condoms, male

circumcision, and HIV testing. Additionally, pre-exposure antiretroviral prophylaxis (PrEP) has been shown to be an effective HIV prevention strategy for at risk individuals.

HIV Treatment

Currently, HIV treatment plans include the use of combination antiretroviral therapy (ART) which attacks the virus, and other medications that aim to treat many other opportunistic infections that occur when the immune system is compromised during HIV infection. Since being introduced in 1996, combination ART has decreased morbidities and mortalities and access to this treatment has risen over the years. Still, only 59% of people living with HIV have access to ART[1]. As a result, only 47% of people with HIV are virally suppressed. While the U.S. government's efforts combined with nearly $21.3 billion spent globally, HIV is still at large[1]. The main focus of these efforts aim to achieve the Joint United Nations Programme on HIV/AIDS (UNAIDS) goal of achieving the "90-90-90" target by 2030[1]. While treatment strategies are complex and continue to be a topic of ongoing investigation, prevention strategies should also be considered.

HIV Prevention

Without an available vaccine, barrier protection such as condom use, is the primary means by which to prevent HIV. In 2004, emtricitabine/tenofovir was approved for medical use under the trade name Truvada. 8 years later, it became the first and only FDA-approved PrEP therapy against HIV infection[1]. HIV-negative individuals could take Truvada before coming into contact with HIV to reduce their risk of becoming infected. Despite consistent efficacy seen in men who have sex with men (MSM), studies in women have produced widely varying results. Microbicide research has focused on male-to-female transmission but has similarly experienced varying efficacy. While researchers have focused on adherence issues, studies have demonstrated no

significant difference in adherence amongst women and MSM despite their outcome differences[3].

Microbicides

Microbicides are unapproved, experimental products which contain drugs that prevent vaginal or rectal transmission of HIV. Microbicide are delivered in a variety of forms including vaginal rings, gels, films, inserts, and enemas[4]. In 2016, the NIH funded the ASPIRE study which looked at the efficacy of a dapivirine vaginal ring across 15 clinical sites spawning 4 different countries. Amongst women 25 years and older, 61% reduced their risk of HIV infection[5]. However, a second trail called The Ring Study only observed an effectiveness of 31%[6]. These varying outcomes match similar varying outcomes from oral Truvada which sparks the question of how much biological factors play a role.

Bacterial Vaginosis

Bacterial vaginosis (BV) is the most common type of vaginitis affecting reproductive age women. Unlike the gut, the bacterial microbiome of a healthy female reproductive tract (FRT) is simple. In healthy premenopausal women, a single *Lactobacillus* species dominates providing the host with protection from pathogenic bacteria and fungi through the production of bacteriocins, lactic acid, and hydrogen peroxide[7]. During BV, changes in the community occur where lactobacilli are replaced by anaerobic species including *Gardnerella*, *Mobiluncus*, *and Prevotella* species[7]. BV is associated with a 2-fold increased risk of acquiring *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis,* and HIV[7]. Despite associations with increased HIV transmission[8], the mechanism(s) by which BV increase these rates are currently unknown.

Inflammation and Neutrophils

Inflammatory cells such as neutrophils are critical for innate immune responses. Neutrophils are the first responders to infection and are crucial to an immune response to bacterial pathogens[9]. Chemotactic factors, like chemokine interleukin-8 (IL-8), recruit neutrophils during inflammation[9]. In order to combat invading pathogens, neutrophils use antimicrobial mechanisms including the use of cytotoxic granules, proteases, and reactive oxygen species[9]. Neutrophils can utilize degranulation which releases these granular contents into extracellular matrices killing pathogens[10,11]. Neutrophils can also use a process known as phagocytosis where neutrophils internalize microorganisms and sequester them within the phagosome[10]. Here they are exposed to granules which can kill pathogens. Lastly, neutrophils can release neutrophil extracellular traps (NETs) which consist of DNA that traps microbes allowing granular contents to kill the pathogen[10]. The antimicrobial function of neutrophils is regulated through recognition of pathogen-associated molecular patterns (PAMPs), which can interact with toll like receptors (TLR) on neutrophil surfaces[12]. These TLRs on neutrophils mediate survival, cytokine release, degranulation, and phagocytosis through expression of TLR 1, 2, and 4-10[12]. While antimicrobial properties of neutrophils is beneficial to the host, unregulated activity from neutrophils can cause barrier damage and additional inflammation. Previous studies have shown an infiltration of neutrophils in the gastrointestinal (GI) tract in treated and untreated infection[13], and recent evidence has shown neutrophil proteases in the FGT associated with inflammation[14]. Understanding the contribution that BV provides and the role of neutrophils in HIV transmission is not currently understood and may be critical in HIV prevention strategies for women. Variability within FRT microbiomes of women and understanding the potential impact of host-microbe interactions may be the next step for personalized medicine. Understanding the potential

impact of host-microbe interactions may be the novel route towards personalized medicine.

Pharmacomicrobiomics

The human microbiome consists of a diverse community of bacteria, fungi, viruses, and Archaea[15]. The microbiota that comprise the microbiome consists of 10-100 trillion microbial cells, primarily located in the gut[15]. A healthy human gut microbiome is quite diverse and largely consists of four phyla, including Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria[16]. With over 10 trillion cells composing the microbiome, an amount roughly equal to the number of somatic cells in a single individual[17], the potential impact of the microbiome on the host is high[18]. It has become increasingly clear that these diverse species and their metabolic products are required for maintaining proper immune health and function as well as overall human health[19]. In particular, drastic microbial changes or perturbations to the community have been linked with various disease states; including cancers[20], diabetes[21], inflammatory bowel diseases[22], the human immunodeficiency virus (HIV)[23], and many others[17,24,25].

During the past several years, efforts have been focused on identifying the impact that our microbiota can have on host health and xenobiotics, and the clinical importance of a healthy microbiome has been demonstrated by several instances. One example is that diversity of the microbiome predicts mortality outcomes after allogenic stem cell transplant[26]. Another example is the successes of fecal microbiota transplants (FMT) to treat *C. difficile*[27] and vancomycin-resistant *Enterococcus* infections[28,29]. In addition, many gastrointestinal (GI) diseases[28], obesity[28], and autism spectrum disorders[30] highlight the potential impact of the microbiome on health. The microbiome is critical for metabolism of dietary fibers and key metabolic products such as short-chain fatty acids; yet the metabolism of other compounds, such as drugs, has been understudied despite

the ability of bacteria to metabolize many xenobiotics[31,32-39]. Additionally, studies have demonstrated that many subgroups of bacteria possess enzymes or enzyme analogs that have been known to play a role in drug pharmacokinetics and metabolism[32]. Our understanding of the link between the human microbiome and disease is rapidly expanding. Improvements in throughput and increasing sensitivity and accuracy of DNA sequencing techniques of microbial communities have vastly advanced our understanding of the microbiome. These improvements are vital in truly understanding the role of the microbiome in pharmacokinetics and drug metabolism.

Variability of Drug Efficacy

Individual responses to specific drugs vary greatly in efficacy and toxicity. Previous reports have identified a 50-75% response rate to common drugs for variety of diseases[2]. These varying efficacy rates are seen in the lack of protection in HIV pre-exposure prophylaxis (PrEP) trials in some women where effectiveness ranged from 75% in the TDF2 trial[40] to as low as 6% in the FEMPrEP trial[41] or even negatively at -49% in the MTN003/VOICE trial[42]. While many of these trials have attributed the variability in drug efficacy to adherence, recent evidence has highlighted the importance of the microbiota, specifically the vaginal microbiota, in the context of HIV prevention[3]. In fact, variations in efficacy go beyond PrEP and even alter antiretroviral drug resistance[43,44]. Studies have shown that 70-80% of individuals with virologic failure experience HIV drug resistance[44]. Beyond HIV, drug resistance and efficacy has been a major obstacle in the fight against malaria[45]. This resistance is seen in *P. falciparum* and extends to multiple drug classes for malaria treatment such as 4-aminoquinolines and antifolates[45]. The tAnGo trial which evaluated the role of adding gemcitabine to anthracycline and taxane chemotherapy for breast cancer also identified unexpected outcomes[46]. Despite preliminary evidence of a beneficial impact, the addition of

gemcitabine offered no therapeutic advantage, but rather increased toxicity potentially due to microbiome effects[46]. This example, amongst others, highlights the growing evidence of increased focus on the relationship between variability in chemotherapy outcomes and the role of the microbiome[47].

Many individuals suffer from adverse drug reactions or serious uncommon toxicities such as those in the tAnGo trial. These idiosyncratic toxicities can occur when bacteria-drug interactions lead to unintended formation of byproducts of drug metabolism by specific bacteria[32,48-52]. Inter-individual heterogeneity in drug response is a serious problem that affects not only patient wellbeing but also poses financial and clinical burdens. As a result, the pharmaceutical industry has recently begun shifting their focus of drug metabolism to include bacteria[53].

Drug Pharmacokinetics and Metabolism

Typical drug metabolism involves the conversion of readily absorbed lipophilic compounds into hydrophilic products for excretion[2]. Microbial modulation of drugs can include both direct and indirect mechanisms. Direct mechanisms include drug activation, detoxification, metabolism, and direct binding. Indirect mechanisms occurs when metabolic or peptide products influence drug bioavailability and response. Metabolism and/or biodegradation of drugs by bacteria and how this contributes to human health remains understudied.

Drugs are introduced into the body by several different routes. Oral administration via liquids, capsules or tablets are the most common. However, these drugs typically move through the digestive tract where absorption begins in the mouth and stomach before the majority is absorbed from the small intestine[54]. Factors such as food or other drugs in

the digestive tract affect how much of and/or how fast a drug is absorbed[54]. Similarly, these drugs are constantly being exposed to the GI microbiome. The gut microbiota is not static, rather it is a dynamic environment that can be affected by antibiotic use, pathology such as GI and systemic infection, diet, circadian rhythm, and other environmental factors[55,56]. All of these contribute to inter-individual and intra-individual clinical outcome variability.

Oral administration of pharmaceuticals have implemented different formulation strategies to increase drug stability in the GI lumen. These strategies, like enteric coating, protect the drug entity from degradation due to low pH in the upper GI tract. However, considering the impact of the distally increasing population of microbes is not fully implemented. Previous reports have suggested that microbiota altering drug pharmacokinetics was primarily from exposure in the distal gut[57]. Therefore, because drug absorption primarily takes place in the small intestine, there would be little interaction with microbes unless the pharmaceutical was a candidate for sustained-release or enterohepatic recirculation. However, as emerging drug candidates come to market, there has been an increase in low solubility and low permeability properties. These result in increased gastrointestinal residence times and therefore increased likelihood of microbial interactions. The need to consider these potential metabolic processes is of growing importance.

Gut and Vaginal Microbiome

The gut microbiome is responsible for various important biochemical functions for the host. Disorders or disruptions within this symbiotic community are associated with many diverse human diseases[28]. It is essential for the future development of personalized healthcare strategies to include quantitative and qualitative analysis of the gut

microbiome as there has been direct implications in pathological states such as obesity[15], circulatory disease[58], inflammatory bowel disease[59], autism[60]. Implications have also been seen in dietary calorific bioavailability[61], immune system response[62], and drug metabolism and toxicity[63]. In most mammals, the gut microbiome is dominated by four bacterial phyla: Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria[16]. Over the long-term, without interventions, the phylotypic composition of the microbiome within the gut is specific and stable within an individual[64]. Commensal bacteria, such as *Lactobacillus* and *Bifidobacterium*, colonize the host shortly after birth[65]. These microbiota are involved in the dietary digestion of foods that otherwise cannot be digested by the stomach and small intestine[66]. These microbes therefore play a key role in maintaining energy homeostasis through dietary fibers and production of short-chain fatty acids, which serve as energy sources for host epitheliums[67]. Throughout development, the host immune systems use signals and metabolites from these microorganisms to learn appropriate physiological responses. While primarily beneficial, some can induce inflammation and other negative immunologic responses[68].

Dysbiosis of the microbiome occurs when the microbiota community differs from the community associated with a healthy control state. With factors such as geographic location, dietary habits, use of probiotics and antibiotics, as well as sleeping or stress patterns, all playing a role in establishing the microbiome, the “healthy” microbiome can vary from individual to individual[69]. Understanding host-specific dysbiosis is vital in appreciating the intricacies and the health of the gut microbiome in relation to disease outcomes. For specific outcomes such as drug pharmacokinetics, identifying the specific taxological changes and their potential impact is necessary for informing patient treatment options both improving efficacy and avoiding toxicity.

Recent studies have shown that gut and vaginal microbiota both influence the metabolism of different drugs. Low diversity in the gut is linked with dysbiosis, but by contrast, within the vaginal microbiome, low diversity is considered to be healthy, and is characterized by the dominance of a single taxa, *Lactobacillus*. These *Lactobacillus* provide a key function in producing lactic acid via fermentation and establishing a low pH within the vagina[70]. Dysbiosis within the female reproductive tract occurs when there is an increase in anaerobic bacteria, notably *Gardnerella vaginalis, Prevotella spp.,* and *Atopobium vaginae* coupled with a decrease in the commensal *Lactobacillus spp*[7].

Microbes that reside in the human gut and vagina can directly or indirectly affect drug pharmacokinetics (PK) and metabolism. Directly, these microbes can metabolize and scavenge for drugs or indirectly, through changing the environment by altering pH levels. Depending on environmental conditions, availability of enzyme substrates, and specific microbiota composition, pharmaceuticals can be metabolized through a variety of different reactions. Typical metabolic reactions are hydrolysis, reduction, and oxidation, but can also include acetylation, deacetylation, deconjugation, proteolysis, functional group removal, thiazole ring breakage, amine formation, denitration, and N-oxide cleavage[71]. Microbiota induced reactions can cause either activation or inactivation of drugs, sequestration of the drug via direct binding to the bacterium, reactivating a drug via enzymes, production of active metabolites, and direct competition for host enzymes[72]. This review will go through potential bacteria-mediated metabolic events and provide examples of each.

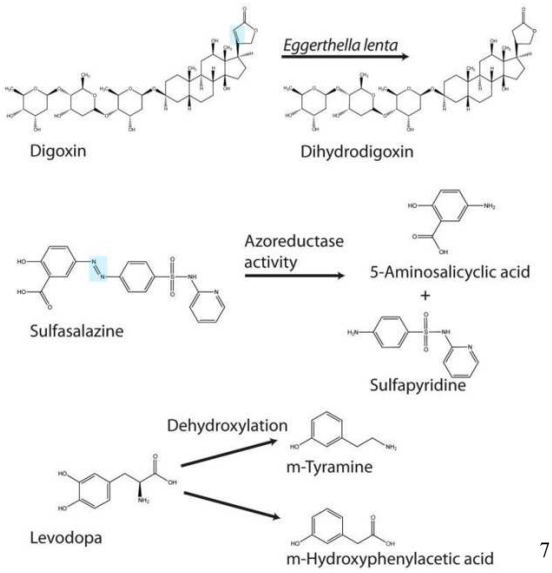

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Figure 1. 1 Key examples of microbial biotransformation (A) digoxin, (B) sulfasalazine, and (C) levodopa

Bacteria-mediated metabolism: Reduction and Hydrolysis

The most common example of a xenobiotic metabolized by gut microbiota enzymes is the reduction of the cardiac glycoside digoxin, where digoxin is inactivated by the Actinobacterium *Eggerthella lenta*[50]. Digoxin is used to treat congestive heart failure and atrial fibrillation. Digoxin has a narrow therapeutic window thus highly susceptible to small changes in bioavailability. Specific strains of *E. lenta* possess the cardiac glycoside reductase operon which can inactivate digoxin. What makes the interactions between *E. lenta* and digoxin metabolism unique is that it is one of the few cases where biotransformation of xenobiotics occurs by a single bacterium in isolation[50]. Thus, before digoxin is given it is critical to test patients for this bacterium. Being listed on the World Health Organization's List of Essential Medicines, the importance of digoxin cannot be understated[73]. With heart disease being the leading cause of death in both men and women[73], highly efficacious cardiac drugs like digoxin are irreplaceable. Identifying bacteria-mediated metabolism, in this case, inactivation caused by reduction, impacts

patient lives in ensuring that therapeutic digoxin levels are being met by identifying at-risk patients like those with *E. lenta.*

Similarly, sulfalazine, which is used to treat gut inflammation, can be re-converted to its pharmacologically active form via amino-salicylic acids by microbial enzymes[74]. Which enzymes produced by which bacteria impact this reactivation? Identifying these microbes and their enzymes can impact patient outcomes by preventing higher than necessary sulfazine concentrations due to re-activation, which can potentially lead to toxicity events. The dopamine precursor Levodopa used to treat Parkinson's disease is an example of a functional group removal with *Helicobacter pylori* performed in rats[75]. For patients with Parkinson's disease, identifying at-risk individuals, those who are highly susceptible to functional group removal by *H. pylori*, may improve variability seen in treatments. Even antibiotics, compounds designed to alter microbiota composition, are susceptible to inactivation by microbiota. Evidence has demonstrated that metronidazole, typically used to treat trichomonal vaginitis, is reduced by intestinal bacteria to form N-oxamic acid, an inactive compound[76]. This could potentially explain why treatment for women with vaginitis is so variable and ineffective with recurrence rates of nearly 50%[77].

Previous studies have identified two major bacterial enzymes that are capable of enzymatic activities relevant to xenobiotic metabolism. Those enzymes, azoreductase and ß-glucosidase, inactivate pharmaceuticals via reduction and hydrolysis, respectively[71]. Azoreductase has been shown to inactivate sulfasalazine into 5-aminosalicyclic acid and sulfapyridine, neither of which carries pharmacologically active properties[75]. The variability in expression of these enzymes differ greatly where Proteobacteria such as *E. coli*, express azoreductase, while it is rare in *Bifidobacterium*

spp[71]. Alternatively, *Bifidobacterium spp.* highly express ß -glucosidase, while Proteobacteria do not[71]. With the dynamic nature of the gut microbiota's composition, further studies are needed to understand enzymatic differences within a microbial community.

Bacteria-mediated metabolism: Denitration and Deconjugation

Additionally, some microbial products can induce metabolic responses within the liver. This would indicate the ability of host-microbe interactions to delay or decrease the detoxification and elimination of these drugs from the body[78]. A common example of this reactivation is seen in the chemotherapeutic drug, irinotecan. As irinotecan undergoes glucuronidation in liver in preparation for elimination, it is then shuttled back into the intestine via bile, where gut bacteria can deconjugate the metabolite back into its parent form where it can exert toxic effects in the colon causing diarrhea[79]. ß -glucuronidase expression is highly variable amongst common cultured gut isolates where it is present in roughly half of the Firmicutes but absent in Actinobacteria such as *Bifidobacterium spp*[71]. Another common mechanism by which flora alter drug PK is through denitration seen in a 1983 study that looked at the metabolism of glyceryl trinitrate in the presence of fecal contents from rats[80]. Their study identified the complete disappearance of the parent drug and the formation of four inactive metabolites[80]. The challenge that healthcare providers and drug companies face lies in the inability to identify at-risk patients for host-microbe drug metabolism. Imagine a scenario where a patient is administered a chemotherapeutic drug such as irinotecan and experiences a toxic event such as diarrhea. The patient presents with an issue and leaves with an additional symptom that has been linked to gut dysbiosis and inflammation[81]. This is an example of how important the microbiome is in the context of drug metabolism and clinical outcomes.

Bacteria-mediated metabolism: Functional Group Removal

The removal of functional groups is another mechanism by which bacteria can alter drug PK and metabolism. This becomes particularly relevant when the resulting metabolites become toxic. The mechanism of toxicity from 5-fluorocytosine chemotherapy is not well understood, however evidence has begun to identify the formation of 5-fluorouracil as metabolite that is potentially involved. *In vitro* culture systems have also found instances of intestinal microflora causing the deamination of 5-fluorocytosine to 5-fluorouracil, which could potentially be the key to finding the mechanism behind this toxicity[82]. Even a recreational drug, such as methamphetamine, can undergo demethylation in the presence of intestinal microflora[83].

While many studies have described the microbiome during HIV-1 infection, the microbial mechanisms that contribute to its progression and pathogenesis are not well understood. In terms of HIV-1 prevention strategies, pre-exposure prophylaxis (PrEP) is a highly effective method when used correctly in men[84], but results have seen varying levels of protection for women[3]. Topical microbicides, such as the 1% tenofovir vaginal gel, are exposed to a different challenge: the vaginal microbiome. Vaginal microbiota associated with bacterial vaginosis have demonstrated not only the ability to modulate host innate immune responses[7], but also the ability to directly impact drug efficacy via metabolism as seen with the drug tenofovir via metabolism to adenine and oxy-methylphosphonic acid[3]. Indeed, this may be a major mechanism by which PrEP drugs are not as efficacious in women. There is a need for a better understanding of the role of the vaginal microbiome, as well as the gut microbiome in modulating HIV prevention efficacy should be further studied.

Bacteria-mediated metabolism: N-oxide Cleavage and Proteolysis

Sequencing advancements such as 16S rRNA and shotgun sequencing techniques have made a great impact on microbial analysis. Coupled with increasing gains in mass spectrometry and metabolite identification, cleavage of N-oxide bonds as seen from ranitidine and colonic bacteria, highlight the impact the gut microbiome can have on drug metabolism and also demonstrate a growing ability to assess these interactions[85]. Similarly, N-oxide cleavage susceptibility is seen in other H_2 receptor antagonists like nizatidine[86]. The impact of these interactions is highly prevalent considering the fact that these drugs are used to treat GI symptoms and microbial dysbiosis yet, they are susceptible to GI bacterial interactions that are not well identified. Studies have even suggested the use of dual therapies, one for treatment and the other for prevention of a potential microbial-mediated metabolic event[87]. For example, this was demonstrated in a study examining the effects of intestinal bacteria on insulin and calcitonin. Both of these drugs were susceptible to degradation by bacteria and it was suggested that protease inhibitors can increase the stability of these drugs thereby increasing absorption to systemic circulation and increasing bioavailability[88].

Bacteria-mediated metabolism: Other

While we have highlighted specific ways bacteria can impact drug PK and metabolism, there have been numerous interactions that have been studied. Most of these studies are done *in vitro* but they highlight the need for larger scale studies that can more accurately assess this understudied field. Bacteria have the ability to undergo various metabolic mechanisms making them particularly adept at causing unusual PK properties when tested in vitro. For example, chloramphenicol can undergo amine hydrolysis[89] and levamisole has seen evidence of thiazole ring-opening[90].

The range of properties bacteria can carry out are largely more variable than that of typical human enzymes that carry out drug metabolism like the cytochrome P450s[91]. In fact, even bacteria themselves contain these P450 enzymes[92] adding another level of potential metabolic events when xenobiotics are introduced. With growing interest in the "pharmacomicrobiomics"[32] field, attention is increasingly directed towards the gut microbiome-brain axis. Evidence has begun to demonstrate the impact the gut microbiome can have on human brain health and that bacterial components such as lipopolysaccharides or bacterial dysbiosis can cause inflammation of the central nervous system[93]. These gut microbes may be responsible for the production of neurotoxic metabolites such as D-lactic acid and ammonia[94]. Similarly, these microbes can produce hormone imbalances because they can secrete neurotransmitters and hormones identical to those produced by humans[94]. Even drugs such as risperidone, used to treat mental disorders, can be impacted by the gut microbiome as shown by previous studies that demonstrate isoxazole scission decreasing the drug efficacy[95].

How can we better treat individuals if the symptoms they exhibit are a result of or related to microbial changes, some of which may render the treatment ineffective due to unforeseen host-microbe drug interactions? Growing technological advances, particularly in microbial ecology[96] and metabolomics[97], may hold the answer. Rapid identification of at-risk patients, those that have microbial communities or metabolites that have shown evidence of impacting treatment, is necessary to move forward in the goal of precision medicine. Imagine a scenario where a patient comes in and has a quick microbiome analysis. Within minutes, the physician can offer a "cocktail" prior to the treatment drug to avoid potential host-microbe drug metabolism or chose another treatment drug that is not susceptible for bacteria-mediated metabolism. While we are certainly far away from these situations, increasing technological

advancements in microbial analysis and awareness of the impact the role of the microbiome can have on drug PK and metabolism is growing. This continued growth will only serve to improve patient outcomes.

Thesis Objectives

This thesis describes studies aimed to identify the impact the vaginal microbiome has on HIV transmission through addressing three objectives: 1) determining the impact of vaginal microbial communities on immune cells essential for pathogen protection and epithelial integrity; 2) identifying which specific bacterial species that are associated with HIV transmission alter PrEP drug concentrations; and 3) characterizing the kinetic rate of PrEP uptake into target cells versus bacteria-mediated drug metabolism.

Chapter II
The Impact of Vaginal Microbial Communities on Immune Cells Essential for Pathogen Protection and Epithelial Integrity

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Background

Bacterial vaginosis (BV) has been linked to an increased risk of STI and HIV acquisition and forward transmission, as well as other adverse outcomes such as preterm delivery and pelvic inflammatory disease[98-103]. BV is the most common cause of vaginal discharge amongst women of reproductive age, and is directly related to the make-up of the vaginal flora[104]. Specifically, clinical and molecular BV are characterized by a diverse community of anaerobes, including *Gardnerella vaginalis, Mobiluncus spp., Prevotella spp., Atopobium vaginae,* and others[98,105,106]. An optimal vaginal microbiome is dominated by *Lactobacillus* spp., although even in this context the species of *Lactobacillus* has important clinical implications, with *L. iners* being more inflammatory and less protective against HIV acquisition than other *Lactobacillus* spp., perhaps due to

differences in hydrogen peroxide production and differing capacity to protect against other bacterial and viral STIs[107].

BV is commonly diagnosed clinically using Amsel's criteria[108]. In Amsel's criteria, BV is diagnosed when 3 out of 4 criterion are met: abnormal discharge, pH>4.5, clue cells present, and fish odor[108]. Nugent score is another method used to diagnose BV, which attempts to capture the bacterial morphotypes via gram staining due to the differentiating *Lactobacillus*-dominating communities compared with small and curved Gram-variable rods [108]. Nugent scoring is widely used to define BV in large cohorts. With increasing correlations to adverse health outcomes, there is a growing need for improvement in BV diagnosis. Due to advances in DNA sequencing technology, molecular methods using sequencing and quantitative PCR, are becoming more readily available as seen with an FDA-approved molecular diagnostic BV test[109]. These techniques have identified even a larger proportion of asymptomatic women at risk for cervicovaginal inflammation and increased STI risk due to the ability of molecular BV testing to identify specific taxon[110].

BV has been found associated with the prevalence and incidence of multiple STIs including chlamydia, gonorrhea, herpes, and trichomoniasis[111-115]. Molecular BV is very common amongst reproductive age women, particularly black women in both North America and Africa, with a prevalence as high as 63% reported in Zambia[8,116]. Amsel's criteria, Nugent score, and molecular techniques, while overlapping, offer different depth in terms of better capturing cervicovaginal microbiota. With increasing associations of BV and inflammation or risk of STIs, including HIV, and the growth in molecular techniques, there is a clear need to better associate specific taxa with these adverse outcomes. BV is associated with a 60% increase in HIV incidence[105,106,117], but the mechanisms underpinning this association are not clearly understood.

The mucosal barrier provides protection against invading bacterial and viral pathogens, including HIV[14], and inflammation-induced reductions in epithelial barrier integrity may be an important mechanism by which BV increases susceptibility to HIV infection[14]. Neutrophils are the first responders to pathogen challenge and play a crucial role in antibacterial and antifungal defense, but they can also contribute to barrier damage and inflammation[13]. Therefore, there is a delicate balance between the anti-microbial activity of neutrophils and potential tissue damage through the release of harmful effector molecules such as reactive oxygen species[13]. This balance could impact HIV and STI infections, which are acquired across mucosal barriers, and the exact role of neutrophils in vaginal health is not understood.

Previous studies have demonstrated accumulation of neutrophils in the gastrointestinal (GI) tract in treated and untreated HIV infection[118,119], and neutrophil proteases in the FGT were associated with GI mucosal inflammation in people living with HIV[14]. There have also been discrepancies in whether neutrophils increase or decrease during BV as seen in Cauci et. al where they saw no increase in neutrophil numbers in women with BV[120]. However, there have been studies to identify increases in IL-8, a potent chemotactic and activating factor for neutrophils, in vaginal fluid from women with BV[121-123]. Even when compared with candidiasis, studies have shown that women with BV have lower levels of neutrophils[124,125]. These discrepancies may be attributed to the aforementioned issues with diagnosing BV. It is this reason that our study utilized molecular techniques to identify specific taxa.

Additionally, penile foreskin neutrophil infiltration was associated with elevated inflammatory cytokines[126]. This inflammation may result in barrier damage and

dysfunction in the gut[127] and other mucosal sites, providing an opportunity for STI penetration, as well as recruiting mucosal CD4+ T cell target cells for HIV infection. Previous work done by Arnold et al. proposed a potential model for how neutrophil proteases can alter epithelial cell differentiation and lead to decreased barrier function[14]. In this study, a set of mucosal proteins associated with mucosal cytokines was identified and linked to specific neutrophil proteases. They present a model that these inflammation-associated neutrophil proteases disrupt epithelial cell differentiation ultimately decreasing barrier function and integrity[14].

Here we analyze the effects of vaginal microbiota composition and specific BV associated bacteria on neutrophil lifespan and functional phenotypes. Based on previous work, we used epithelial barrier assays to fully evaluate the effect of neutrophils on barrier integrity in the presence of these taxa, revealing potential mechanisms for increased HIV susceptibility amongst women with BV.

Methods

Study procedures

Informed written consent was obtained from all participants prior to enrolment, and the study was approved by Institutional Review Boards at St. Michael's Hospital (Toronto) and the University of Toronto. The described studies were conducted according to the principles expressed in the Declaration of Helsinki. For analysis of endocervical neutrophil populations, female participants were recruited from the Colposcopy Clinic at St. Michael's Hospital, Women's Health Care Centre in Toronto, Canada. Recruited participants self-reported to be HIV-negative and were not actively menstruating at the time of sample collection; women with/without clinical findings of BV were recruited using a convenience-based cross-sectional sampling frame. For the blood samples for

bacterial stimulations, HIV- study participants (n=6) were recruited through the University of Washington Center for AIDS Research.

Samples collection

Two cervical cytobrushes were collected from each participant after insertion of a cytobrush into the endocervical os and rotation through 360°. Cytobrushes were then transferred into 1 mL of RPMI in a 15mL conical tube and vortexed for 30 seconds prior to removal and disposal of the cytobrush. The cytobrush media was centrifuged at 1900RPM for 6 minutes at 4°C. Cytobrush supernatant was then aliquot into 2 cryovials (500µL/vial) and stored immediately at -80°C. Vaginal swabs were inserted into the vaginal speculum and rotated 360° three times prior to collection. Swabs were then placed back into the original container and immediately stored at -80°C.

Flow cytometry

Cytobrush cell pellets were immediately re-suspended in 500 µL PBS prior to flow cytometry staining. Whole blood samples were stained fresh or following co-culture with various bacteria as outlined below. Cytobrush cells were stained using the following surface antigen mouse anti-human antibodies: CD32 FITC (Becton Dickinson, BD), CD66b PerCP-Cy 5.5 (BD), CD64 Ax700 (BD), CD11b APC Cy7 (BD), CD3 PE (BD), CD45 PE CF594 (BD), Caspase-3 V450 (BD), CD16 BV605 (BD), CD15 BV650 (BD), HLA-DR BV711 (BD), CD14 BV786 (BD), CD20 BUV395 (BD), CD49d BV421 (BD), CD89 APC (Biolegend), CD62L PerCP Efluor 710 (Ebioscience), CD 274 (PD-1L) PE Cy7 (Biolegend), and Aqua L/D/eBio506 L/D (LifeTech). Blood from the bacteria stimulations were stained using the following Becton Dickinson (BD), Biolegend, Ebioscience, and LifeTech surface antigen mouse anti-human antibodies: CD32 FITC (BD), CD45 PerCP (Biolegend), CD11b APC Cy7 (BD), CD3 PE (BD), CD45 PE CF594

(BD), CD62L PerCP Efluor 710 (Ebioscience), CD 274 (PD-1L) PE Cy7 (Biolegend), Caspase-3 V450 (BD), CD20 BV570 (Biolegend), CD16 BV605 (BD), CD15 BV650 (BD), HLA-DR BV711 (BD), CD14 BV786 (BD), and Aqua L/D/eBio506 L/D (LifeTech). Both cells from cytobrushes and blood were permeabilized using Cytofix/Cytoperm (BD) after surface staining. After staining, samples were fixed in 1% paraformaldehyde and collected on a Fortessa X20 (BD) and an LSR II (BD) for the cytobrush samples and in-vitro stimulations, respectively. FlowJo version 9.7.6 was used for analysis.

Bacterial strains and culture conditions

Lactobacillus iners, ATCC 55195, *Lactobacillus crispatus*, ATCC 33197, *Gardnerella vaginalis*, ATCC 14018 (group C), *Prevotella bivia,* ATCC 29303, and *Atopobium vaginae,* ATCC BAA-55, were obtained from the American Type Culture Collection (ATCC). *Lactobacillus* spp. and *G. vaginalis* were maintained on Human Bilayer Tween Agar (BD) plates and New York City III (NYCIII) medium according to the manufacturer's instructions. *P. bivia* and *A. vaginae* were maintained on ATCC medium 260: Trypticase soy agar/broth with defibrinated sheep blood and ATCC medium 1377: Haemophilus ducreyi medium, respectively. Agar plates and liquid cultures were incubated at 37°C with anaerobic gas mixture, 80% N_2, 10% CO_2, and 10% H_2. Frozen stocks of strains were stored at -80°C in 40% (v/v) glycerol.

In-vitro bacteria stimulations

For the in-vitro stimulations, 100µL of whole blood from healthy individuals was stimulated at a ratio of 2.5 bacteria per leukocyte in 1mL R10 media (RPMI 1640 with 2.05mM L-glutamate and 10% fetal bovine serum). Incubations were done at 37°C for 18 hours. Following incubation, blood was centrifuged and washed with 1mL PBS prior to flow cytometry analysis as described above.

TEER w/ Neutrophil Isolation

HeLa cells (ATCC CCL-2) were cultivated and seeded into transwell inserts (Corning). Cells were monitored microscopically to evaluate detachment. Wells were seeded with 50,000 HeLa cells in 300µL of Dulbecco's Modified Eagle's Medium (DMEM, ATCC 30-2002) and 10% fetal bovine serum. The wells were filled with 1mL of the same medium and placed at 37°C until stable TEER values and a monolayer was formed. Triplicate technical replicates were taken for each well. Nine biological replicates were assessed per condition. Cutoff values were <400Ω. Resistance was measured using an EVOM2 Epithelial Voltohmeter (World Precision Instruments). Neutrophils were isolated from whole blood using the MACSxpress Whole Blood Neutrophil Isolation Kit (Miltenyi Biotec) and Red Blood Cell Lysis solution. Neutrophil purity was validated after staining an aliquot of sample fraction with anti-CD15 and assessed via flow cytometry. Neutrophils were then counted and $2x10^5$ were added to the wells. Bacterial were added at a 2:1 ratio with neutrophils and the wells were placed at 37°C. TEER readings were done at 0, 4, and18 hours.

Lucifer Yellow Transport Assay

Transport studies were performed on HeLa cell monolayers with 100µg/mL lucifer yellow (Thermo Fisher) (LY). An experiment without a sample added to the apical side was performed as a negative control. Fresh DMEM media was added to both sides of the transwell. After a 15-minute rest period, 0.5mL DMEM media containing LY was added to the apical chamber while 1.5mL of DMEM media without LY was added to the basolateral side. After incubation for 1 hour, transport rates were determined by measuring fluorescence from LY on the basolateral side. The LY was determined fluorometrically at 430nm excitation and 540nm emission using a SpectraMax Gemini

XS (Molecular Devices). HeLa cell tight junction formation was confirmed using LY fluorometric data. HeLa cells had an average permeability of 0.010±0.001 nmol/min/cm^2 while media alone had an average permeability of 0.062±0.011 nmol/min/cm^2.

Analysis of 16s rRNA gene sequencing data

Vaginal swabs were added to 200µL of the Qiagen's DNeasy Blood and Tissue kit lysis buffer (ATL). Samples were heated at 65°C for 10 minutes before lysozyme solution was added to a final concentration of 10mg/ml. An 1-hour incubation at 37°C then occurred followed by adding 5% SDS to a final concentration of 1% w/v. 10-minute incubation at 56°C was then done. 25µL of Proteinase and 200µL of Buffer AL from the Qiagen's DNeasy Blood and Tissue kit was then added and incubation of 30 minutes at 56°C took place. The swabs were removed and 200µL of EtOH was added into the solution. The rest of the DNA extraction was performed according to Qiagen's DNeasy Blood and Tissue Kit protocol. The Earth Microbiome Protocol for 16S Illumina sequencing with primers discussed in Caporaso et al. (2012) was used to amplify the V4 region of the 16S SSU rRNA. Amplicon concentrations were normalized, pooled, and cleaned followed by a KAPA quantification. A 2x150 bp Illumina MiSeq run was used to sequence the pooled library. 16S sequencing reads were processed using QIIME2 version 2018.2; taxonomic determination in QIIME2 utilized the Silva 119 classifier. Taxonomic plots were created in part within Rstudio utilizing the phyloseq package.

Statistical Analysis

GraphPad Prism statistical software (version 6; GraphPad Software, San Diego, CA) was used for all statistical analysis. Differences in active Caspase-3, neutrophil

frequencies, and CD62L between *Lactobacillus* dominant and non-*Lactobacillus* dominant communities from the cervicovaginal cytobrushes were determined by Mann-Whitney test. A one-way ANOVA was used to assess differences between groups stimulated with different bacteria and controls followed by a Tukey's multiple comparisons. A one-way ANOVA was also used to assess differences between groups stimulated with different bacteria and neutrophils for the TEER assays followed by a Tukey's multiple comparisons. Relative abundance plots focused on the top 21 most abundant genera.

Results

Microbial composition of BV positive and BV negative women

Nugent score is effective in diagnosing BV by assessing bacterial morphology using gram staining through the absence of *Lactobacillus*-dominant rods. However, with some BV-associated bacteria resulting in gram-variable staining, it is difficult to identify specific non-*Lactobacillus* taxon[108]. Similarly, the scoring system allows for intermediate diagnosing where combinatory morphology types exist. 16S sequencing allows for the complete characterization of the microbiota using deep sequencing of the 16s rRNA gene and in doing so, allows the measurements of relative abundance of bacteria taxa.

Vaginal swabs from 22 women were analyzed using 16S ribosomal RNA (rRNA) sequencing, which identified 229 different bacterial genera. 10 women were diagnosed as BV negative, with a Nugent score under 7, and 11 women were diagnosed as BV positive based on a Nugent score above 7. One woman's diagnosis was inconclusive by Nugent scoring. All of these women were healthy, HIV negative individuals. Two major bacterial community groups were identified: one in which *Lactobacillus* represented greater than 50% of the total bacterial composition (*Lactobacillus* dominant, LD, n=10

women) and the other dominated by non-*Lactobacillus* microbiota (nLD, n=12 women) (Figure 1).

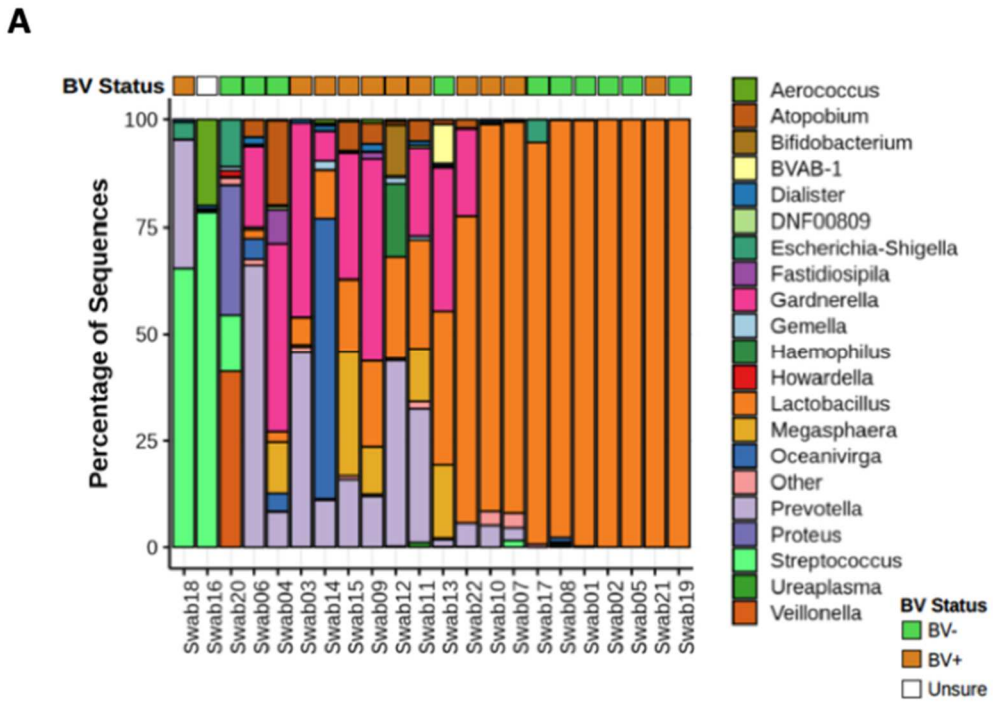

Figure 2. 1: Microbial composition from vaginal swabs

(A) Relative abundance of bacteria from vaginal swabs from women with and without diagnosed BV. The 21 most abundant genus are shown. Green, BV negative by Nugent score at time of collection; Orange, BV positive by Nugent score at time of collection; White, BV inconclusive by Nugent score at time of collection.

Effect of vaginal dysbiosis on neutrophil lifespan and accumulation

To identify the effects of vaginal microbial communities on neutrophil frequencies and lifespan, we further analyzed cervicovaginal cytobrushes using a multicolor flow cytometry-based approach to assess the frequency of neutrophils as a percentage of live CD45+ leukocytes. In our nLD samples, we observed a significantly higher frequency ($p<0.0001$) of total neutrophils amongst live CD45+ cells compared to our LD samples (Figure 2a). To assess increased neutrophil lifespan as a potential mechanism for this accumulation, we evaluated levels of active Caspase-3 and levels of CD16 in neutrophils to determine the frequency of surviving, functional neutrophils

(CD16high,Active Caspase-3Low). In our nLD samples, we observed a significant increase in the number of neutrophils that had downregulated both CD16 and Caspase 3 ($p<0.0001$) compared to LD samples (Figure 2b), indicating that they were not in apoptosis but would survive, likely the mechanism underlying accumulation in the FGT.

To further identify the effects of vaginal dysbiosis on neutrophils, leukocytes from cervicovaginal cytobrushes were analyzed using a multicolor flow cytometry staining panel designed to specifically assess neutrophil phenotype. We identified neutrophils previously reported as having suppressive function[128] by assessing the frequency of CD62LlowCD16High neutrophils. In our non-*Lactobacillus* (nLD) samples, we observed a significant increase ($p<0.0001$) in these potentially suppressive neutrophils compared to samples from women with a *Lactobacillus* dominant (LD) community (Figure 2c). As such, we also observed significantly more ($p=0.0002$) CD62LhighCD16 high neutrophils, representing a less activated neutrophil population, in the LD samples when compared to the nLD samples (Figure 2d).

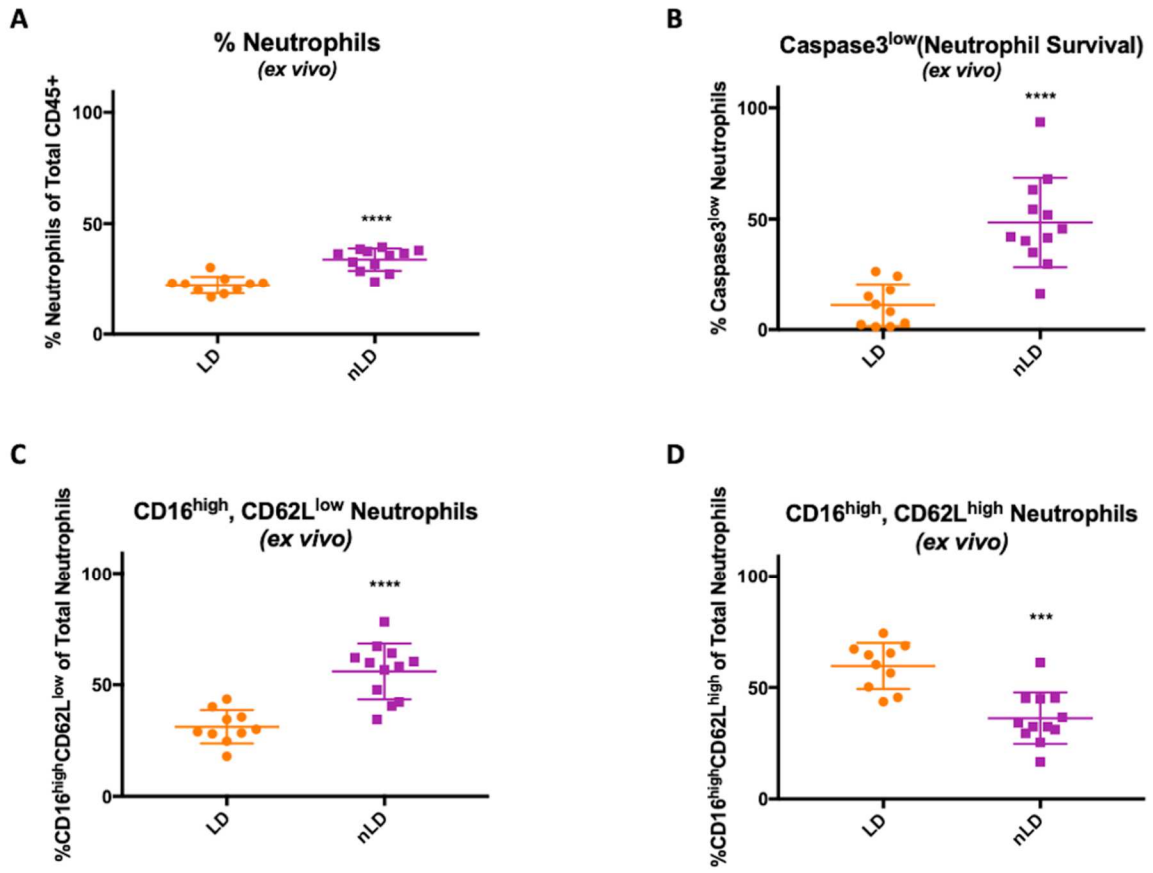

Figure 2. 2: Effects of vaginal bacteria from cervico-vaginal cytobrushes on neutrophil frequency and phenotype.

(A) Total neutrophils as a percentage of total CD45+ leukocytes from vaginal cytobrushes as measured by flow cytometry. p<0.0001.
(B) Active Caspase-3low, CD16highCD16high neutrophils as a percentage of total neutrophils from vaginal cytobrushes as measured by flow cytometry. p<0.0001.
(C) CD16high, CD62Llow neutrophils as a percentage of total neutrophils from vaginal cytobrushes as measured by flow cytometry. p<0.0001.
(D) CD16high, CD62Lhigh neutrophils as a percentage of total neutrophils from vaginal cytobrushes as measured by flow cytometry. p=0.0002.

Statistical differences in neutrophil frequencies between non-*Lactobacillus* dominant and *Lactobacillus* dominant samples were determined by Mann-Whitney test with a p-value<0.05 considered significant. Lines represent mean and SD.

Bacteria associated with vaginal dysbiosis impact neutrophil lifespan and accumulation

Given the important interactions between bacteria and neutrophils, we hypothesized that the mechanism underlying increased neutrophil lifespan and accumulation was dysbiotic bacteria. To test this, we cultured whole blood from healthy individuals (n=6) with

bacteria associated with BV and non-BV vaginal microbiomes and assessed neutrophils via flow cytometry after co-culture. *G. vaginalis, P. bivia,* and *A. vaginae* were used to represent bacteria associated with BV and *L. iners* and *L. crispatus* represented non-dysbiotic bacteria. Lipopolysaccharide (LPS) and peptidoglycan (PGN) were used as positive controls for gram negative and gram-positive bacteria, respectively. We observed a higher frequency of neutrophils (percentage of live CD45+ leukocytes) in co-cultures with *G. vaginalis, P. bivia,* and *A. vaginae* compared to samples cultured with *L. crispatus* ($p<0.0001$, $p<0.0001$, and $p=0.0018$, respectively) and *L. iners* ($p<0.0001$, $p=0.0015$, and $p=0.0457$, respectively) (Figure 3a). Neutrophil frequencies in the samples cultured with *G. vaginalis, P. bivia,* and *A. vaginae* were similar to the LPS ($p=0.9616$, $p=0.9987$, and $p=0.5930$, respectively) and PGN positive controls ($p>0.9999$, $p=0.7947$, $p=0.1344$, respectively), and neutrophils frequencies in samples with *L. iners* and *L. crispatus* were similar to the negative control ($p>0.9999$, $p=0.9824$, respectively). We observed no significant difference between samples co-cultured with *L. iners* and *L. crispatus* ($p=0.9321$).

Additionally, we assessed the impact of these specific bacteria on neutrophil lifespan by measuring non-apoptotic, functional neutrophils (active Caspase-3 low, CD16 high, Figure 3b). Consistent with our observations *in vivo*, we observed a higher percentage of non-apoptotic neutrophils in samples co-cultured with *G. vaginalis, P. bivia,* and *A. vaginae* compared to samples cultured with *L. crispatus* ($p=0.0021$, $p=0.0126$, and $p=0.0452$, respectively) and *L. iners* ($p=0.0010$, $p=0.0067$, and $p=0.0255$, respectively). The neutrophils from samples with *G. vaginalis, P. bivia,* and *A. vaginae* were similar to the positive controls LPS ($p>0.9999$, $p=0.9995$, and $p=0.9709$, respectively) and PGN positive controls ($p>0.9999$, $p=0.9999$, $p=0.9844$, respectively), while neutrophils from samples with *L. crispatus* and *L. iners* were comparable to the negative control

(p>0.9999, p>0.9999, respectively). Additionally, there was no significant difference between samples cultured with *L. crispatus* and *L. iners* (p>0.9999). Thus, supporting our observations *in vivo*, these data demonstrate that dysbiotic bacteria can directly promote neutrophil survival and may underlie accumulation of neutrophils.

We also assessed the functional phenotypes of neutrophils. In the samples co-cultured with *G. vaginalis, P. bivia,* and *A. vaginae*, the frequency of CD62lowCD16high neutrophils was similar to those observed for the positive controls LPS (p>0.9999, p=0.5031, and p=0.1944, respectively) and PGN (p=0.8926, p>0.9999, and p=0.9826, respectively). We did, however, identify an increase in CD62lowCD16 high neutrophils when compared to neutrophils in samples co-cultured with *L. crispatus* (p<0.0001, p<0.0001, and p=0.0004, respectively) and *L. iners* (p<0.0001, p=0.0059, and p=0.0293, respectively) indicating neutrophils in the presence of BV associated bacteria are more prone to suppressive functionality (Figure 3c). The *L. crispatus* and *L. iners* were similar to negative controls (p>0.9999 and p=0.8099, respectively), and we observed no significant difference between *L. crispatus* and *L. iners* (p=0.7876).

We also observed more CD62LhighCD16high, non-activated neutrophils in our samples co-cultured with *L. crispatus* and *L. iners* compared to samples co-cultured with *G. vaginalis* (p<0.0001 and p<0.0001, respectively), *P. bivia* (p<0.0001 and p<0.0001, respectively), and *A. vaginae* (p<0.0001 and p<0.0001, respectively) (Figure 3d). Similarly, neutrophils from co-cultures with *G. vaginalis*, *P. bivia,* and *A. vaginae* were similar to the positive controls LPS (p=0.8281, p=0.8182, and p=0.6745, respectively) and PGN (p=0.8934, p=0.7356, and p=0.5782, respectively) while *L.crispatus* and *L. iners* samples resembled the negative controls (p=0.9942 and p>0.9999, respectively). We observed no difference in the non-activated neutrophils in samples co-cultured with

L. crispatus and *L. iners* (p=0.9996). Collectively, these data demonstrate that dysbiotic bacteria can directly induce the same phenotype of neutrophils observed in women with BV.

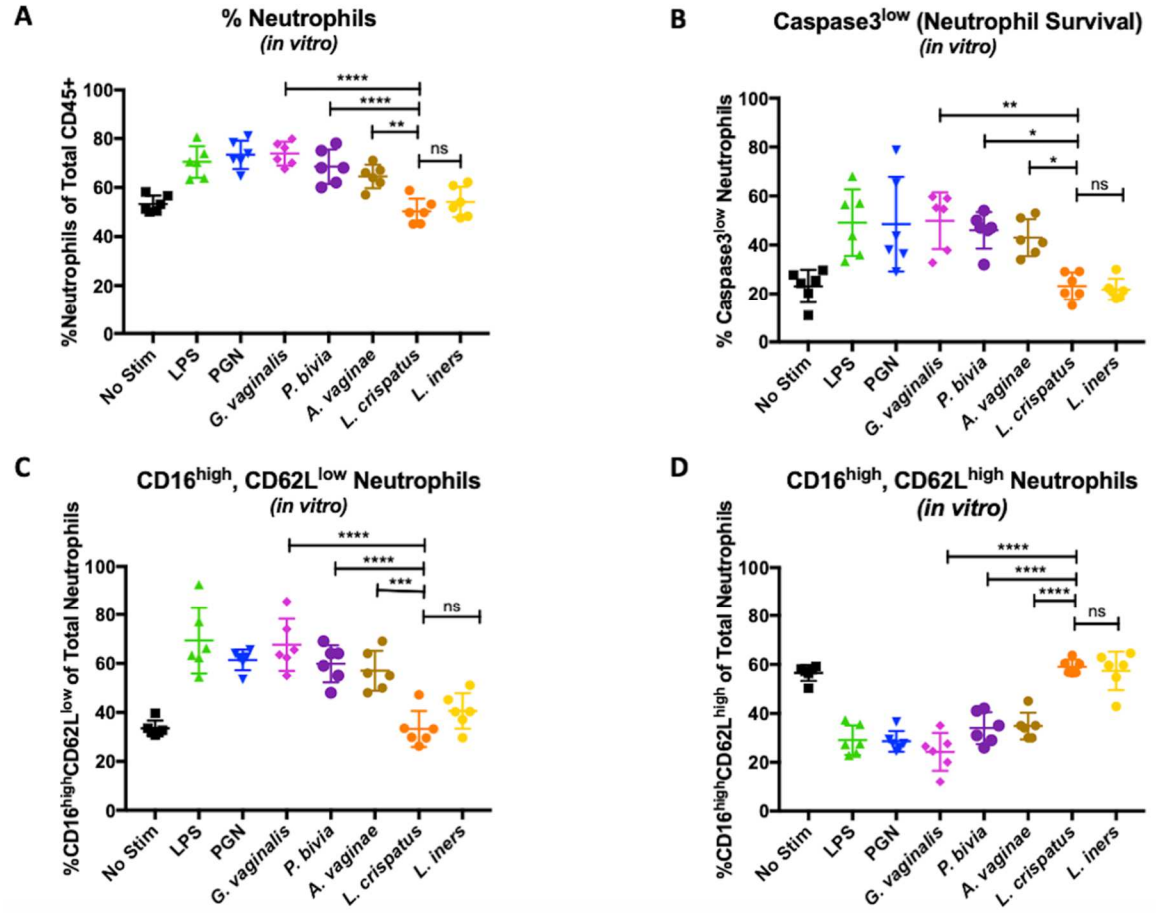

Figure 2. 3: Effects of vaginal bacteria on neutrophil frequency and phenotype.

(A) Total neutrophils as a percentage of total CD45+ leukocytes from whole blood as measured by flow cytometry. p<0.0001, p<0.0001, p=0.0018, p=0.9321.
(B) Active Caspase-3low, CD16high as a percentage of total neutrophils from whole blood as measured by flow cytometry. p=0.0021, p=0.0126, p=0.0452, p>0.9999.
(C) CD16high, CD62Llow neutrophils as a percentage of total neutrophils from whole blood as measured by flow cytometry. p<0.0001, p<0.0001, p=0.0004, p=0.7876.
(D) CD16high, CD62Lhigh neutrophils as a percentage of total neutrophils from whole blood as measured by flow cytometry. p<0.0001, p<0.0001, p<0.0001, p=0.9996.

Each symbol represents a biological replicate. Statistical differences in neutrophil frequencies between experimental conditions were determined by ANOVA with a p-value<0.05 considered significant. Lines represent mean and SD. Additional statistical differences are listed below.

Comparison for (A)	Summary	P value
L. crispatus vs. No Stim	ns	p=0.9824
L. crispatus vs. LPS	****	p<0.0001

L. crispatus vs. PGN	****	p<0.0001
L. iners vs. No Stim	ns	p>0.9999
L. iners vs. LPS	***	p=0.0002
L. iners vs. PGN	****	p<0.0001
L. iners vs. *G. vaginalis*	****	p<0.0001
L. iners vs. *P. bivia*	**	p=0.0015
L. iners vs. *A. vaginae*	*	p=0.0457

Comparison for (B)	Summary	P value
L. crispatus vs. No Stim	ns	p>0.9999
L. crispatus vs. LPS	**	p=0.0029
L. crispatus vs. PGN	**	p=0.0040
L. iners vs. No Stim	ns	p>0.9999
L. iners vs. LPS	**	p=0.0015
L. iners vs. PGN	**	p=0.0021
L. iners vs. *G. vaginalis*	**	p=0.0010
L. iners vs. *P. bivia*	**	p=0.0067
L. iners vs. *A. vaginae*	*	p=0.0255

Comparison for (C)	Summary	P value
L. crispatus vs. No Stim	ns	p>0.9999
L. crispatus vs. LPS	****	p<0.0001
L. crispatus vs. PGN	****	p<0.0001
L. iners vs. No Stim	ns	p=0.8099
L. iners vs. LPS	****	p<0.0001
L. iners vs. PGN	**	p=0.0023
L. iners vs. *G. vaginalis*	****	p<0.0001
L. iners vs. *P. bivia*	**	p=0.0059
L. iners vs. *A. vaginae*	*	p=0.0293

Comparison for (D)	Summary	P value
L. crispatus vs. No Stim	ns	p=0.9942
L. crispatus vs. LPS	****	p<0.0001
L. crispatus vs. PGN	****	p<0.0001
L. iners vs. No Stim	ns	p>0.9999
L. iners vs. LPS	****	p<0.0001

L. iners vs. PGN	****	p<0.0001
L. iners vs. *G. vaginalis*	****	p<0.0001
L. iners vs. *P. bivia*	****	p<0.0001
L. iners vs. *A. vaginae*	****	p<0.0001

Bacteria associated with vaginal dysbiosis impact epithelial barrier integrity

To determine if activated neutrophils with reduced homeostatic apoptosis can damage epithelial barrier integrity, we isolated neutrophils from whole blood from healthy individuals and co-cultured them with BV-associated bacteria and non-BV associated bacteria as described above. Transepithelial electrical resistance (TEER) is a widely used method to functionally analyze tight junctions within physiological barriers of cell culture models and measures electrical resistance across a cellular monolayer. Combined with Lucifer Yellow transport assay, tight junction formation was verified and allowed us to assess barrier function in the presence of these neutrophil/bacteria co-cultures. Following incubation, we saw a significant decrease in fold change in wells containing the BV associated bacteria: *G. vaginalis, P. bivia,* and *A. vaginae* when compared with wells just containing *L. iners* ($p<0.0001$, $p<0.0001$, and $p=0.0026$, respectively) and *L. crispatus* ($p=0.0001$, $p=0.0001$, and $p=0.0104$, respectively) (Figure 4a). Differences in changes in TEER values were negligible between wells containing neutrophils only and wells with just *L. crispatus* or *L. iners* ($p=0.2773$ and $p=0.5439$, respectively) (Figure 4a). We saw no statistically significant difference when comparing the different *Lactobacillus* species ($p=0.9993$). We observed a significant decrease in fold change in TEER value in wells containing neutrophils and *G. vaginalis, P. bivia,* and *A. vaginae* compared to co-cultures containing neutrophils and *L. iners* ($p<0.0001$, $p<0.0001$, and $p<0.0001$, respectively) and *L. crispatus* ($p<0.0001$, $p<0.0001$, and $p<0.0001$ respectively) (Figure 4b). We observed no significant difference between wells containing neutrophils and two different *Lactobacillus* species ($p=0.9904$).

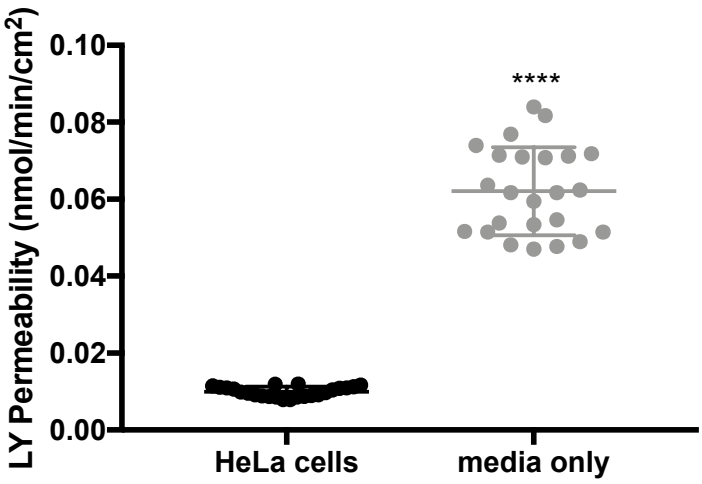

Figure 2. 4: Luciferase Yellow Permeability

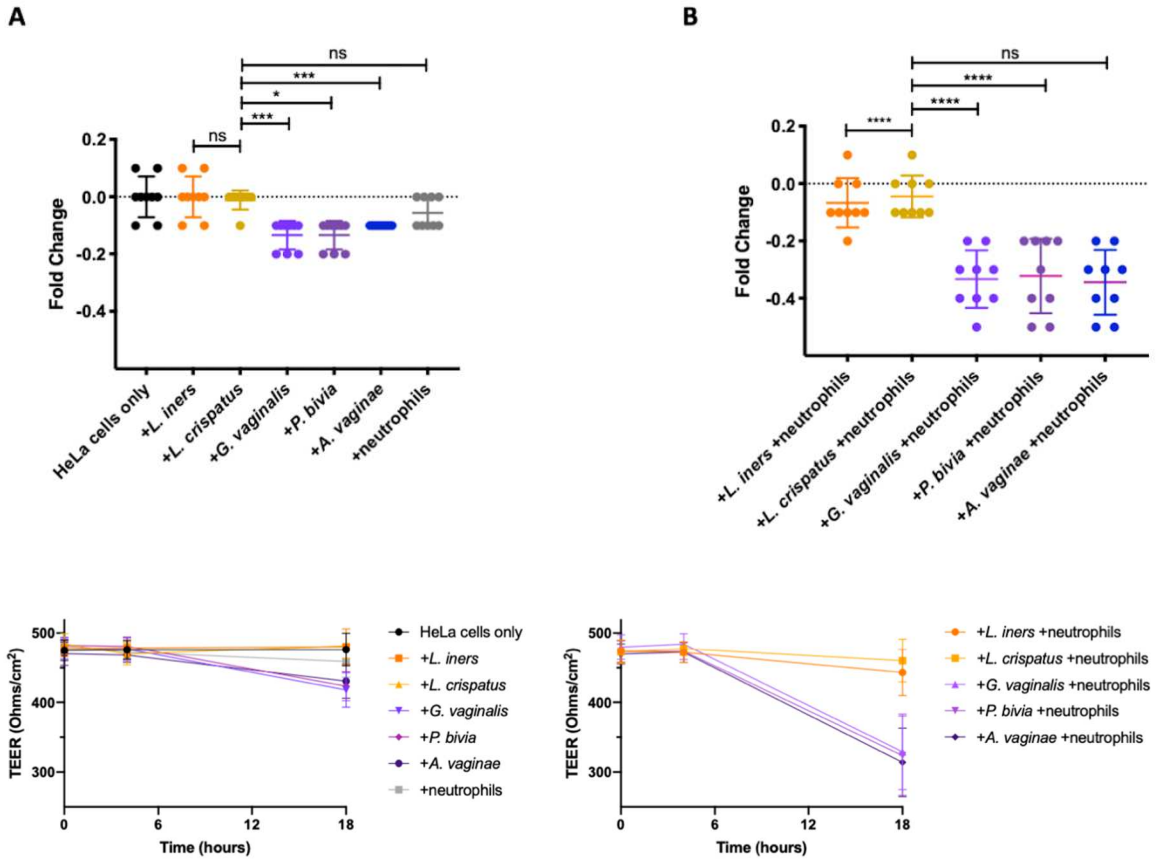

Figure 2. 5: Effects of vaginal bacteria and neutrophils on HeLa cells using a TEER breakdown assay.

(A) Fold change from baseline of TEER breakdown assay in the presence of neutrophils or vaginal bacteria. p=0.9993, p<0.0001, p<0.0001, p=0.0026, p=0.2773.
(B) Fold change from baseline of TEER breakdown assay in the presence of neutrophils and vaginal bacteria. p=0.9904, p<0.0001, p<0.0001, p<0.0001.
(C) Time course of TEER breakdown in the presence of neutrophils or vaginal bacteria.

(D) Time course of TEER breakdown in the presence of neutrophils and vaginal bacteria.

Statistical differences in TEER values between experimental conditions were determined by ANOVA with a p-value<0.05 considered significant. Lines represent mean and SD.

Discussion

This study provides a detailed characterization of the impact of the vaginal microbiome on neutrophils in the female reproductive tract and the potential role of neutrophils in barrier damage. Using ex-vivo cytobrushes and vaginal swabs, we confirmed that neutrophil phenotype is altered in women with dysbiosis and the mechanisms by which this occurs. Indeed, we found that increased neutrophil frequency is associated with decreased apoptosis and altered functionality. Furthermore, we identified via co-culture systems that the mechanism underlying the alterations in neutrophils is microbial dysbiosis, as we show dysbiotic bacteria can directly induce the equivalent alterations in neutrophils.

Neutrophils are critically important for pathogen protection; however recent studies have summarized the importance of balance within neutrophil function[13,118,129-133]. For example, studies have linked increased neutrophil proteases with increased inflammatory cytokines and with barrier function[14,134-137]. Furthermore, studies have even identified specific immune signatures from vaginal epithelial cells elicited from specific species of BV-associated bacteria, including *Prevotella bivia* and *Atopobium vaginae*[138-140]. Our data allude to specific host responses to pathogenic bacteria, such as neutrophil accumulation, as a mechanism for increased HIV susceptibility during BV. This study expands on previous work identifying CD16 downregulation consistent with prolonged neutrophil survival and activity within the vagina of women with vaginitis[141]. Uncontrolled, neutrophils may cause tissue damage through the release of reactive oxygen species as

well as neutrophil proteases, both of which may damage the mucosal barrier and contribute to increased susceptibility to HIV and other STIs.

Our study also highlights the impact vaginal dysbiosis can have on neutrophil lifespan and accumulation. Maintaining the sensitive balance of neutrophils requires controlled neutrophil clearance. Typically, neutrophils have a relatively short half-life ranging from 8 hours to 5 days[142]. To prevent unintended tissue damage, neutrophils undergo apoptosis to control their accumulation[143]. Caspase-3 mediates the final steps of apoptosis[143], and the decreased frequency of neutrophils expressing active caspase-3 in the tissue in women with nLD vaginal microbiota highlights the impact vaginal dysbiosis can have on neutrophil lifespan. Additionally, we observed increases in total neutrophils in the *in vitro* cultures with *G. vaginalis* and in the cytobrushes form women with vaginal dysbiosis. Therefore, these data and the supporting *in vitro* data demonstrate that pathogenic bacteria are likely the mechanism underlying neutrophil accumulation in the FGT.

Recent evidence has shown that neutrophils can act as myeloid-derived suppressor cells (MDSCs) with the ability to suppress the adaptive immune response[144]. These suppressive neutrophils are associated with T cell exhaustion[145] and may contribute to diseases such as HIV infection, and may be important in promoting the latent HIV-1 reservoir[146].[147]. MDSCs have shown evidence of decreasing T cell function[148] and that MDSC expansion contributes to immune suppression through cytokine and cellular responses[149]. Here, using primary cervicovaginal cytobrushes, we confirmed that nLD communities are associated with an increase in neutrophils with a suppressive phenotype. Our *in vitro* experiments demonstrated that specific taxa, such as *G. vaginalis*, *P. bivia,* and *A. vaginae* can also drive this phenotype, and our 16S analysis and *in vitro* experiments indicated that healthy commensal *Lactobacillus spp.* are less

likely to induce a suppressive phenotype. Together, these data provide evidence that BV-associated bacteria may be contributing to an altered neutrophil response that may weaken the local immune system, further contributing to increased susceptibility to infection.

Of note, the similar neutrophil phenotypes observed in response to culture with *L. iners* and *L. crispatus* was particularly interesting given that *L. iners* has been previously demonstrated to be more inflammatory[107]. Maintaining the balance between anti-microbial function while preventing uncontrolled inflammation is critical, particularly in the context of HIV transmission in women with BV. These data allude to the activation of neutrophils and the favoring of a suppressive phenotype as a potential mechanism for how BV-associated bacteria increase HIV and STI transmissions through the role of neutrophils within the FRT. Further studies are needed to elucidate the exact mechanisms by which these BV associated bacteria delay apoptosis and induces phenotypic changes in neutrophils.

Importantly, this study is the first to directly demonstrate the ability of specific vaginal bacteria to reduce epithelial barrier integrity through neutrophils. Epithelial cells form dense layers with tight cell to cell junctions when cultured in-vitro[150]. Using our TEER system, we identified decreased barrier function in the presence of neutrophils and *G. vaginalis, P. bivia,* and *A. vaginae*. Typically, pathogens invade and traverse the mucosal epithelium resulting in damaging intercellular junctions. The decrease observed in wells with these BV associated bacteria provide evidence for specific pathogenic vaginal bacteria contributing in invading the mucosal epithelium. While the combination of neutrophils and BV associated bacteria induce the most significant damage to the barrier (Figure 4B), it is noteworthy that the presence of BV bacteria alone do induce

mild changes in TEER values (Figure 4A). Due to this, we cannot dissect specifically if it is the bacteria themselves or neutrophils that are inducing the additional damage observed when co-cultured together versus when cultured individually. Future studies to dissect the cause of the increased damage could include measuring neutrophil protease activity in the presence and absence of BV associated bacteria and whether these neutrophil proteases alone could induce TEER changes. A previous study from our lab similarly identified *G. vaginalis*' impact on wound healing and how toxins produced from *G. vaginalis* significantly reduce wound healing after 24 hours[151].

Combined with damage caused by neutrophil proteases[14], our study provides evidence of a potential mechanism for how BV increases HIV and STI susceptibility through promoting damage to the tight epithelial barrier.

Lastly, our study provides additional evidence for the need to improve diagnostic tools for BV. Amongst our 22 samples, we identified four women who were diagnosed with BV by Nugent score yet had *Lactobacillus* dominant communities by 16S sequencing. Similarly, we identified four women that were not diagnosed with BV by Nugent score, but had highly diverse, non-*Lactobacillus* dominant communities that represent molecular BV and contribute to increased STI and HIV infections. Further studies are needed to elucidate the observed difference between current BV diagnosis methods and 16S rRNA sequencing, and how these differ in resulting BV symptoms, inflammation, and HIV/STI risk[152].

Limitations of this study include the use of *ex vivo* samples from women that were not screened for STIs. STIs are known to cause increased inflammation[153] and could contribute to alterations in neutrophil phenotypes. Additionally, we focused on

representative bacteria in our *in vitro* experiments and further studies investigating the effect of other bacteria associated with BV, and potentially mixed microbial populations, on neutrophils and barrier damage would further increase our understanding of how the complex vaginal microbiome and neutrophils together contribute to mucosal barrier damage and transmission risk. These additional studies would further elucidate the mechanisms by which BV alters female health.

Conclusion

Overall, this study provides increased understanding of relationships between neutrophils, vaginal dysbiosis, and the mucosal barrier and elucidates potential mechanisms of increased HIV and STI susceptibility in women with BV. Women's health is vastly understudied and understanding the role of BV in HIV and STI acquisition is critical.

Declaration

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Author Contributions

R.K.C. and C.J.M designed and performed all in vitro experiments. R.K.C., T.H.M, N.R.K. analyzed all flow data. A.M., M.Y., and R.K collected all ex-vivo cytobrushes and performed flow cytometry. C.B. and C.B.D. performed all 16S rRNA sequencing experiments and analyzed the data. R.K.C., T.H.M., R.K., and N.R.K. edited and wrote the manuscript. R.K. lead all clinical studies. N.R.K. designed and lead the overall study.

Competing Interests

The authors have no competing interests.

Chapter III

Vaginal bacteria modify HIV tenofovir microbicide efficacy in African women

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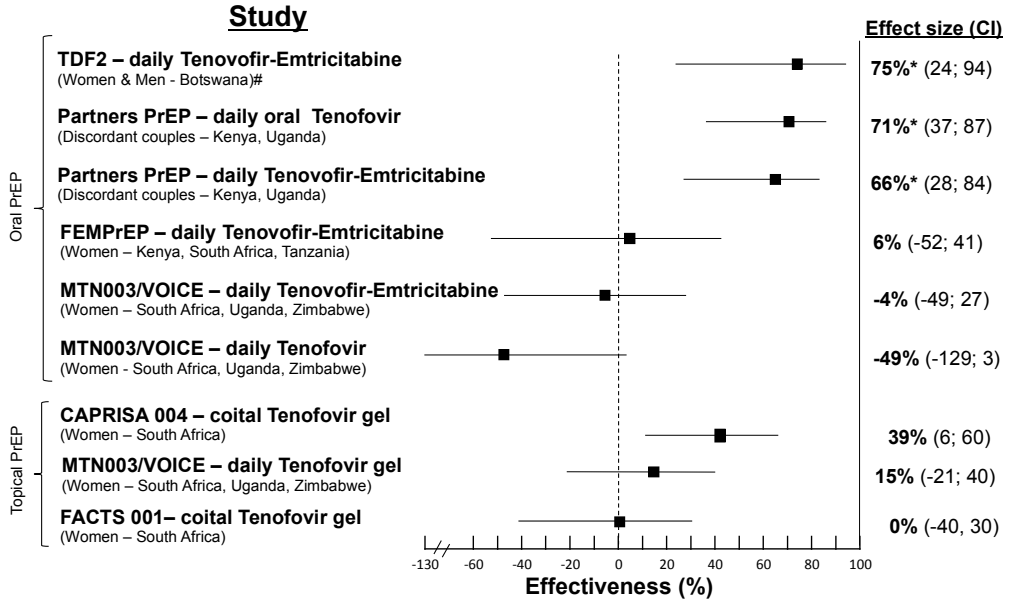
Introduction

More than one million women are infected with HIV annually, and the majority of these new infections occur in young women in sub-Saharan Africa, with South Africa having amongst the highest incidence rates [154,155]. Antiretroviral-based clinical trials in men who have sex with men (MSM) have consistently demonstrated effectiveness in preventing HIV infection [69,156,157], however studies in women have produced widely varying results. In clinical trials of women, the efficacy of antiretroviral drugs to prevent HIV infection ranged from -49% (VOICE) to 75% (TDF2) for daily oral tenofovir or tenofovir-

emtricitabine and from 0% (FACTS 001) to 39% (CAPRISA004) for daily or coital vaginally-applied tenofovir gel (Fig. 3.1). Variability in the levels of adherence [158] has been shown to be a major contributing factor for the diverse trial outcomes in women. However, little is known about what biological factors may also contribute to the variability in these results, and why higher adherence is required for antiretroviral-based prevention efficacy in women [159].



#(Study population and countries where the study was conducted)
**Effect size calculated from the incidence rate ratio for women only*

Figure 3. 1: Results of all Placebo-controlled Oral and Topical Pre-exposure Prophylaxis (PrEP) Trials in Women Testing Tenofovir or Tenofovir-emtricitabine for Protection from HIV Infection. Data was generated from these references [69,160-165].

The vaginal compartment contains many microbial species critical for the health of the vaginal mucosa, and dysbiosis of vaginal bacteria, clinically known as bacterial vaginosis (BV), can result in severe health consequences [166,167]. The recent advent of advanced molecular tools has redefined our understanding of vaginal bacteria communities [168,169], where the most frequently observed "community state types" (CST) have been

described [7,169-171]. Although significant heterogeneity exists, a key commonality is that CST fall into two clear groups: 1) *Lactobacillus*-dominant, where one or more species of *Lactobacillus* make up >90% of total copy number or sequencing reads,(*L. iners*, *L. crispatus*, *L. jensenii* and *L. gasseri*), and 2) non-*Lactobacillus* dominant, with *Lactobacillus* making up <30% of total copy number or sequencing reads. The non-*Lactobacillus* dominant group typically contains high abundance of *Gardnerella vaginalis* alone or co-dominant with other facultative and obligate anaerobic bacteria including *Prevotella* ssp., *Mobiluncus* ssp. and/or several *Clostridia* species. BV occurs following a shift from *Lactobacillus*-dominate to these more diverse communities [169,170], and is often asymptomatic, and can often go undiagnosed using traditional Amsel's criteria [172] and/or the Nugent's score used to diagnose BV [173].

Bacterial vaginosis is associated with poor reproductive health outcomes and increased HIV infection risk in women [174], by as much as 60% in some meta-analyses of women with BV [175]. BV likely increases HIV risk through multiple mechanisms, including increased inflammation and target cells, as well as vaginal epithelial barrier disruption and wound-healing impairment, however the mechanisms are not entirely understood [7,176,177]. Given that women from sub-Saharan Africa have high prevalence rates of bacterial vaginosis [116], and that HIV prevention strategies are being targeted for women in these areas, it raises the question of whether and how vaginal microbial communities may impact the efficacy of antiretroviral based prevention technologies, especially those which are topically applied to the vaginal surface.

Characterization of the vaginal microbiome using unbiased metagenomic, metatranscriptomic, and metaproteomic approaches represent a potential paradigm shift in understanding host-microbial interactions at the mucosal surface *in vivo*. We recently

utilized metaproteomics to gain insight into host-bacterial interactions in vaginal microbial dysbiosis [177]. As this method simultaneously collects unbiased information on microbial and host proteomes, this provides systems level information on microbial communities and mucosal surfaces not available with other techniques.

Here we utilized a metaproteomic approach to assess whether vaginal bacteria modulate the efficacy of the topical microbicide tenofovir in preventing HIV infection, and *in vitro* systems to determine mechanisms of microbiome influence on tenofovir.

Vaginal microbial diversity in women using tenofovir or placebo microbicide gels

Samples from 688 HIV-negative women that were assigned to either tenofovir or the placebo gel arm, were analyzed by protein mass spectrometry as outlined in the Materials and Methods section (Fig. 3.2). Proteomic analysis identified 3,334 unique bacterial proteins from 188 different species in the cervicovaginal lavage (CVL) of 688 women. Two major vaginal bacterial community groups were identified, including one where *Lactobacillus* was the predominant genus (Group I) (n=423, 61.5%) and the other dominated by non-*Lactobacillus* microbiota (Group II) (n=265, 38.5%) (Fig. 1). Approximately 11% of individuals had no single dominant species (defined as >50% community composition), and the majority of these individuals fell into group II.

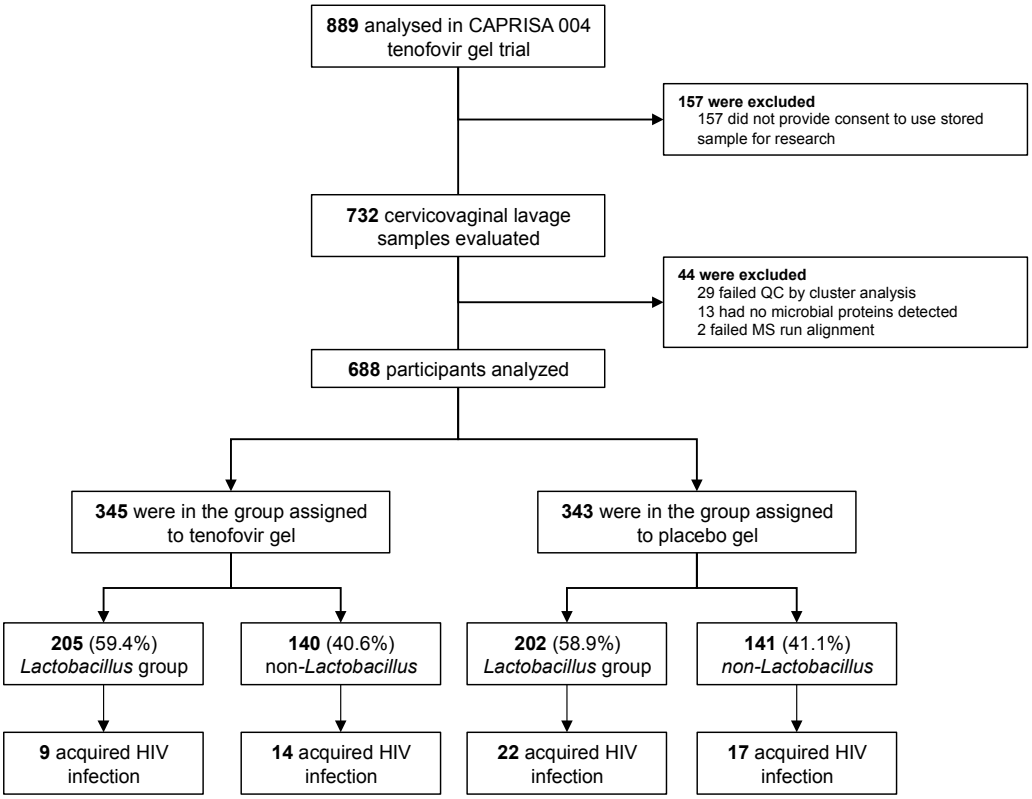


Figure 3. 2: Study Design and Metaproteomic Analysis of CAPRISA 004 Participants

In comparing the mass spectrometry proteomic approach with 16S rRNA sequencing, we found concordance with respect to classifying women into Group I and II (91.5% agreement) and measurements of bacterial abundance, including major taxa *Lactobacillus*, *G. vaginalis*, *Prevotella,* and others (P<0.001) (Fig. 3.3). A limitation of this study was that BV data was not collected during the CAPRISA 004 trial, and thus precluded comparisons to Nugent score or other BV criteria.

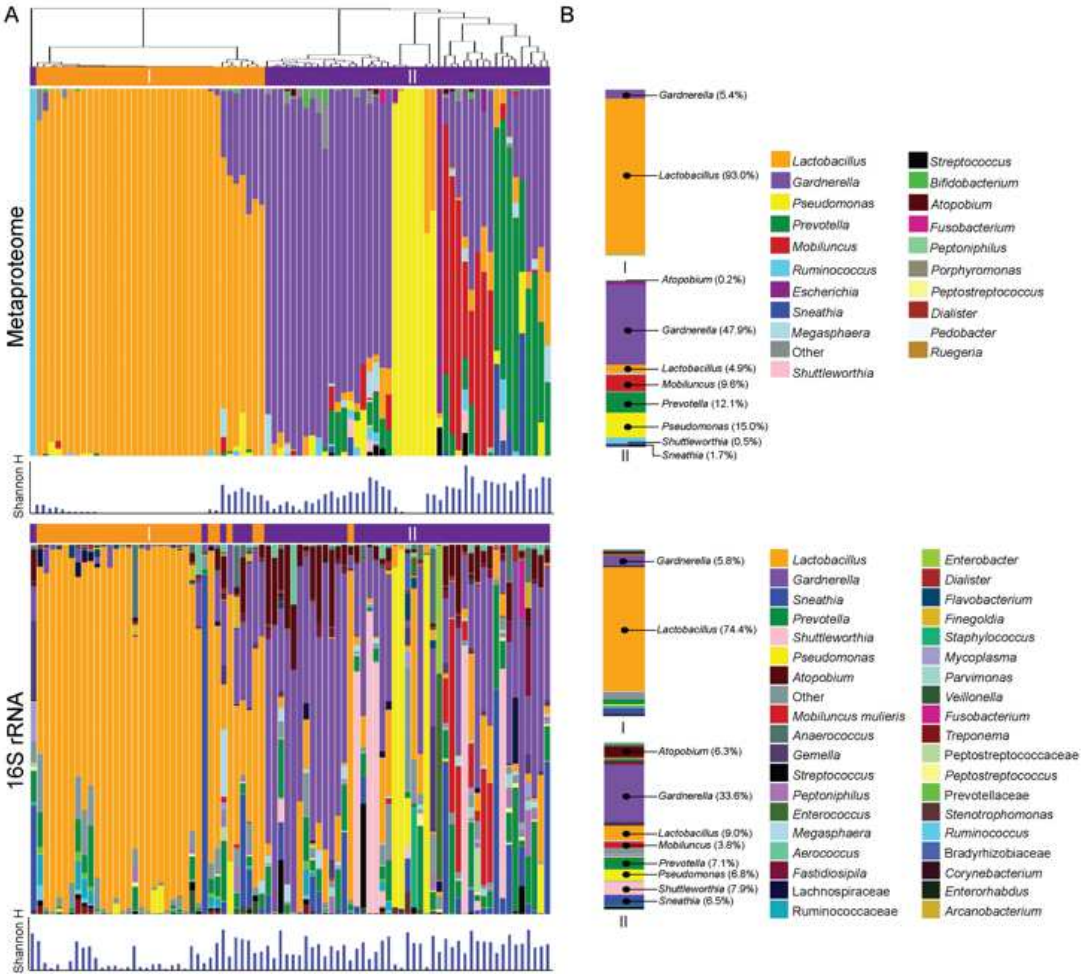


Figure 3. 3: Composition of Mass Spectrometry and 16S rRNA Gene Sequence Analysis of Cervicovaginal Secretions to Characterize Bacterial Proportions

(A) Bacterial diversity plots of major genera detected using mass spectrometry (MS) (top) and 16S rRNA gene sequencing (bottom).

(B) Average bacterial community group structure for each of the two major profiles - Group I (*Lactobacillus* dominant) and Group II (non-*Lactobacillus* dominant). Samples analyzed by MS underwent unsupervised hierarchical clustering (average Euclidean linkage as the distance metric) using bacterial proportions and subsequently matching the 16s rRNA painter's plots (shown below). Classification of samples as either Group I or Group II showed 91.5% overlap by either technique. In Group I, *Lactobacillus spp.* comprised 93.0% and 74.4% of the proteome and 16S rRNA reads, respectively, while in the non-LD group, *G. vaginalis* was predominant comprising 47.9% and 33.6% of the proteome and 16S rRNA reads, respectively. While a higher number of major taxa (>0.1%) were observed at the 16S (36) than the protein level (20), increased diversity in Group II over Group I are apparent by either method (H index (MS): 0.69 vs. 0.19 (P<0.0001); H index (16S): 1.42 vs. 0.80 (P<0.0001), respectively). Spearman correlation analysis showed close concordance between the two methods to quantify predominant taxa proportions (those >5.0% detected by either method), including *Lactobacillus* (r=0.75, P<0.0001), *Gardnerella* (r=0.79, P<0.0001), *Mobiluncus* (r=0.52, P<0.0001), *Prevotella* (r=0.35, P=0.0012), and *Pseudomonas* (r=0.36, P=0.0008). Milder correlations were observed with *Shuttleworthia* (r=0.28, P=0.01), S*neathia* (r=0.23, P=0.01), and *Atopobium* (r=0.27, P=0.01), which were detected at higher levels by 16S.

Group I showed the lowest diversity (Shannon H diversity index median: 0.05) with the majority (70.5%) having *L. iners* as the dominant species, followed by *L. crispatus* (15.1%), and other lactobacilli, such as *L. jensenii,* and *L. gasseri* (3.0%). In Figure 3.4B, the first subgroup (A) was homogeneous with clear dominance of *Lactobacillus* (54.7%) with very low diversity (H-index median: 0.035), while a minority of these individuals (B, 6.8%), showed an intermediate amount of diversity (H-index median: 0.87) where small amounts of *G. vaginalis*, *Pseudomonas*, and other bacteria were detected. A sub-analysis of Group IA at the *Lactobacillus* species level clearly illustrates *L. iners* as predominant.

Women in Group II had overall higher bacterial diversity (H-index: 0.78) with several distinct subgroups. The largest subgroup (C) was dominated by *G. vaginalis* (n=163, 23.7%, H-index: 0.66) that contained multiple taxa including, *Prevotella,* and minor amounts of *Lactobacillus* and *Mobiluncus* (Fig. 3.4B). The second largest subgroup (D) was the most diverse and had no clearly dominant taxa (n=78, 11.3%, H-index: 1.15), where *G. vaginalis, Prevotella* and *Mobiluncus* were predominant. Finally, the smallest subgroup (E) was low diversity containing either *Pseudomonas or Escherichia* (n=24, 3.5%, H-index: 0.14) (Fig. 3.4B). Grouping sub-groups A and B, which showed relative homogeneity in a single group (Group I), with the remaining 3 sub-groups C, D and E, which had more variability and diversity into a single group (Group II) was supported by principal component analysis (Fig. 3.4C). Overall the majority (96.2%, n=407) of women in Group I had >50% *Lactobacillus* by abundance.

For downstream comparisons of topical tenofovir efficacy, we chose a *Lactobacillus* dominant (>50%) (LD) or non-*Lactobacillus* dominant ($\leq$50%) (non-LD) classification, as >50% *Lactobacillus* at proteome level corresponds with a practical clinical marker of

vaginal pH below 4.5 (interquartile range [IQR]) pH value of 4.0-4.5 [177]); and it accurately identified 96.2% of Group I individuals. Importantly utilizing LD and non-LD definitions did not significantly change any outcomes reported based on Group I vs. Group II comparisons.

LD and non-LD women had similar baseline clinical, behavioral, and demographic characteristics as well as sexual behavior and gel adherence during the trial. Presence of an STI, which included *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Neisseria gonorrhea, Mycoplasma genitalium*, *Treponema pallidum*, and HSV-2 were not different between groups. Although not statistically significant, women within the non-LD group were marginally younger (mean age 23.6 vs. 24.1 years, P=0.092). Within each of the groups of LD and non-LD women, women assigned to tenofovir gel and placebo gel was similar.

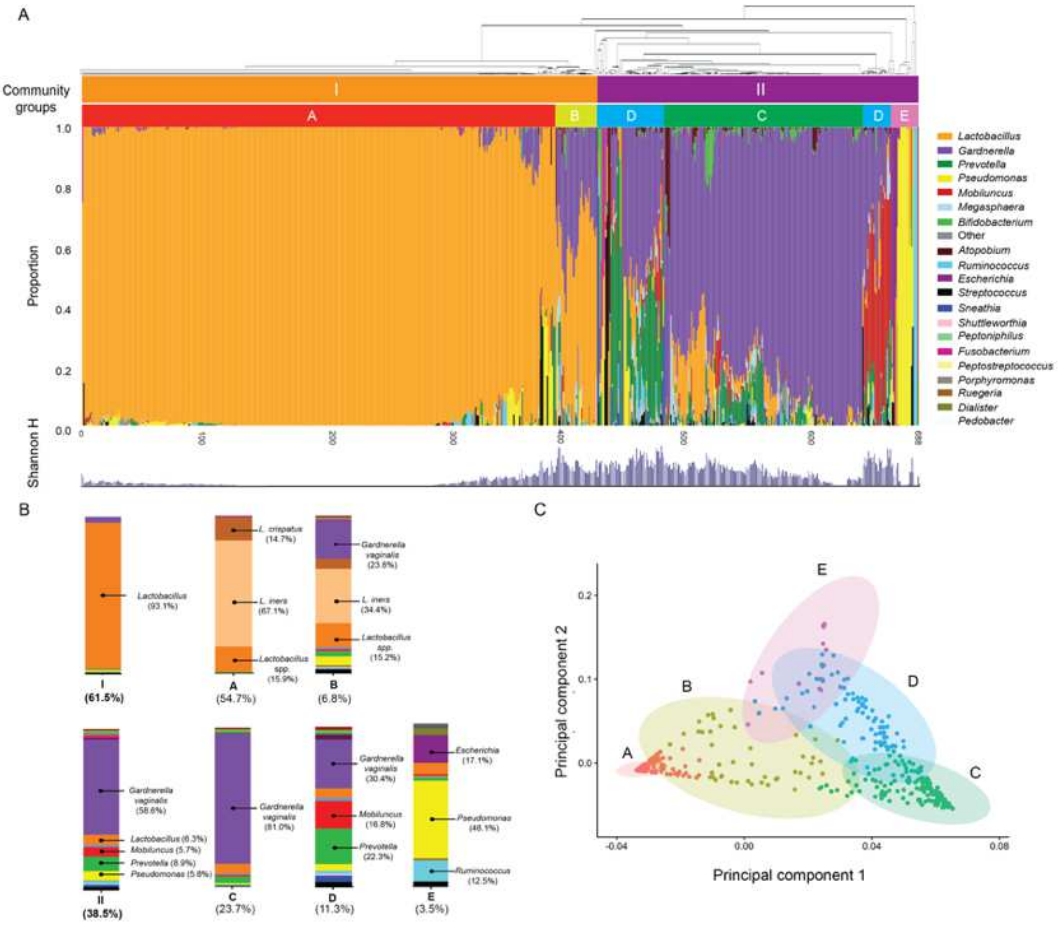


Figure 3. 4: Bacterial Profiling by Mass Spectrometry using Cervicovaginal Lavage Samples from 688 Women from the CAPRISA 004 Trial

(A) Overall bacterial diversity plot of major genera of all women profiled.
(B) Average bacterial community group structure for each of the two major profiles - group I (A, B) and group II (C, D, E).
(C) Principal component analysis of 688 women using bacterial proportion data showing the 5 subgroups.

Vaginal microbial profiles and tenofovir gel efficacy

In the LD group, the HIV incidence rate was 61% lower (95% CI: 0.16-0.89) in women assigned to tenofovir gel compared to women assigned to the placebo gel (2.7 vs 6.9 per 100 women-years; IRR=0.39; *P*=0.013) (Fig. 3.5A). In contrast, in non-LD women, the HIV incidence rate was only 18% lower (95% CI: 0.37-1.77) in those assigned to tenofovir gel compared to those assigned to placebo gel (6.4 vs. 7.8 per 100 women-years; IRR=0.82; *P*=0.644) (Fig. 3.5B). Adjusting for STIs, HSV-2 infection, antibiotic

usage, DMPA usage, and sexual behaviours (frequency of sex, number of partners, condom usage) did not impact these findings.

Lactobacillus, particularly *L. crispatus*, has been associated with reduced HIV infection. Although the HIV incidence rate of 4.8 per 100 women-years in LD women was 32% lower (95% CI: 0.4-1.12) than the 7.1 per 100 women-years in non-LD women, this was not statistically significant (*P*=0.127). Comparing the sub-group of all 63 *L. crispatus* dominant (>50% abundance) to non-LD women showed a 57% lower HIV incidence (95% CI: 0.13-1.41) that was not statistically significant (3.1 vs. 7.1 per 100 women-years; *P*=0.167). Comparing *L. crispatus* dominant women to all others as a single group (*L. iners* dominant and non-LD women) had similar findings (50% lower HIV incidence; 3.1 vs. 6.0 per 100 women-years; 95% CI: 0.16-1.60 *P*=0.237). Similarly, within the placebo group, the 31 *L. crispatus* dominant women compared to non-LD women showed a 48% lower HIV incidence (95% CI: 0.12-1.27) that was not statistically significant (4.17 vs. 7.8 per 100 women-years; *P*=0.387). This is likely attributed to insufficient power due to the low numbers of women with *L. crispatus* dominance. However, since the proportion of women with *L. crispatus* dominance was similar in women assigned to tenofovir gel and placebo gel (51% vs. 49%), the HIV incidence differences between these two groups of women are not due to *L. crispatus*. In addition, considering just *L. iners* dominant women, the efficacy of tenofovir was maintained, where the HIV incidence rate was 67% lower (95% CI: 0.13-0.83) in those assigned to tenofovir gel compared to those assigned to placebo gel (2.5 vs. 7.7 per 100 women-years in tenofovir and placebo arms, respectively; *P*=0.0118).

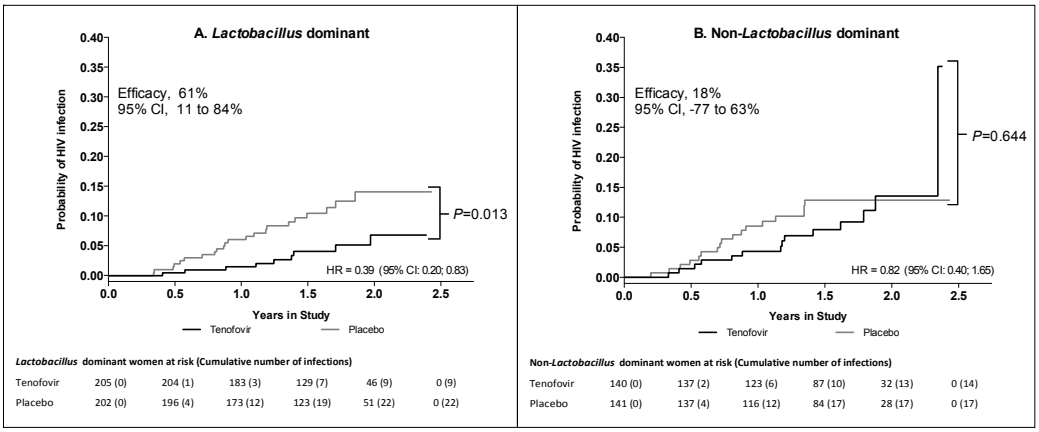


Figure 3. 5: Cumulative HIV Infection Probability by Treatment Assignment in Women

(A) Vaginal *Lactobacillus* dominance (*Lactobacillus* >50%) (n=407 women) and (B) non-*Lactobacillus* bacterial dominance (*Lactobacillus* ≤50%) (n=281 women). The table below shows the cumulative number of HIV infections in each study arm, corresponding HIV incidence rates, and efficacy of tenofovir gel to prevent HIV acquisition for each additional 6 months of follow-up. The protective efficacy of tenofovir gel was > 3-fold higher in women with *Lactobacillus* dominance (A) compared to non-*Lactobacillus* dominance (B).

Microbicide gel adherence and tenofovir efficacy in LD and non-LD women

Gel adherence as assessed by monthly empty applicator returns [164] was similar in both groups; 60.0% (244/407) of LD women compared to 61.4% (172/280) of non-LD had >50% gel adherence (where >50% sex acts were covered by two applications of the gel as recommended in the trial) (Table 3.1). Stratifying LD and non-LD women separately on adherence demonstrates that gel adherence >50% was associated with higher efficacy in preventing HIV in LD women than in non-LD women (Table 3.1). The efficacy of tenofovir gel in preventing HIV infection in the sub-group of women with >50% adherence was 78% (95% CI: 29%, 95*%; P*=0.003) in the LD group, but only 26% (95% CI: -98%, 73*%; P*=0.558) in the non-LD group.

Vaginal tenofovir concentrations are lower in non-LD versus LD women

Tenofovir concentrations (n=270) were assessed in a random sample of CVLs from HIV-negative women and from the first post-infection visit CVL from HIV seroconvertors.

Although gel adherence was not different between the LD and non-LD groups, and time since last gel application was similar (*P*=0.558), tenofovir was only detectable in CVL in 29.8% (34/114) of non-LD women compared to 46.2% (72/156) of LD women (*P*=0.008). Genital tenofovir concentrations were also significantly lower in non-LD (upper quartile=24.3 ng/ml) compared to LD women (upper quartile=8020 ng/ml) (*P*=0.0077). A sub-analysis showed that genital tenofovir concentrations negatively correlated with *G. vaginalis* protein abundance (r=-0.19, *P*=0.0014) and other anaerobic bacteria (*Prevotella*, r=-0.14, *P*=0.023), suggesting a relationship between BV-associated bacteria and tenofovir levels.

		No. of HIV infections/women years			**HIV incidence (95% CI)**				
Group	**Gel adherence**	**Tenofovir**	**Placebo**	**n**	**Tenofovir**	**Placebo**	**Incidence rate ratio**	**Efficacy**	***P*-value (logrank)**
All Participants	*Lactobacillus* Dominant	9/331	22/318	407	2.7 (1.2; 5.2)	6.9 (4.3; 10.5)	0.39 (0.16; 0.89)	61%	0.013
	Non-*Lactobacillus* dominant	14/219	17/218	281	6.4 (3.5; 10.7)	7.8 (4.5; 12.5)	0.82(0.37; 1.77)	18%	0.644
All Participants	Greater than 50% adherence	13/349	26/304	416	3.7 (2.0; 6.4)	8.6 (5.6; 12.5)	0.44 (0.21; 0.88)	56%	0.013
	Less than 50% adherence	10/200	13/232	271	5.0 (2.4; 9.2)	5.6 (3.0; 9.6)	0.89 (0.35; 2.21)	11%	0.771
Lactobacillus Dominant	Greater than 50% adherence	4/209	15/176	244	1.9 (0.5; 4.9)	8.5 (4.8; 14.1)	0.22 (0.05; 0.71)	78%	0.003
	Less than 50% adherence	5/122	7/142	163	4.1 (1.3; 9.6)	4.9 (2.0; 10.2)	0.83 (0.21; 3.05)	17%	0.735
Non-*Lactobacillus* Dominant	Greater than 50% adherence	9/141	11/128	172	6.4 (2.9; 12.2)	8.6 (4.3; 15.4)	0.74 (0.27; 1.98)	26%	0.558
	Less than 50% adherence	5/78	6/90	108	6.4 (2.1; 15.0)	6.7 (2.4; 14.5)	0.96 (0.23; 3.79)	4%	0.935

Table 3. 1: Impact of Adherence on the HIV Prevention Efficacy of 1% Tenofovir Gel in Women Participating in the CAPRISA 004 Trial Stratified by *Lactobacillus* Dominance in the Female Genital Tract

Metabolism by G. vaginalis and BV-associated bacteria leads to tenofovir depletion

Given the decreased levels of mucosal tenofovir in non-LD dominant women with *G. vaginalis* and other BV-associated bacteria vs. LD women, we aimed to determine whether interactions between microbes and tenofovir may underlie altered drug levels. We used an *in vitro* culture system to assess potential biodegradation of tenofovir by the major bacterial species present in this cohort. We found that tenofovir concentrations in culture with *G. vaginalis* decreased rapidly within 4 hours by 50.6% compared to marginal changes in either *L. iners (P*=0.0037), *L. crispatus* (*P*=0.0019) or abiotic (same NYCIII media without bacteria) control (*P*<0.0001; Fig. 3.6A). The differential decline continued; by 24 hours, tenofovir concentrations in the culture medium had dropped 67.4% with *G. vaginalis* but only 14.0% with *L. iners* (*P*<0.0001), and 9.4% with *L. crispatus* (*P*<0.0001; Fig. 3.6A). Of interest, *G. vaginalis* used here was a subtype C strain (ATCC type strain 14018) but repeating these methods with *G. vaginalis* with Kenyan clinical isolates from three different subtypes demonstrated that all subtypes of *G. vaginalis* metabolized tenofovir (*P*<0.005). Concomitantly to tenofovir loss, intracellular tenofovir concentrations rose sharply in *G. vaginalis* but not in *L. iners* or *L. crispatus* cultures *(P*<0.0001; Fig. 3.6B). Predicted metabolites at m/z 136.06, 206.10, 159.07, and 59.05 showed a sharp increase at 136.06 m/z in *G. vaginalis* cultures, indicating adenine production via cleavage of oxy-methylphosphonic acid (Fig. 3.6C), the side chain component of tenofovir (*P*<0.0001 compared to *L. crispatus, L. iners* and abiotic). Residual tenofovir plus the intracellular metabolite adenine made up >80% of

recovered products indicating adenine is the major metabolite of tenofovir metabolism by *G. vaginalis* (Fig. 3.6D). Finally, to determine whether other major bacterial species in non-LD women were capable of metabolizing tenofovir, we tested the ability for *Prevotella amnii, Prevotella bivia, Mobiluncus mulieris* and *Escherichia coli* to deplete tenofovir. We found that both *Prevotella* species and *M. mulieris* significantly depleted tenofovir compared to abiotic (Wilkins Chalgren media) controls (P=0.0007 for all at 24 hours), and *E. coli* trended towards depletion (P=0.100 as compared to abiotic Tryptic soy media) though not to the same extent or as rapidly as *G. vaginalis* (Fig. 3.6E).

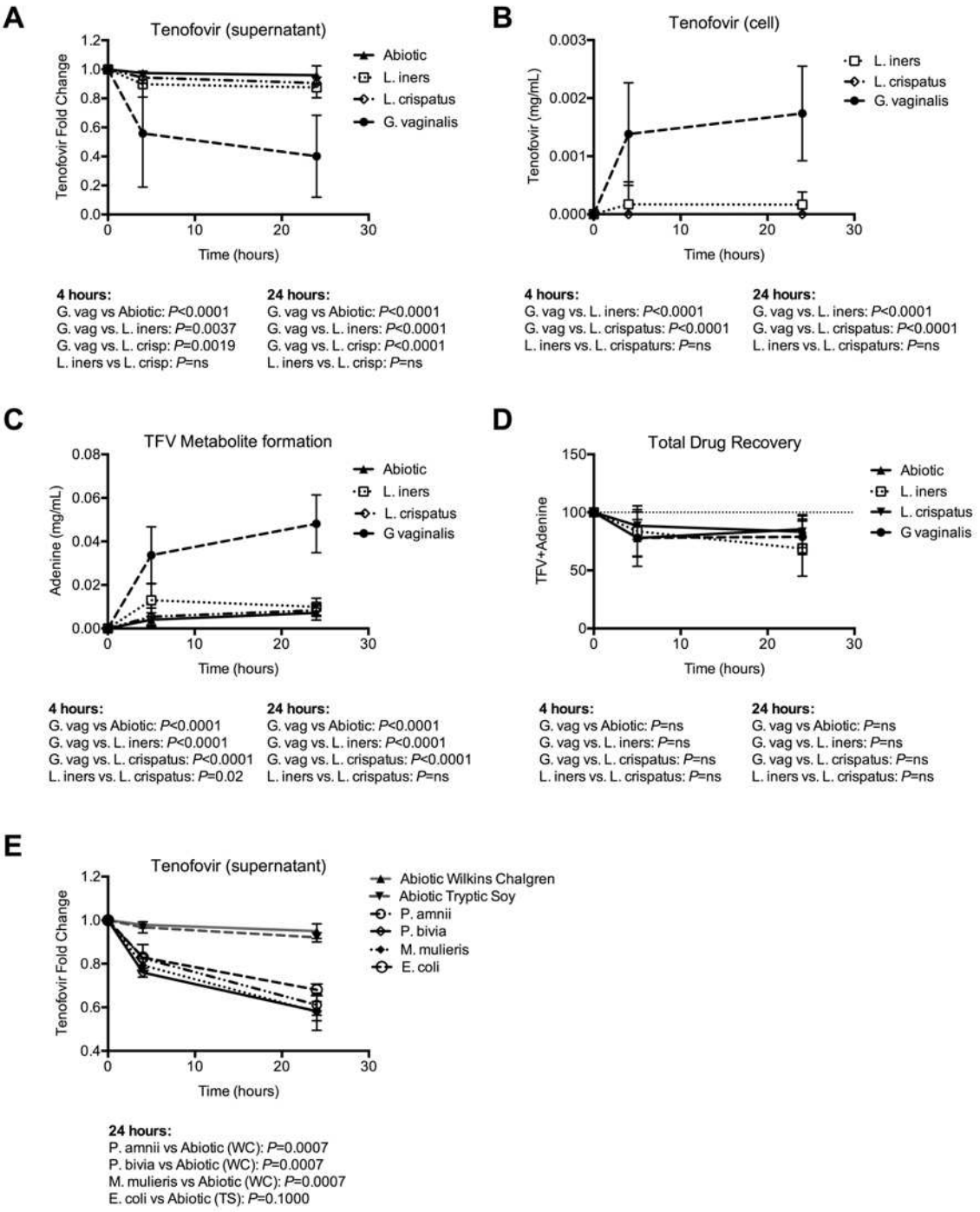


Figure 3. 6: Metabolism of Tenofovir (TFV) by *G. vaginalis* and BV-associated Bacteria

(A) Tenofovir fold-change was calculated in supernatant after 1mg/mL tenofovir was added to cultures of *G. vaginalis*, *L. iners*, *L. crispatus* or abiotic controls in NYCIII media. Tenofovir levels were measured by mass spectrometry at 0, 4 and 24 hours. Data show average +/- SEM of 18 replicate experiments for *L. iners* and *G. vaginalis* cultures compared to 15 replicates of abiotic controls.

(B) Total intracellular tenofovir detected in cell pellets from cultures.

(C) Predicted tenofovir metabolite adenine was measured in cultures.

(D) Total drug recovery of tenofovir and adenine metabolite.
(E) Tenofovir fold-change in supernatant after 1mg/mL tenofovir was added to cultures of *P. amnii, P. bivia, M. mulieris* and *E. coli*, or abiotic controls in Wilkins Chalgren (WC) media or tryptic soy (TS) broth (for *E. coli*). Data show average +/- SEM of 6 replicate experiments for *P. amnii, P. bivia, M. mulieris*, and 4 replicates for *E. coli*, compared to 5 replicate abiotic controls. *P*-values are calculated by Mann-Whitney U Test.

Metabolism by vaginal bacteria affects uptake and conversion of tenofovir to active drug in target cells

We next assessed whether tenofovir metabolism by bacteria affects the kinetics or ability of target cells to uptake tenofovir and convert to pharmacologically active tenofovir-diphosphate. We performed co-cultures of Jurkat cells (HIV targets) in the presence of tenofovir, with *G. vaginalis, L. iners*, *L. crispatus* or abiotic controls (both tenofovir alone or tenofovir plus Jurkat cells), to assess overall tenofovir depletion/uptake in culture. We found that tenofovir is most rapidly depleted in Jurkat cells in the presence of *G. vaginalis*, relative to Jurkat cells alone (*P*=0.0001) or those with *L. iners* (*P*=0.0022) or *L. crispatus (P*=0.0238) (Fig. 3.7A). To assess microbial tenofovir metabolism, we measured adenine levels in the cell pellets, and found that adenine was only created in cultures with *G. vaginalis* (*P*=0.0022 relative to all conditions, Fig. 3.7B). A critical component is whether *G. vaginalis* can metabolize tenofovir more rapidly than target cells can convert tenofovir to pharmacologically active tenofovir-diphosphate. To assess this, we measured tenofovir-diphosphate in cell pellets, and found that while tenofovir-diphosphate was made at equal levels in the presence of *Lactobacillus spp.,* it was significantly decreased in the presence of *G. vaginalis (P*=0.0002, *P*=0.0022 and *P*=0.0238 relative to TFV+Jurkat alone, TFV + Jurkat + *L. iners*, or TFV + Jurkat + *L. crispatus,* respectively, Fig. 3.7C). Total drug recovery measurements (tenofovir + tenofovir-diphosphate + adenine) demonstrated that all components of tenofovir were fully recovered (Fig. 3.7D). These data importantly demonstrate that *G. vaginalis* is

capable of decreasing pharmacologically active tenofovir-diphosphate by metabolizing tenofovir prior to drug uptake by target cells.

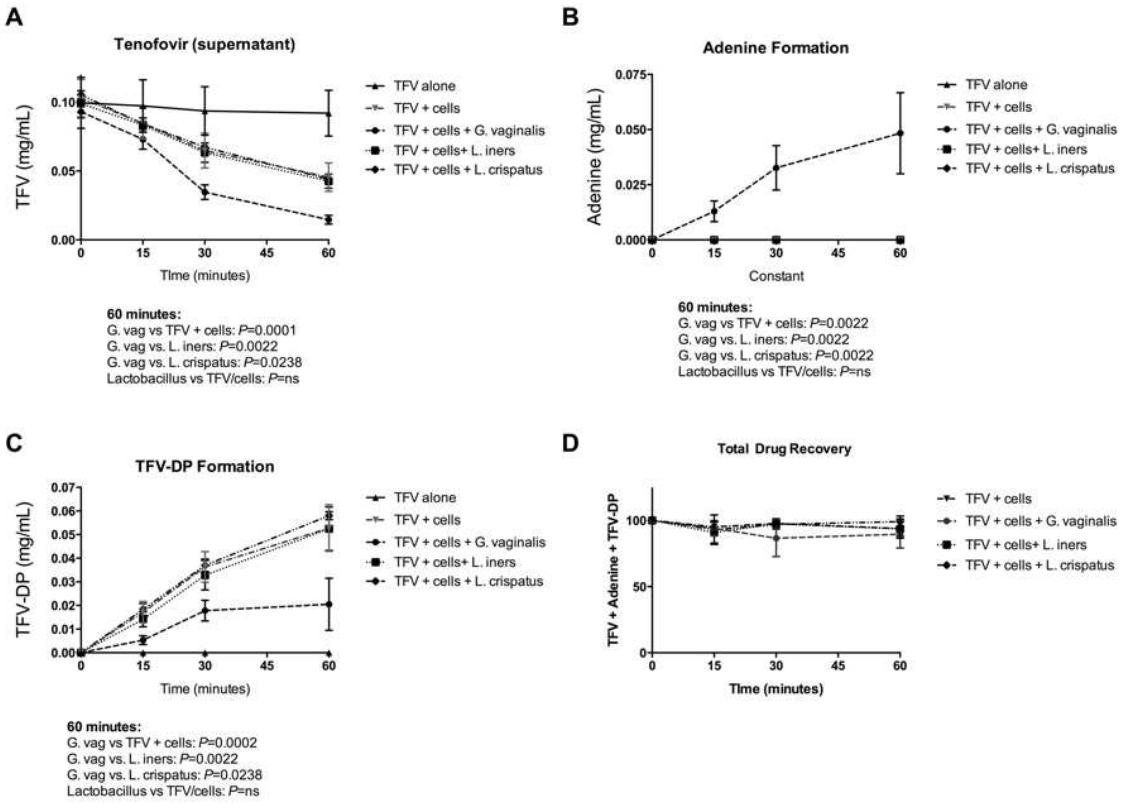


Figure 3. 7: Kinetics of Tenofovir Depletion by Bacteria Relative to Conversion to Pharmacologically Active Tenofovir-diphosphate (TFV-DP)

In vitro cultures of Jurkat cells ("cells") with tenofovir, co-cultured with *L. crispatus*, *L. iners*, or *G. vaginalis*.

(A) Tenofovir is rapidly decreased in supernatant of cultures including Jurkat cells, and most rapidly in cultures with *G. vaginalis.*

(B) The metabolite of bacterial metabolism of tenofovir, adenine, is only formed in the presence of *G. vaginalis.*

(C) Tenofovir-diphosphate is produced in Jurkat cells in the presence of *L. iners* and *L. crispatus*, but significantly lower in the presence of *G. vaginalis.*

(D) Total drug recovery demonstrates that tenofovir, tenofovir-diphosphate and adenine make up major tenofovir based drug recovery.

Implications for antiretroviral-based HIV prevention

The efficacy of tenofovir-containing topical microbicide to prevent HIV infection varied more than three-fold depending on vaginal bacterial profiles; tenofovir gel reduced HIV incidence by 61% in LD women but only by 18% in non-LD women. These efficacy differences between LD and non-LD women were consistently present in the most gel adherent women (78% vs. 26%) as well as in the least adherent women (17% vs. 4%). *In vitro* studies demonstrate that metabolism of tenofovir occurs by *G. vaginalis, P. bivia, P. amnii, M. mulieris,* and slightly by *E. coli,* but not *L. iners* or *L. crispatus*, indicating a putative mechanism for the differences in topical microbicide efficacy observed. The modifying effect of *Lactobacillus* dominance on tenofovir gel efficacy underscores the importance of both high adherence and LD vaginal bacterial communities for women to benefit maximally from topical microbicides for HIV prevention.

In searching for an underlying cause for the discordance between applicator adherence and detectable vaginal tenofovir concentrations between the LD and non-LD groups, we found that non-*Lactobacillus* dominant bacteria associated with BV rapidly metabolized tenofovir, thereby likely reducing extracellular drug availability. The rapid loss of tenofovir by *G. vaginalis* and other bacteria may also have an impact on gel adherence estimates based on vaginal drug levels in non-LD women, particularly considering that we demonstrated *Gardnerella* metabolizes bacteria more rapidly than target cells uptake and convert the drug to active form. Thus, while adherence has been demonstrated to be important for efficacy [163,165], the lack of *Lactobacillus* dominance may also be a contributing factor. These data indicate that women with bacterial vaginosis may have to be more rigorous in their adherence to tenofovir gel in order to be protected against HIV infection due to the rapid metabolism of tenofovir by dysbiotic bacteria. Due to the potential interplay between vaginal bacteria and adherence in topical microbicide

efficacy, a useful next step could be to evaluate the proportion of non-LD women in other topical microbicide trials of tenofovir conducted in Africa such as VOICE and FACTS [163,165]. These findings may have broader implications for other topical antiretroviral delivery strategies, like vaginal rings, for tenofovir-based HIV prevention.

The prevalence of *Lactobacillus* dominance in women in CAPRISA 004 (59%) was comparable to that observed in self-described black women from a North American study (58%) [170], but considerably lower than that reported in Caucasian women (90%). However, the extent of *Lactobacillus* dominance may differ even within the same country or region, the *Lactobacillus* species and overall community-state types in our study participants were similar to those described in a previous South African study [7,178], but was somewhat different from those reported recently from the FRESH cohort in KwaZulu-Natal in South Africa [7]. The reasons for these differences in the prevalence *Lactobacillus* dominance are not known. Some notable differences were that the CAPRISA 004 women were slightly older (24 vs. 21 years) and had higher hormonal contraceptive usage (97% vs. 54% on DMPA, NET-EN, or COC), which have previously been associated with higher *Lactobacillus* [179,180].

While *Lactobacillus* has been associated with lower HIV-incidence in previous observational studies we did not find a strong relationship between LD status and HIV protection in the placebo arm. This may be due to the predominance of *L. iners* in the women in our study, rather than *L. crispatus*, which has been associated with lower HIV incidence [176,181], and *L. iners* has been associated with increased HIV risk [182], or simply being underpowered with few *L. crispatus* dominant women. Nevertheless, we did observe a non-significant trend of reduced HIV incidence with *L. crispatus* compared to

women with non-*Lactobacillus* dominant profiles, regardless of tenofovir or placebo gel assignment.

The ecological diversity in the vaginal microbiome has been previously linked to changes in mucosal immunity in the female genital tract, including elevated cytokine levels, increased HIV target cells ($CD4^+CCR5^+$ T-cells), as well as epithelial barrier disruption [177,183,184]. Thus, the presence of BV-associated inflammatory bacteria could have influences at the vaginal mucosa that may add collectively to a multifactorial mechanism impacting microbicide efficacy. The relative contributions of drug depletion and these potential host modulatory effects on microbicide efficacy would be an important avenue of future investigation.

Some limitations need to be taken into account when interpreting these data. Lack of clinical BV measurements such as Nugent score or Amsel's criteria precludes our ability to associate these findings with clinical BV criteria, an important factor in vaginal health. Instead, these data are limited to associations with metaproteomics and metagenomics characterization of bacterial vaginosis. Another caveat of this study is the assumption that a single random sample represents the vaginal microbiome for the duration of the trial. Previous longitudinal evaluations of women show that some bacterial communities shift significantly in short periods of time, while others, including those dominated by *Lactobacillus* species, show considerably more stability, where outgrowths of anaerobic bacteria are less frequent (~10%) over time [70]. In a sensitivity analysis to address this question, unrealistically high levels of misclassification would be required to double the HIV incidence rate observed in this study. In randomly selected LD women (n=21), 76% maintained LD status at previous visits (median days=301), and a 24% misclassification had minimal impact on overall incidence rate. Further, as neither the trial participants nor

investigators were aware of LD status at randomization, overall random allocation in the CAPRISA 004 trial is expected to lead to comparable groups assigned to study arms of the LD and non-LD strata.

While it is a limitation of the data that the metabolism of tenofovir was only demonstrated *in vitro* here, the decreased tenofovir levels in women with vaginal dysbiosis supports this as a potential *in vivo* mechanism. Further, the *in vitro* studies were performed with a range from 1-10 mg/mL, representing predicted *in vivo* tenofovir concentrations after gel use [185], and we observed similar kinetics of tenofovir metabolism despite tenofovir concentration. Since multiple BV-associated bacteria could biodegrade tenofovir *in vitro* future studies of mixed cultures on tenofovir metabolism would be an important next step. While we have not yet assessed whether *Pseudomonas* species metabolize tenofovir, they (Group II-E) represent a small fraction of women (3.5%), and removal of this group in LD vs. non-LD comparisons did not impact outcomes reported. Other BV-associated bacteria not evaluated in this study may also metabolize tenofovir and would be important to explore. We demonstrated that clinical isolates of *G. vaginalis* are also capable of degrading tenofovir, albeit at reduced rates than type-strains, which was likely due to substantially decreased growth rates in the clinical isolates. In addition, this mechanism is supported by several studies showing *in vivo* metabolism of drugs by microbiota, just never in the context of HIV infection and antiretroviral metabolism (reviewed in [186]). Furthermore, the kinetics of bacterial metabolism of tenofovir relative to host cellular uptake and conversion of tenofovir to the pharmacologically active phosphorylated form (tenofovir-diphosphate) demonstrated that *G. vaginalis* can actually metabolize tenofovir more rapidly that target cells can convert it to tenofovir-diphosphate. Previous studies in CAPRISA 004 demonstrated that low levels of tenofovir-diphosphate was associated with infection [187], and a recent study by Hillier *et al*

demonstrated that in the FAME study both genital and plasma levels of tenofovir-diphosphate negatively correlated with *G. vaginalis* levels in the vagina, as well as markers of BV, supporting that our results translate clinically [188].

This study utilized mass spectrometry-based proteomics to characterize vaginal bacteria, which is an emerging technology to study mucosal systems. Both mass spectrometry and standard 16S rRNA sequencing had high concurrence in the classification of *Lactobacillus* dominant profiles. There were some differences in the proportion of detected taxa. In particular, the predominant genera, such as *Lactobacillus, Gardnerella, and Prevotella* more frequently dominate the bacterial proteome load than found by 16S, similar to previous observations [177]. This could be due to sensitivity differences as the proteome spans a larger dynamic range; post-transcriptional regulatory mechanisms that modulate protein translation from genes; a reflection of greater diversity of metabolic states; or divergence of functionality between the same taxa in different individuals or community state-types. Future studies of the bacterial metaproteome may provide unique insights into *in vivo* microbial systems.

We assessed whether LD and non-LD women differed with respect to clinical symptoms associated with STI and found that reported vaginal symptoms did not differ between LD and non-LD women. This concurs with other descriptions which suggest that the presence of *G. vaginalis* is often asymptomatic [169,170], making it unlikely that *G. vaginalis* led to behavioral changes impacting gel use. While previous results showed that tenofovir is protective against HSV-2 [189], our sub-analysis did not find an effect of *Lactobacillus* dominance on efficacy of tenofovir against HSV-2 acquisition. However, given the many differences between HIV and HSV-2, including the target cells in the genital tract, and the unknown levels required for protection *in vivo*, it is challenging to

compare these two infections in this context. Future studies aimed at assessing tenofovir in decreased HSV-2 infection relative to vaginal microbiota will be very important.

Since *Lactobacillus* dominance corresponds with a relatively low vaginal pH, typically below 4.5 [170], vaginal pH testing may be a pragmatic approach to identify women most likely to benefit from topical tenofovir-containing microbicides and potentially other prevention strategies. A caveat of this, however, is that current treatment strategies for BV may not be sufficiently efficacious [190], and better strategies that can both deplete anaerobic bacteria and support recolonization with *Lactobacillus* may be required. Importantly, if validated in other trials, this could be a compelling reason for integrating topical microbicide implementation with sexual and reproductive health (SRH) services so that vaginal health becomes an integral component of topical microbicide initiation.

Materials and Methods

Study Design

A total of 688 women from KwaZulu-Natal in South Africa, who had been enrolled and followed-up from 2007 to 2010 in the Centre for the AIDS Program of Research in South Africa (CAPRISA) 004 trial, were included in this study. Of the 889 women in the trial, 22.6% could not be included in this study; specimens were not available for 157 women who had not consented to sample storage; and samples from 44 women did not pass quality control criteria (fig. S2). Unavailable participants samples were evenly distributed between study arms (50.8% tenofovir, 49.2% placebo).

Cervicovaginal lavage (CVL) samples were collected during the CAPRISA 004 trial at quarterly study visits when pelvic examinations were performed. The last HIV-

seronegative CVL available from the 62 HIV seroconvertors and a randomly selected CVL sample from the 626 HIV-negative women that were assigned to either the tenofovir or placebo gel, were then analyzed by mass spectrometry (MS) as outlined below.

The MS experiments and data generation were performed blinded, such that the study samples were indistinguishable to individuals performing the mass spectrometry experiments, and those generating the final dataset were unaware of the study-group assignments until after study completion. CVL samples were randomized using a number generator for sample preparation steps as well as analysis sequence by mass spectrometry. Quality of each sample was assessed using total protein intensity and cluster analysis to identify outliers as described in detail below. Replicates of reference samples (every 10 samples) were included and evenly distributed throughout MS analysis to monitor consistency of MS performance and utilized for downstream normalization.

Sample preparation for mass spectrometry

Protein content of CVL samples was determined by BCA assay (Novagen, Bilerica, MA). Twenty-five micrograms of protein from each sample was then individually denatured with urea exchange buffer (8M Urea GE HealthCare, 50mM HEPES Sigma, pH 8.0) for 20 minutes at room temperature. These mixtures were then placed into 10 kDa cutoff Nanosep filter cartridges (Pall, Port Washington, NY), followed by centrifugation. Proteins were then reduced with 25mM dithiothreitol (Sigma) for 20 minutes, alkylated with 50mM iodoacetamide (Sigma) for 20 minutes at room temperature, and washed three times with urea exchange buffer and two times with 50mM HEPES buffer. Protein samples were then digested by addition of 2µg of Trypsin (Promega) and the samples were incubated at 37˚C overnight in the cartridge. Peptides were eluted from the filter

with 50 mM HEPES, and dried using vacuum centrifugation. Peptides were cleaned of detergents by reversed-phase liquid chromatography (high pH RP, Agilent 1200 series micro-flow pump, Water XBridge column) using a step-function gradient as previously described[191]. Eluted peptide fractions were dried using vacuum centrifugation and stored at -80°C. Peptide fractions were resuspended in MS-grade water (Sigma) and had peptide concentration determined by fluorescence (LavaPep) to further normalize peptide concentration used for MS analysis.

Mass spectrometry analysis

Each sample was analyzed using a nano-flow Easy 1000 in-line to a Q-Exactive Plus mass spectrometer with a nano-electrospray ion source at 2.0 kV (Thermo Fisher Scientific). The peptide fractions were loaded (1µg) on a C_{18}-reversed phase Easy-Spray column ES803 (Thermo Fisher Scientific, 50 cm long, 100 µm inner diameter, 2.0 µm particles) with 100% nano-LC buffer A (2% Acetonitrile, 0.1% formic acid). Peptides were eluted using a 120-min linear gradient of 2-30% nano-LC buffer B (98% acetonitrile, 0.1% formic acid) at a constant flow rate of 200 nl/min. Total nano-LC/MS/MS run-time was 180 minutes, including the loading, linear gradient, 15-min column wash at 95% buffer B, and the column re-equilibration (5 µl nano-LC buffer A). The full-MS survey scans were acquired in the Orbitrap over *m/z* 300-1700 with a target resolution of 70,000 at *m/z* 200, AGC target of 3e6, and maximum injection time of 80 ms. Data-dependent acquisition method was used, dynamically choosing the top 15 abundant precursor ions from each survey scan with an isolation width of *m/z* 3 for fragmentation by HCD (28% normalized collision energy). The intensity threshold for selecting a precursor ion for fragmentation was 1e5 ions, with charge state recognition of 2-5, and a dynamic exclusion for 10 s. The fragment ion MS2 scans were also acquired in the Orbitrap over a dynamic *m/z* range with a target resolution of 17,500 at *m/z* 200,

AGC target of 2e5, and maximum injection time of 100 ms. Lock Mass was used with the m/z's 445.120 and 371.101 from polysiloxane. CVL peptide samples were randomized and run in 18 separate batches of 50 samples. Reference samples were included and evenly distributed (every 10) to monitor LC consistency, MS performance, and utilized for downstream normalization. Quality of each sample was assessed by evaluating the total protein intensity in comparison to median abundance of all samples within each batch run. Those that fell outside 1.5 IQR were excluded. Final sample quality was assessed by cluster analysis, where samples that exhibited visible batch effects were removed.

MS Database Construction & Taxonomic Search Details

Initial searches were performed using the Mascot search engine (v2.4, Matrix Science) against the TrEMBL database (Aug-2015) that was restricted to only include bacterial proteins both from reviewed and non-reviewed sources (Database construction parameters: taxonomy:bacteria AND reviewed:no, downloaded from Uniprot) on a sub-population of our cohort (n=521). Using these results, we generated a new, curated database (Curated_CAP004_vagbact_Mar2016.fasta, 6,435,458 proteins) containing genera identified from our initial search that each accounted for ≥0.2% of the total microbial protein abundance measured (Most abundant genus to least: *Lactobacillus, Gardnerella, Pseudomonas, Mobiluncus, Ruminococus, Prevotella, Ruegeria, Bifidobacterium, Chlamydia, Megasphaera, Pedobacter, Streptococcus, Escherichia*). Genera identified in our initial search that are also commonly identified by 16S were also included despite having levels below the 0.2% threshold: *Atopobium, Dialister, Fusobacterium, Peptoniphilus, Peptostreptococcus, Porphyromonas, Shuttleworthia, Sneathia*, and merged with the UniProtKB/SwissProt Human database to account for homologous proteins between the bacterial genera and humans. The "OR" function was

also set as a database construction parameter to limit the inclusion of homologous proteins among the bacterial genera included. Individual samples were searched a second time against this curated database along with a decoy database to determine the number of false discoveries. Search results were imported into Scaffold (v 4.4.1, Proteome Software). Confidence thresholds were set to ≤1% FDR protein identification, requiring at least 2 unique peptides and ≤0.1% FDR peptide identification. Accession number protein reports were further monitored to remove any protein identifications that were assigned to more than one genus, which represented 16.3% of the initial protein list, and were not included in downstream analysis. Criteria for assigning presence of microbial proteins included those that had at least 1 peptide in its samples, and at least two peptides identified per protein across all samples. Microbial abundance was calculated by summing normalized total spectral counts for all proteins associated with each genus. Relatedness between microbial groups was determined by unsupervised hierarchical clustering using average Euclidean linkage as the distance metric. Graphical representations were generated in GraphPad Prism (version 6.05) and Shannon diversity index scores calculated in MatLab (v2015b, MathWorks).

Bacterial strains and culture conditions

Lactobacillus iners ATCC 55195 and *Gardnerella vaginalis* ATCC 14018 (group C) were obtained from the American Type Culture Collection (ATCC) and maintained on Human Bilayer Tween Agar (BD) plates and New York City III (NYCIII) medium according to the manufacturer's instructions. Clinical *G. vaginalis* isolates from Kenyan women [192], including N72 (subtype A), N144 (subtype B), and N165 (subtype C) were cultured with the same protocol. Agar plates and liquid cultures were incubated at 37°C with 5% CO_2 atmosphere. *Prevotella amnii* DSMZ 23384 was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and maintained in Wilkins Chalgren

Anaerobe Media according to the manufacturer's instructions. *Prevotella bivia* ATCC 29303 and *Mobiluncus mulieris* ATCC 35243 were obtained from the ATCC and were cultured with the same protocol. Liquid cultures were incubated at 37°C in anaerobic conditions (80% N_2, 10%CO_2, 10%H_2). *Escherichia coli* ATCC25922 was obtained from ATCC and maintained on Tryptic Soy Agar plates and Tryptic Soy Broth according to manufacturer's instructions. Agar plates and liquid cultures were incubated at 37°C with 5% CO_2 atmosphere. Frozen stocks of both strains were stored at -80°C in 40% (v/v) glycerol.

Tenofovir biodegradation experiments

For each experiment, 15 mL of overnight liquid cultures of bacteria were centrifuged at 4,000 RPM, 4°C for 10 minutes and resuspended in 1.5 mL fresh NYCIII medium. 5 mLs of abiotic medium with 1 mg/mL Tenofovir (Gilead) was inoculated with 150 µL of the concentrated inoculum and time 0 samples were collected within 3 minutes. Negative controls (matching media with no bacteria; abiotic) without the addition of tenofovir, and abiotic controls of media with 1 mg/mL tenofovir, but no bacteria, were also included. Tenofovir concentrations were assessed at 0, 4, and 24 hours. Tenofovir biodegradation experiments with *G. vaginalis* and *L. iners* were conducted in 18 replicate experiments, and *L. crispatus* in 12 replicate experiments, compared to abiotic controls conducted in 15 replicate experiments, cultured at 37°C in aerobic conditions with 5% CO_2 atmosphere. Tenofovir biodegradation experiments with *P. amnii, P. bivia, M. mulieris* (6 replicates each, grown anaerobically in Wilkins Chalgren (WC) media, compared to anaerobic abiotic WC media control) and *E. coli* (4 replicates, grown anaerobically in tryptic soy (TS) broth, compared to anaerobic abiotic TS media control) were conducted and compared to abiotic controls conducted in 5 replicate experiments for each TS and WC medias, cultured at 37°C in anaerobic conditions.

Tenofovir and Tenofovir-DP kinetics

Jurkat cells (ATCC clone ES-1TIB-152) were placed in cultures at $5x10^5$ cells/well in RPMI and were rested for 37°C for 10 minutes. *L. iners, L. crispatus* or *G. vaginalis* was added at 2.5 bacteria cells/Jurkat cell, and tenofovir was added at 0.1 mg/mL. Supernatant was collected and cells spun at 0, 15, 30 and 60 minutes. Samples were prepared and analysed for tenofovir, tenofovir-diphosphate and adenine as described below.

Tenofovir quantification by mass spectrometry

Tenofovir concentrations were determined by validated LC-MS/MS assays. Tenofovir was extracted from media via protein precipitation with acetonitrile. Chromatographic separation was achieved using a gradient elution with a Chromolith Performance RP-C_{18} column. The column was maintained at 25°C throughout. Samples were subjected to positive electrospray ionization (ESI) and detected via multiple reaction monitoring (MRM) using a LC-MS/MS system (Agilent Technologies 6460 QQQ/MassHunter). Calibration standards were prepared and ranged from 10-1000ng/mL with an inter- and intra-day precision and accuracy of ≤10.1% with an r^2 value of 0.9981±0.0017. Quantification was performed using MRM of the transitions of m/z 288.1→176.1 and 294.1→182.1 for tenofovir and the internal standard (IS) -d6, respectively. Each transition was monitored with a 150-ms dwell time. Stock solutions of tenofovir and the IS were prepared at 1mg/mL in acetonitrile-water and stored at -20°C. Mobile phase A is 0.1% acetic acid in H_2O and mobile phase B is 0.1% acetic acid in ACN, and chromatographic separation was achieved using a gradient elution with a Chromolith Performance RP-C18 column from 0-4.6 minutes, B% 0-100, with 0.5 flow and 200 max pressure. During pre-study validation, calibration curves were defined in multiple runs on the basis of triplicate assays of spiked media samples as well as QC samples. The

method was validated for its sensitivity, selectivity, accuracy, precision, matrix effects, recovery, and stability. Tenofovir-diphosphate (TFV-DP) was measured as above only using Tenofovir-diphosphate Triethylamine salt as a standard, with m/z 448.0 → 270.0 for TFV-DP.

For analysing tenofovir metabolites, a Q-TOF LC/MS system (Agilent 6520 Accurate-Mass Quadrupole Time-of-Flight) was used. The mass spectrometer was operated in positive ion electrospray mode at a desolvation temperature of 350°C. Data was acquired in the continuum mode from *m/z* 50-1000 with a scan time of 0.3s and processed using MassHunter Data Acquisition and Processing. A single predominant quantitative ion for tenofovir was 135.1021 (adenine). We also conducted an MS/MS analysis of three other likely precursor ions, *m/z* 206.1037, 159.0663, and 59.0491 based on the proposed molecular formula for each fragment using a fragmentation predictor tool (ChemDoodle). Tenofovir was extracted from media via protein precipitation with acetonitrile. Chromatographic separation was achieved using a gradient elution with a Chromolith Performance RP-C18 column.

DNA extraction and 16S rRNA gene sequencing

Total genomic DNA was extracted from cervicovaginal mucosal samples using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with modifications to enhance lysis, as previously described[193]. The V3-V4 region 16S rRNA gene sequencing was using the Illumina MiSeq sequencing platform. Paired-end reads were combined using the PANDAseq assembler[194] and 16S rRNA gene sequence data was analyzed using the QIIME software package[195]. Sequences were initially clustered into operational taxonomic units (OTUs) at 97% similarity and a representative sequence was selected

for each OTU. Taxonomy was then assigned to the representative sequences by aligning sequences to the SILVA 111 release database[196].

Statistical analysis

Mann-Whitney test and Fisher's exact test were used for continuous and categorical data, respectively. Duration of time on study was calculated from randomization to estimated date of HIV infection or date of withdrawal, whichever occurred first. Incidence rate ratios (IRRs) were calculated. Cox proportion hazard regression models were used to estimate risk of HIV infection between bacterial groupings while adjusting for potentially confounding covariates (SAS v. 9.4). Hazard ratios (95% confidence intervals (CI)) and log-rank p values are reported. All reported *P*-values are two sided. A Poisson distribution was assumed for CIs of incidence rates and IRRs.

Ethics

The study was approved by the University of Manitoba and University of KwaZulu-Natal's Human Research ethics committees. This study only includes women who provided written informed consent during the CAPRISA 004 trial (NCT00441298), for storage of their specimens for future research.

Chapter IV
Impact of Vaginal Microbiome Communities on HIV Antiretroviral-Based PrEP Drug Metabolism

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Introduction

Women account for more than half of the 35 million people living with human immunodeficiency virus (HIV) and young women are among the most vulnerable to HIV acquisition worldwide. Women contribute to over 1 million new infections annually, with the majority of these new infections located in sub-Saharan Africa[197]. In 2016, close to 18 million women were living with HIV[104]. With no efficacious vaccine, HIV prevention strategies are important for prevention of HIV transmission. While oral PrEP with Tenofovir/Emtricitabine (TDF/FTC) has demonstrated efficacy in men who have sex with men (MSM)[198], both oral and topical PrEP have yielded highly variable efficacy results for women[3], highlighting the need for better prevention strategies for women. While much of this has been attributed to adherence or presence of semen, other biological issues, such as the vaginal microbiome, can play a role. Recent studies have highlighted the role these microbes can play in diminishing the efficacy of HIV prevention for women[3].

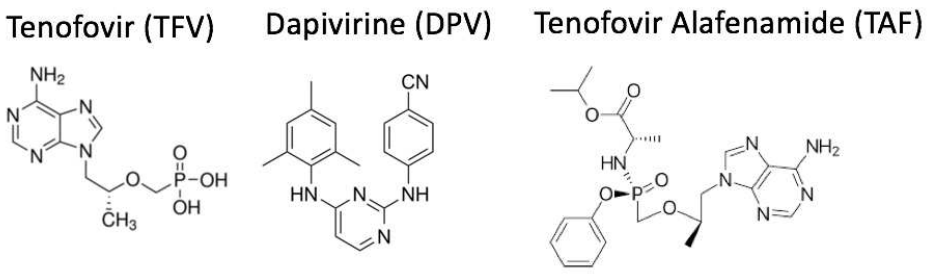
In the female reproductive tract (FRT), *Lactobacillus* spp. prevent colonization by pathogenic bacteria and are associated with protection from HIV[7,105,182]. However, colonization of the FRT

by more diverse communities of anaerobic bacteria, notably *Gardnerella vaginalis*, is common[98] and is associated with the development of bacterial vaginosis (BV)[98]. Recent studies have demonstrated that low *Lactobacillus spp.* and high diversity bacteria in the lower FRT strongly correlate with increased pro-inflammatory cytokines as a potential mechanism by which cervicovaginal microbiota may increase the risk of HIV acquisition in women via vaginal intercourse[199]. BV is highly prevalent, affecting 4-58% of women globally; with rates as high as 55% in sub-Saharan Africa[105,106,117]. In fact, a study in Zambia demonstrated 63.3% of women had BV[8]. BV is associated with significant obstetric and gynecological poor health outcomes, and with increased susceptibility to STI's[105] and HIV[98,200]. BV is associated with a 60% increase in HIV acquisition rates[105,106,117] by mechanisms that are not clearly understood. In a study of young, healthy South African women with *G. vaginalis*-containing high diversity anaerobic dysbiosis identified a 4-fold higher risk of acquiring HIV and had elevated genital CD4+ T cells compared with *Lactobacillus spp.* dominant women[182]. Of note, amongst *Lactobacillus* spp., *L. iners* has been shown to be more pro-inflammatory and when compared with *L. crispatus/jensenii*, differ in their ability to produce hydrogen peroxide and roles in protecting against STIs[107]. Maintenance of the mucosal barrier is critical for preventing invading microorganisms, including HIV, and bacterial diversity in the FRT has been strongly associated with FRT inflammation that negatively impacts FRT vulnerability to HIV infection[130].

Another potential role of the vaginal microbiome on HIV acquisition in the context of PrEP could be via alteration of pharmacokinetics of PrEP drugs. PrEP is a strategy aimed at preventing HIV infection by using ART to prevent viral replication prior to infection of HIV receptor cells. While PrEP has demonstrated efficacy in preventing the acquisition of HIV infection in men who have sex with men (MSM), studies in women have produced suboptimal outcomes. Studies evaluating topical PrEP with 1% tenofovir have shown low levels of protection (0% in FACTS to 39% in the Center for AIDS Program of Research in South Africa CAPRISA 004 trial)[201,202]. Daily

oral PrEP (Truvada) demonstrated results ranging from -49% reduction (VOICE) to 75% reduction (TDF2)[201]. Differences in outcomes between these prevention trials were attributed to adherence but recent data suggest that other biological factors at the mucosal level and the composition of the vaginal microbiome likely contributed to the variability in PrEP efficacy as well[3,203]. A secondary analysis of the CAPRISA 004 trial demonstrated differences in efficacy based on vaginal microbial communities and identified taxa responsible for bacteria-mediated drug metabolism that likely contributed to efficacy[3].

Currently, Truvada is only one FDA drug available for PrEP use. This therapy is a combination of tenofovir disoproxil fumarate (TDF) and emtricitabine. TDF is the prodrug form of tenofovir (TFV), containing an additional ester group to improve drug absorption. Topical microbicides, like the TFV vaginal ring are not in the prodrug formulation and thus our study uses TFV in its original form. While we focused on this specific drug, our study also includes the use of additional PrEP drugs such as tenofovir alafenamide (TAF) and dapivirine (DPV). TAF is the next generation oral PrEP drug that is also a pro-drug of TFV. TAF contains an amide group that aims to achieve therapeutic levels through lower dosage forms due to increasing lipophilicity. DPV is also a topical microbicide being tested in clinical trials as a vaginal ring. Unlike TAF and TFV, DPV is a non-nucleotide reverse transcriptase inhibitor (NNRTI). Due to the absence of an adenine group, we were interested in using this additional PrEP therapy to see if *G. vaginalis* would still participate in bacteria-mediated drug metabolism.



Structures of PrEP drugs TFV, TAF, and DPV.

In order to improve efficacy of prevention strategies for HIV in women, it is critical to define the mechanisms by which vaginal microbial dysbiosis affect PrEP drug levels. In this study, we investigate how vaginal bacteria alter PrEP drug concentrations through drug metabolism. Based on previous work[3], we hypothesize that variability in PrEP efficacy is a result of drug metabolism by vaginal bacteria, specifically how BV associated bacteria, such as *G. vaginalis*, decrease extracellular drug levels and prevent intracellular accumulation of active drug metabolites. If correct, the variability seen in PrEP drug trials may not only be due to varying adherence, but the vaginal microbial composition.

Methods

Study Procedures

The study utilized a cross sectional design, enrolling women with HIV or at risk for HIV infection, and it was conducted at the AIDS Clinical Research Unit at the University of Miami. The study was undertaken in cooperation with the Miami Women and HIV Interagency Study (WIHS) and the Miami Center for AIDS Research (CFAR). Eligibility criteria for participation were women 18 to 45 years of age and being sexually active in the 3 months previous to study enrollment. Women included also had no history of cervical dysplasia, were not taking immunosuppressant medications, and had no malignancies. In order to avoid the effect of hormones on the mucosa, pregnant women or women using contraceptive medications or intrauterine devices were excluded.

HIV testing

The OraQuick ADVANCE® Rapid HIV- 1/2 Antibody Test was used to determine HIV status for women without documentation of HIV status. Positive results were followed by a confirmatory HIV

western blot. Participants known to be infected with HIV prior to the study, presented documentation of positive HIV status, such as HIV Western blot results, medical records, or two laboratory results with detectable HIV viral loads greater than 1000 copies/ml, and had a rapid test performed as confirmation.

STD testing

Chlamydia and gonorrhea were tested in urine samples using nucleic acid amplification tests (NAAT). All women included were negative for these tests.

Genital samples collection

FRT cervicovaginal lavages were collected by inserting a vaginal speculum and utilizing a procedure that has previously been described[204]. A vaginal swab was used Gram stained for Nugent scoring. Cervicovaginal lavage (CVL, 10 ml) was collected and immediately placed on ice and aliquoted and stored at -80^{o} C. Samples were shipped to Dr. Klatt's laboratory at the University of Washington in dry ice.

WIHS CVLs and PrEP drug experiments

CVLs were thawed and centrifuged at 4,000 RPM, 4C for 10 minutes and resuspended into 1.5mL fresh NYCIII medium. Each CVL was divided into 3x300uL aliquots. Each aliquot was labeled with the sample name and #1, #2, and #3 respectively. Each aliquot was allowed to rest in anaerobic conditions (80% N_2, 10% CO_2, 10% H_2) for 4 hours prior to drug degradation experiments. Once thawed and rested, 5ug/mL of TFV (Selleckchem) was added to aliquot #1. Similarly, 5ug/mL of TAF (Selleckchem) and 5 ug/mL of DPV (Selleckchem) were added into aliquots #2 and #3 respectively. Once the drugs were added, HIV target cells (Jurkat, ATCC) were added at a concentration of $1x10^5$ cells into each aliquot. Samples were then incubated under anaerobic conditions for 24 hours. 50uL sample time points were taken at t=0, t=4 hours, and t=24 hours.

Samples were compared to just bacteria inoculum from CVLs and drug alone (no Jurkat cells) as well as bacteria inoculum alone (No Jurkat cells or drug). Samples were prepared for mass spectrometry as written below and aliquots saved for microbiome analysis as written below. Figure 4.1 demonstrates there is no difference in Jurkat cell growth (n=9) in aerobic versus anaerobic conditions.

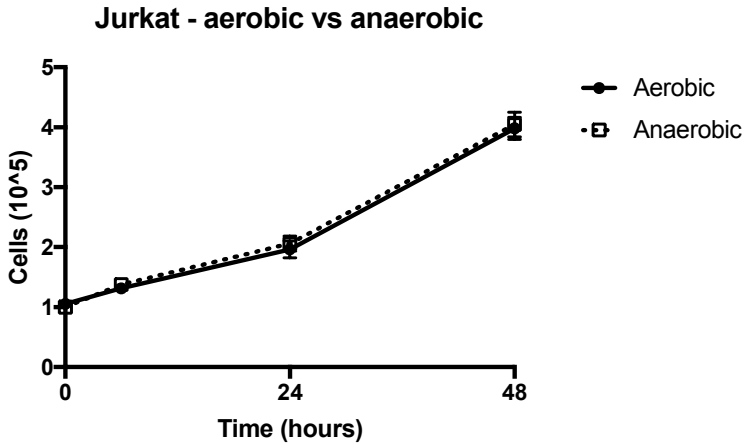


Figure 4. 1 Jurkat cell growth curves in aerobic vs anaerobic conditions.

Bacterial Vaginosis by Nugent Criteria

Bacterial vaginosis was diagnosed by Gram staining techniques of slides with vaginal secretions at the University of Miami microbiology laboratories within 24 hours of collection. Staff were trained on proper procedures prior to analyzing the slides by the chief microbiologist and the same trained technicians were used to read the slides. Unclear cases were reviewed and confirmed by a second technician and the laboratory chief once a consensus was reached. The slides were judged based on Nugent criteria. A Nugent score of 7 or above was classified as bacterial vaginosis.

ART Drug degradation kinetic experiments

Jurkat cells (ATCC clone ES-1TIB-152) were placed in cultures at $5x10^5$ cells/well in RPMI and were rested at 37C for 1 h. *L. iners*, *L. crispatus* or *G. vaginalis* were added at 2.5 bacteria cells/Jurkat cell, while drugs were added at 0.1 mg/mL. Supernatant was collected, and cells saved at time 0, 30, 60, and 120 minutes. Cells were separated from bacterial cells using Miltenyi Biotec CD3 Microbeads isolation kit. Samples were prepared and analyzed for mass spectrometry as described below.

DMA inhibitor experiments

Jurkat cells (ATCC clone ES-1TIB-152) were placed in cultures at $2.5x10^5$ cells/well in RPMI and were exposed to DMA (100uM) at 37C for 30 minutes. *L. iners* and *G. vaginalis* was added at 2.5 bacteria cells/Jurkat cell, while drug levels were added at 1 mg/mL. Supernatant was collected, and cells saved at time 0 and 24 hours. Samples were prepared and analyzed for mass spectrometry as described below.

Bacteria-drug HIV infection experiments

CEM-GFP cells are non-adherent and were obtained from the NIH AIDS Reagent Program and were cultured in RPMI 1640 medium with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% 200mM L-glutamine. HIV-1_{LAI} was titered by terminal dilution on CEM-GFP cells at different multiplicities of infection for 4 h at 37C in the presence of 2ug/mL polybrene. Cells were then subjected to a wash with sterile PBS 2x and re-suspended in 2mL culture medium without penicillin-streptomycin and incubated at 37C in 5% CO2. 200uL aliquots were taken daily for 6 days and fixed in 1% PFA prior to flow cytometry. CEM-GFP cells ($2.5x10^5$) were incubated for 30 minutes with bacterial inoculum, then for 1.5 h at 37C with TFV, TAF, or DPV before infection at a MOI of 0.1 with a T-cell tropic strain of HIV-1_{LAI} ($5x10^6$ $TCID_{50}$/mL)[205]. An aliquot of $2x10^5$ cells for each condition was harvested daily for 4 days after infection. Cells were then subjected to an Aqua Live/Dead stain, washed with PBS and fixed with 1% PFA prior to flow cytometry

analysis. Conditions included CEM-GFP cells alone, CEM-GFP cells + TFV, CEM-GFP cells + TAF, CEM-GFP cells + DPV, CEM-GFP cells + TFV + *G. vaginalis,* CEM-GFP cells + TAF + *G. vaginalis,* CEM-GFP cells + DPV + *G. vaginalis,* CEM-GFP cells + TFV + *L. iners,* CEM-GFP cells + TAF + *L. iners,* and CEM-GFP cells + DPV + *L. iners.* 200uL aliquots of sample media were also taken for drug degradation analysis using methods described below. Both bacterium did not alter their growth curves using the CEM-GFP media without penicillin-streptomycin. There were no significant difference in *G. vaginalis* or *L. iners* proliferation in R10 vs. NYCIII media.

Analysis of 16S rRNA gene sequencing data

Bacteria from cervicovaginal lavage samples were pelleted by centrifugation and resuspended in 200µl of lysis buffer (30mM Tris-HCl, 10mM EDTA, 200mM sucrose, pH 8.2). Samples were heated at 65°C for 10 minutes prior the addition of 10mg/ml lysozyme solution. Samples were then incubated for 1 hour at 37°C. 5% SDS was then added to a final concentration of 1% w/v and incubated at 56°C for 10 minutes. DNA extractions were then performed using Qiagen's DNeasy Blood and Tissue Kit. Extracted DNA was amplified following the Earth Microbiome Protocol for 16S Illumina sequencing utilizing 515F-806R primers[195] to target the V4 region of the 16S SSU rRNA. Amplicon concentrations were normalized, pooled, and cleaned prior to KAPA quantification. The pooled library was sequenced using a 2x150 bp Illumina MiSeq run. 16S sequencing reads were processed using QIIME2 version 2018.2; taxonomic determination in QIIME2 utilized the Silva 119 classifier. Taxonomic plots were created in part within Rstudio utilizing the phyloseq package.

ART concentrations by Mass Spectrometry

TFV, TAF, DPV, and TFV-DP concentrations were determined by validated LC-MS/MS assays. Drugs was extracted from media via protein precipitation with acetonitrile. Stock solutions of

TFV, TAF, DPV, TFV-DP and both IS were prepared at 1mg/mL in acetonitrile-water and stored at -20°C. Mobile phase A is 0.1% acetic acid in H2O and mobile phase B is 0.1% acetic acid in ACN, and chromatographic separation was achieved using a gradient elution with a Chromolith Performance RP-C18 column (1.15μm, 100x4.6 mm) from 0-4.6 minutes, B% 0-100, with 0.5μL/min flow rate and 400 bars max pressure. The column was maintained at 25°C throughout. Samples were subjected to positive electrospray ionization (ESI) and detected via multiple reaction monitoring (MRM) using a LC-MS/MS system (Agilent Technologies 6460 QQQ/MassHunter). Quantification was performed using MRM of the transitions of m/z 288.1→176.1, 477.1→270.1, 330.4→158.1, 448.0→270.0, 294.1→182.1, and 343.4→171.1 for TFV, TAF, DPV, TFV-DP, TFV internal standard (IS) -d6, and DPV IS -d13 respectively. Each transition was monitored with a 150-ms dwell time.

Calibration curves were prepared and run in separate analytical runs by plotting the peak area ratio of the TFV/TFV-d6 and DPV/DPV-d13 against the analyte concentration. Concentrations for each calibration level were back-calculated and the slope and coefficient of determination were calculated. Calibrations curves ranged from 10 to 6000ng/mL. Accuracy and precision were determined by the analysis of quality control samples (QCs) in 3 separate analytical runs with three replicates. Accuracy was within ±15% at the LLOQ and ±10% at all other concentrations. Stability studies included 3 freeze/thaw cycles and 4-day time studies at room temperature where accuracy was within ±10%. The lowest limit of quantitation (LLOQ) was 10ng/mL for TFV, TFV-d6, and TFV-DP. The LLOQ for TAF was 15ng/mL and the LLOQ for DPV and DPV-d13 was 20ng/mL. Precision was determined by calculating the coefficient of variation (CV) for the inter- and intra-day replicates (±15% CV). Degradation analysis was performed blinded, such that study samples were indistinguishable to individuals performing the experiments. Replicates of reference samples were included every 4 samples and evenly

distributed throughout the MS analysis to monitor consistency and performance and to utilize for downstream normalization.

Bacterial Strains and culture conditions

Lactobacillus iners ATCC 55195, *Lactobacillus crispatus*, ATCC 33197, and *Gardnerella vaginalis* ATCC 14018 (group C) were obtained from the American Type Culture Collection (ATCC) and maintained on Human Bilayer Tween Agar (BD) plates and New York City III (NYCIII) medium according to the manufacturer's instructions. Agar plates and liquid cultures were incubated at 37°C with 5% CO_2 atmosphere. Liquid cultures were incubated at 37°C in aerobic conditions. Frozen stocks of strains were stored at -80°C in 40% (v/v) glycerol.

Statistical Analysis

GraphPad Prism statistical software (version 6; GraphPad Software, San Diego, CA) was used for all statistical analysis. Results obtained at the pre-incubation time point were compared with those obtained at 24 hours for the WIHS CVL samples. Similarly, in vitro experiments with Jurkat cells compared pre and 1-hour post incubation time points. CEM-GFP experiments were compared between pre-incubation and 24, 48, and 72 hours post incubation. The significance between time points was evaluated using Mann-Whitney tests. *P* values of <0.05 were considered significant. Rates of degradation versus *Lactobacillus*, formation of metabolites versus *Lactobacillus*, and rates of degradation versus infection were calculated using linear regression analysis. Goodness of fit was evaluated using R square as well as *P* values to assess non-zero slope significance. To generate relative abundance plots, we focused on the top 19 abundant genera. The significance between multiple experimental conditions was evaluated using one-way ANOVA Tukey's multiple comparison test. *P* values of <0.05 were considered significant.

Results

Cervicovaginal lavage samples from 44 women were analyzed using 16S ribosomal RNA (rRNA) sequencing which identified 352 different bacteria genera. Twenty-nine women were diagnosed with BV based on Nugent score above 7, while fifteen were BV negative by Nugent score under 7. Thirty-three of these women were HIV positive while 23 were HIV negative. Two major bacterial community groups were identified: one in which *Lactobacillus* dominated (>50%, n=18 women) and the other dominated by non-*Lactobacillus* microbiota (<50%, n=26 women).

Effect of vaginal dysbiosis on bacteria mediated drug metabolism

To identify the effects of vaginal dysbiosis on bacteria-mediated drug metabolism, CVL samples were incubated with Jurkat cells with 3 different ART-based PrEP therapies, and bacteria communities were determined by 16S rRNA sequencing. Since some participants were HIV positive taking ART drugs, all CVLs were tested for residual ART drug levels prior to analysis. No CVLs had detectable drug levels prior to drug metabolism assays (data not shown). Bacteria were tested and confirmed for viability using selective media for *Lactobacillus* spp. and *G. vaginalis.* Soluble drug levels were measured after samples were subjugated to centrifugation and the supernatants were assessed. Figure 4.2 identifies the effects dysbiotic bacteria can have on TFV. Figure 4.2A ranks the samples in order from least amount of TFV degraded and shows the bacterial community composition for each sample. To identify the effects of *Lactobacillus* on drug biodegradation, we looked at differences in % TFV remaining between *Lactobacillus* dominance versus non-*Lactobacillus* dominant. In samples with less than 50% *Lactobacillus*, we observed only 43.8±11.9% remaining of TFV ($p<0.0001$) after 24 hours when compared to 79.6±7.5% remaining for *Lactobacillus* dominant samples (Figure 4.2B). Our control group without bacteria from the CVLs demonstrated 82.2±8.4% remaining, which was not statistically significantly different from the *Lactobacillus* dominant samples ($p>0.9999$),

Figure 4.2B). Our study also found that rates of degradation for TFV exhibited a significant negative correlation relative to *Lactobacillus* abundance (R^2=0.8518, p<0.0001; Figure 4.2C).

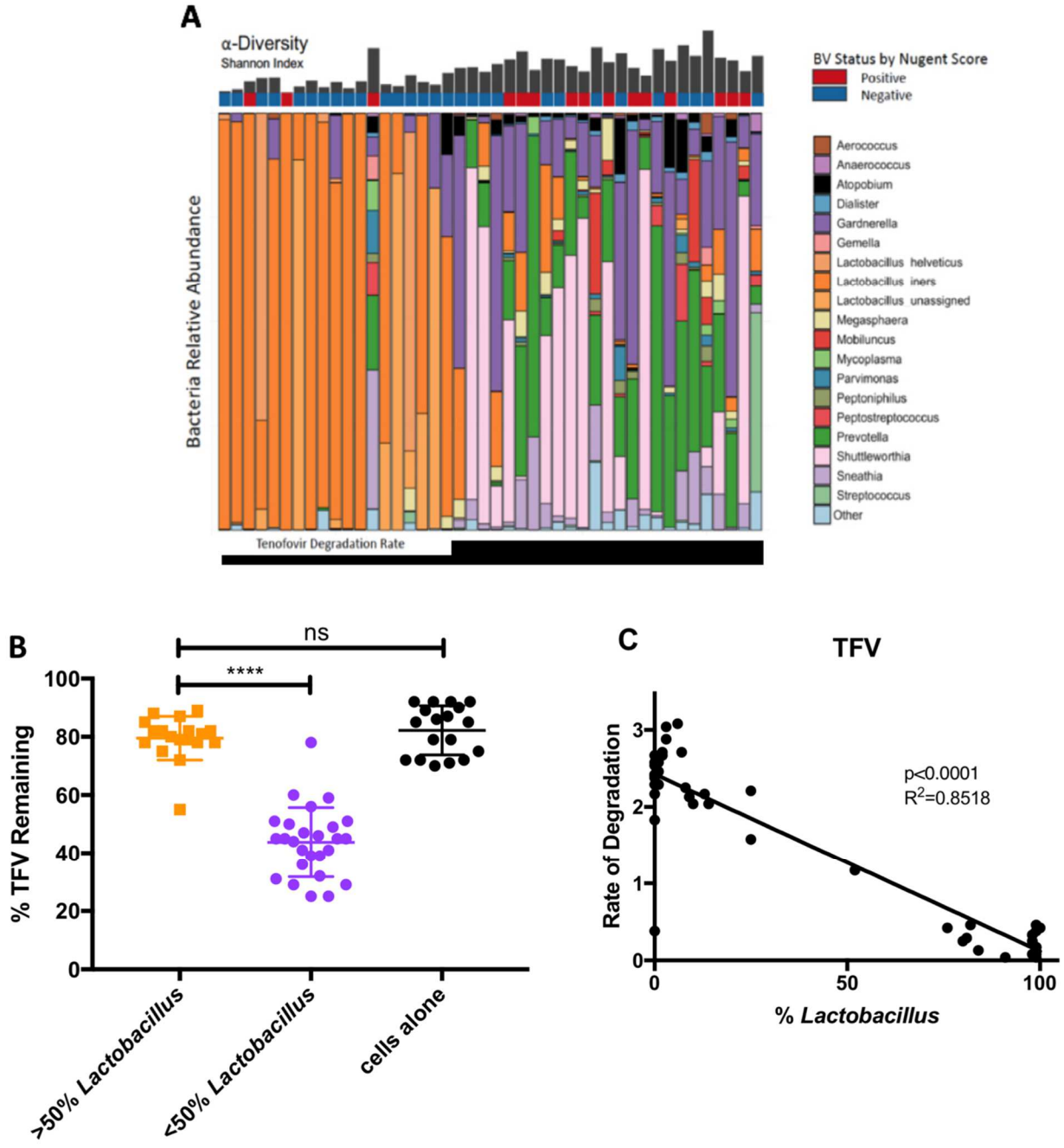


Figure 4. 2: Effects of Vaginal Bacteria on TFV Degradation

(A) Relative abundance of CVLs from 44 women with and without diagnosed BV ranked in order of increasing TFV degradation rates. The 19 most abundant phyla are shown. Shannon diversity plots showing alpha diversity in CVLs. Blue, BV negative by Nugent score at time of collection; Red, BV positive by Nugent score at time of collection.

(B) % TFV remaining after 24 hours at 37C in *Lactobacillus* dominant vs. non-*Lactobacillus* dominant CVLs. Orange, greater than 50% dominance; Purple, less than 50% dominance; Black, cells + TFV control.

(C) Rate of TFV degradation (% lost/hour) vs. %*Lactobacillus* in CVLs after 24-hour incubation. Rate of degradation calculated as % remaining/24 hours.

We next evaluated the effects of dysbiotic bacteria on the pro-drug tenofovir alafenamide (TAF), given that this is a next generation drug being assessed for PrEP. We found no significant difference in degradation rate of TAF, regardless of bacteria community state (Figure 4.3A). Similarly, we found no difference in TAF remaining between our *Lactobacillus* dominant (79±7.0%) and non-*Lactobacillus* dominant samples (77.7±7.6%, p=0.1156; Figure 4.3B) as well as no association between rate of degradation and fraction of *Lactobacillus* (Figure 4.3C). Our control group matched both experimental in %TAF remaining (80.4±6.2%).

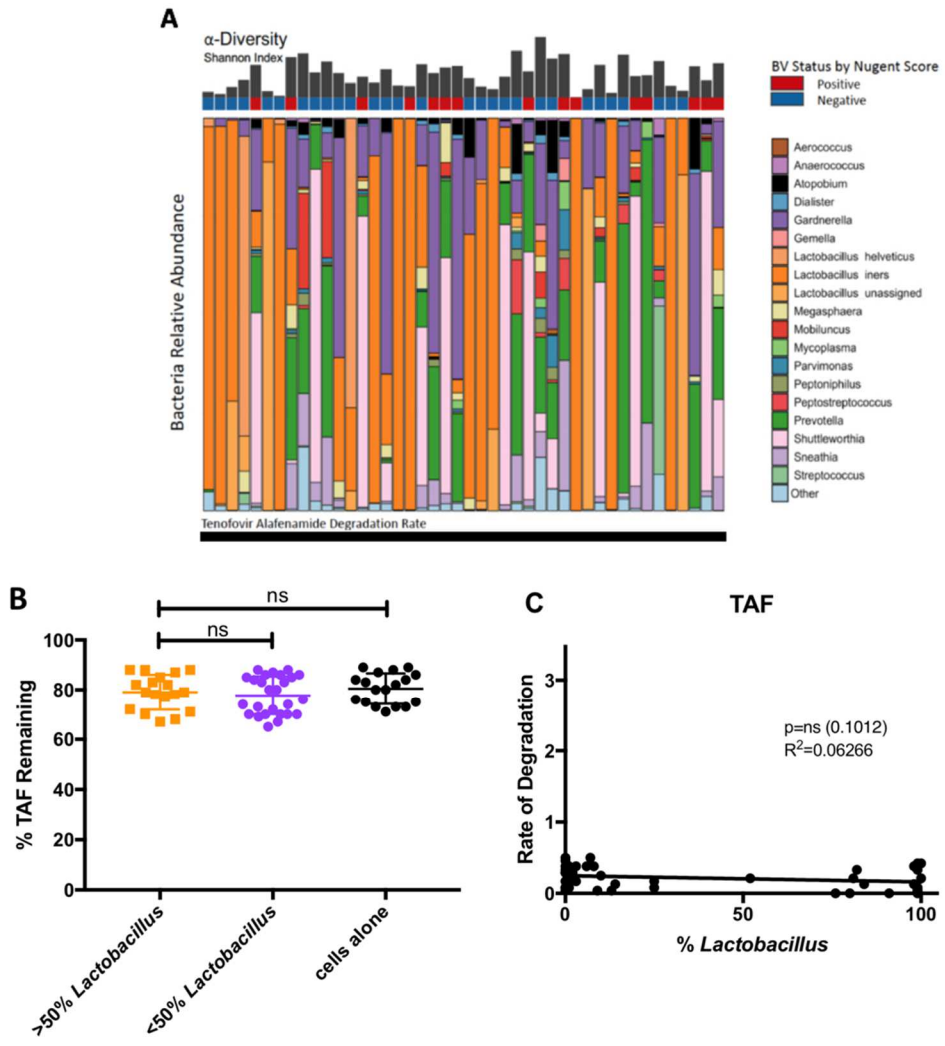


Figure 4. 3: Effects of Vaginal Bacteria on TAF Degradation

(A) Relative abundance of CVLs from 44 women with and without diagnosed BV ranked in order of increasing TAF degradation rates. The 19 most abundant phyla are shown. Shannon diversity plots showing alpha diversity in CVLs. Blue, BV negative by Nugent score at time of collection; Red, BV positive by Nugent score at time of collection.

(B) % TAF remaining after 24 hours at 37C in *Lactobacillus* dominant vs. non-*Lactobacillus* dominant CVLs. Orange, greater than 50% dominance; Purple, less than 50% dominance; Black, cells + TFV control.

(C) Rate of TAF degradation (% lost/hour) vs. %*Lactobacillus* in CVLs after 24-hour incubation. Rate of degradation calculated as % remaining/24 hours.

Dapivirine (DPV) is used in trials of vaginal ring-based PrEP[5,6]. Figure 4.4A highlights the effects that dysbiotic bacteria have on DPV degradation, with a clear association between diversity of vaginal bacterial communities and increased DPV degradation. When categorizing samples between *Lactobacillus*-dominant versus non-*Lactobacillus* dominant, we identified a significant increase in DPV remaining after incubation in samples with *Lactobacillus* dominance ($p<0.0001$;

Figure 4.4B). We saw an average of 73.2±7.96% DPV remaining in our *Lactobacillus*-dominant samples versus only 38.8±11.9% in our non-*Lactobacillus* dominant samples. Our control group without bacteria found 71.3±6.1% remaining after incubation. Our study also found that rates of degradation for DPV was also negatively associated with *Lactobacillus* abundance (R^2=0.7274, p<0.0001s; Figure 4.4C).

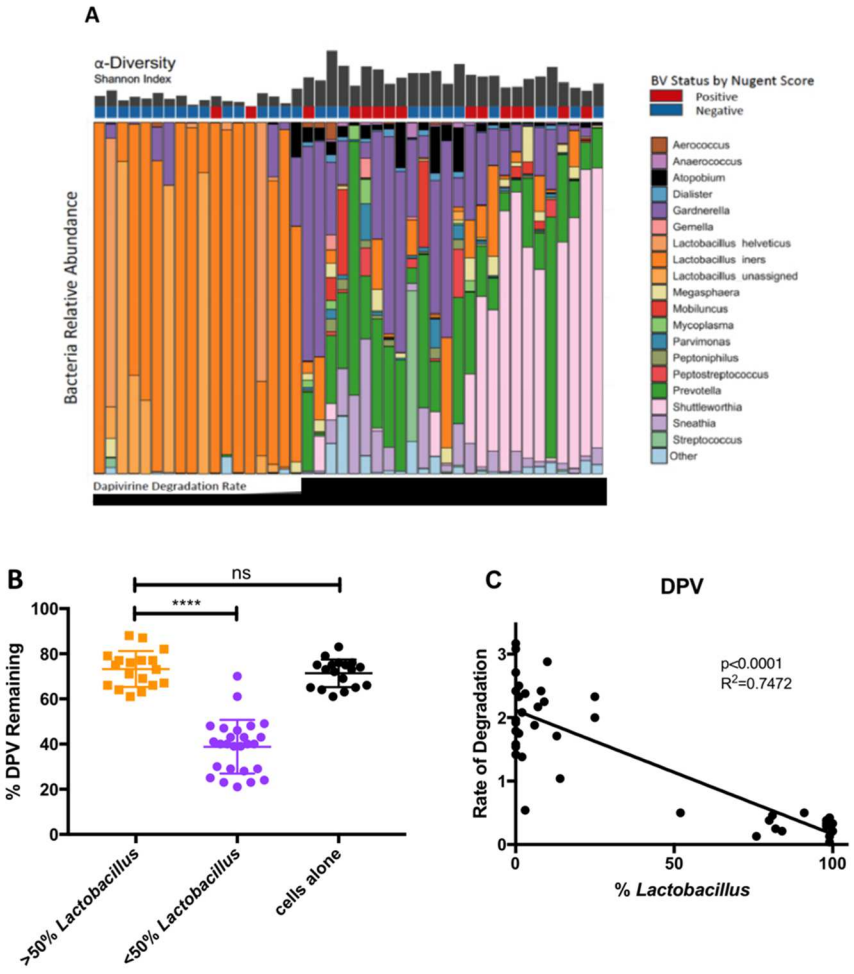


Figure 4. 4: Effects of Vaginal Bacteria on DPV Degradation

(A) Relative abundance of CVLs from 44 women with and without diagnosed BV ranked in order of increasing DPV degradation rates. The 19 most abundant phyla are shown. Shannon diversity plots showing alpha diversity in CVLs. Blue, BV negative by Nugent score at time of collection; Red, BV positive by Nugent score at time of collection.

(B) % DPV remaining after 24 hours at 37C in *Lactobacillus* dominant vs. non-*Lactobacillus* dominant CVLs. Orange, greater than 50% dominance; Purple, less than 50% dominance; Black, cells + TFV control.

(C) Rate of DPV degradation (% lost/hour) vs. %*Lactobacillus* in CVLs after 24-hour incubation. Rate of degradation calculated as % remaining/24 hours.

Of note, we compared vaginal dysbiosis measured by 16S rRNA sequencing compared to clinical BV diagnosis by Nugent score. Amongst CVLs from women without diagnosed BV, almost 27% (n=13 women) had less than 50% *Lactobacillus* (black dots) and instead had a much more diverse community profile. These samples also exhibited the most degradation amongst non-BV samples and matched the samples from BV-positive women without *Lactobacillus* dominance (Fig. 4.5A-C). Similarly, amongst CVLs from women with diagnosed BV, roughly 13% (n=2 women) had *Lactobacillus* dominance (gray dots) and were amongst the highest fraction of drug remaining within the BV group (Fig. 4.5A-C). These women matched degradation profiles similar to women without BV and had *Lactobacillus*-dominance. When ranked by Nugent score, we observed a statistically significant association where increasing Nugent score correlated with more TFV degradation (data not shown). Similarly, we observed a significant association with increasing DPV degradation and Nugent score (data not shown). Of note, due to lack of TAF degradation, there was no significant trend with Nugent score (data not shown). Sub-analysis of all experiments did not show a significant difference when comparing HIV infected vs uninfected (data not shown).

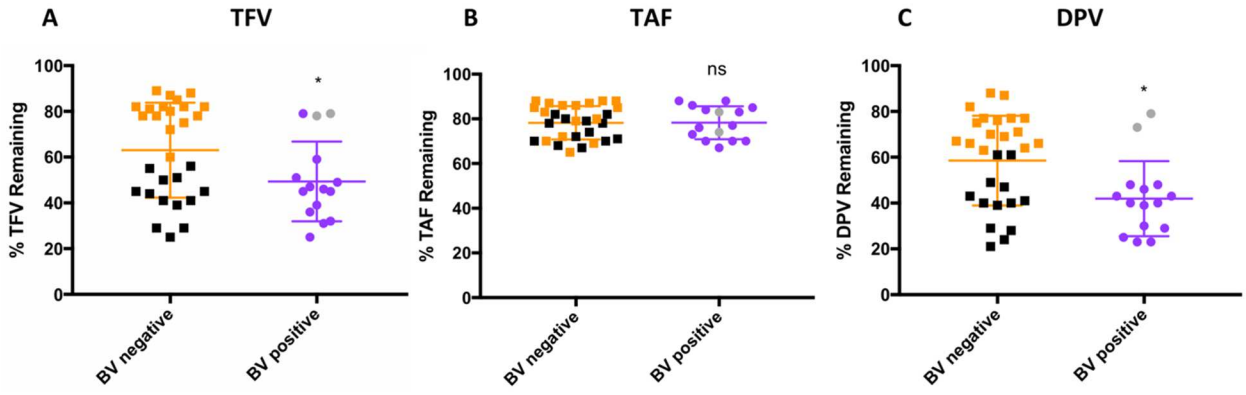


Figure 4. 5: % Drug Remaining after 24 hours based on Nugent Score Diagnosed BV+/- Women from the WIHS Cohort

(A) % TFV remaining after 24 hours at 37C in BV diagnosed vs non-BV diagnosed CVLs. Orange, BV negative; Purple, BV positive. Black dots indicate less than 50% *Lactobacillus* dominance despite BV negative diagnoses. Gray dots indicate greater than 50% *Lactobacillus* dominance despite BV positive diagnoses.

(B) % TAF remaining after 24 hours at 37C in BV diagnosed vs non-BV diagnosed CVLs. Orange, BV negative; Purple, BV positive. Black dots indicate less than 50% *Lactobacillus* dominance despite BV negative diagnoses. Gray dots indicate greater than 50% *Lactobacillus* dominance despite BV positive diagnoses.
(C) % DPV remaining after 24 hours at 37C in BV diagnosed vs non-BV diagnosed CVLs. Orange, BV negative; Purple, BV positive. Black dots indicate less than 50% *Lactobacillus* dominance despite BV negative diagnoses. Gray dots indicate greater than 50% *Lactobacillus* dominance despite BV positive diagnoses.

Effect of drug metabolism by vaginal bacteria from CVLs on drug uptake

In order to determine if metabolism by vaginal bacteria alters drug uptake into target cells, we assessed intracellular pharmacologically active drug compounds from the Jurkat cell pellets after centrifugation of samples. Cell pellets were re-suspended in 1mL of R10 media and a 10 uL aliquot was taken for cell counting. For TFV and TAF, we assessed intracellular TFV-DP, and for DPV, we assessed intracellular DPV, normalized for differences in cell counts. We evaluated the amount of active drug compounds formed in *Lactobacillus*-dominant versus non-*Lactobacillus* dominant samples. In our *Lactobacillus*-dominant samples, we observed 0.973±0.224ug/mL of TFV-DP versus only 0.367±0.104ug/mL of TFV-DP in our non-*Lactobacillus* dominant samples ($p<0.0001$) from TFV administration after 24 hours (Figure 4.6A). In our negative control Jurkat cells + TFV alone, we saw 0.897±0.267ug/mL of TFV-DP formed, which was not statistically significant from our *Lactobacillus* dominant samples ($p>0.9999$, Figure 4.6A). The difference in TFV-DP formation from TAF administration was not significantly different ($p>0.9999$) with an average of 1.53±0.298ug/mL versus 1.53±0.341ug/mL for *Lactobacillus* and non-*Lactobacillus* dominant samples, respectively (Figure 4.6B). In our negative control group containing Jurkat cells and TAF alone, we measured 1.646±0.285ug/mL of TFV-DP formed, which was not statistically significantly different from either experimental group ($p>0.9999$). In samples with DPV, intracellular DPV was significantly higher in *Lactobacillus* dominant samples versus non-*Lactobacillus* dominant samples ($p<0.0001$; Figure 4.6C). The median amount of intracellular DPV was 2.288±0.590ug/mL in *Lactobacillus* dominant samples versus only 1.241±0.263ug/mL in non-*Lactobacillus* dominant samples. Our

negative control group of Jurkat cells alone + DPV saw 2.057±0.497ug/mL of intracellular DPV, which was not statistically significantly different from the *Lactobacillus* dominant samples (p>0.9999). When evaluating the intracellular formation of TFV-DP from TFV we saw a significant correlation between active drug formation in cells and abundance of *Lactobacillus* (R^2=0.685, p<0.0001; Figure 4.6D). Similarly, the presence of intracellular DPV exhibited a significant correlation between active drug formation and *Lactobacillus* abundance (R^2=0.6365, p<0.0001; Figure 4.6F). The rate of TFV-DP formation after TAF administration did not correlate with *Lactobacillus* abundance however (R^2=0.0005671, p=0.8781; Figure 4.6E).

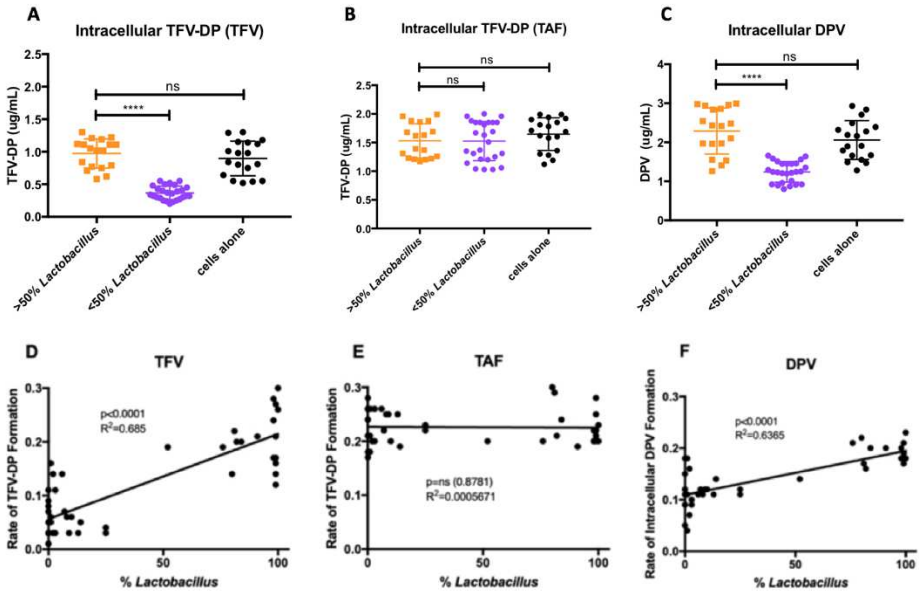


Figure 4. 6: Intracellular Active Metabolite Formation after 24 hours Grouped by *Lactobacillus* Dominance or Non-dominance from the WIHS Cohort

(A) Amount of intracellular TFV-DP formed post-TFV administration after 24 hours at 37C in *Lactobacillus* dominant versus non-*Lactobacillus* dominant CVLs. Orange, greater than 50% dominance; Purple, less than 50% dominance.
(B) Amount of intracellular TFV-DP formed post-TAF administration after 24 hours at 37C in *Lactobacillus* dominant versus non-*Lactobacillus* dominant CVLs. Orange, greater than 50% dominance; Purple, less than 50% dominance.
(C) Amount of intracellular DPV formed post-DPV administration after 24 hours at 37C in *Lactobacillus* dominant versus non-*Lactobacillus* dominant CVLs. Orange, greater than 50% dominance; Purple, less than 50% dominance.
(D) Rate of TFV-DP formation (amount/hour) vs. %*Lactobacillus* in CVLs after 24-hour incubation with TFV. Rate of degradation calculated as amount formed/24 hours.
(E) Rate of TFV-DP formation (amount/hour) vs. %*Lactobacillus* in CVLs after 24-hour incubation with TAF. Rate of degradation calculated as amount formed/24 hours.
(F) Rate of DPV formation (amount/hour) vs. %*Lactobacillus* in CVLs after 24-hour incubation with DPV. Rate of degradation calculated as amount formed/24 hours.

The effect of vaginal microbiota on uptake efficiency of ART drugs

Given the decreased TFV and DPV levels after incubation with CVLs from women with non-*Lactobacillus* dominant microbiomes, we aimed to determine interactions between major vaginal bacterial taxa that may contribute to drug degradation. Using an *in vitro* co-culture system, we assessed potential bacteria-mediated metabolism using *G. vaginalis* and *Lactobacillus iners* and *Lactobacillus crispatus*. Despite *G. vaginalis* not being the pre-dominant taxa in the non-*Lactobacillus* samples, previous studies have identified this specific bacterium amongst African women as well as playing a role in bacteria-mediate drug metabolism[3]. We found that in culture with *G. vaginalis*, there was a significant reduction in the amount of intracellular active drug formed versus drug administered (uptake efficiency) when compared with cultures with *Lactobacillus* spp. after 1 hour ($p<0.0001$; Figure 4.7A). Of note, when co-cultured together, the negative effects of *G. vaginalis* are somewhat ameliorated by the presence of *Lactobacillus*, albeit still significantly lower than *Lactobacillus* alone ($p<0.0001$), but higher efficiency than *G. vaginalis* alone ($p<0.0001$). When comparing the uptake efficiency after TAF administration, *G. vaginalis* had the greatest negative impact when compared with controls ($p<0.0001$) and *Lactobacillus* spp. ($p<0.0001$). However, intriguingly, when co-cultured with both *G. vaginalis* and *Lactobacillus* spp. together, TAF degradation was no longer observed and there is no statistically significant difference when compared with *Lactobacillus* spp. alone ($p=0.8143$) and controls ($p=0.0780$; Figure 4.7B). The presence of *G. vaginalis* also caused a significant reduction in uptake efficiency of DPV when compared with controls and *Lactobacillus* spp. alone ($p<0.0001$; Figure 4.7C). When co-cultured with both *Lactobacillus* and *G. vaginalis*, there was a significant increase in DPV uptake efficiency when compared with *G. vaginalis* alone ($p<0.0001$), but still significantly lower than *Lactobacillus* alone ($p<0.0001$). We did not see any observable difference uptake efficiency with any drug when co-cultured with *L. iners* vs. *L. crispatus* (Figure 4.7). Although we observe less formation of TFV-DP from TAF administration

in cultures with *G. vaginalis*, the reason we do not see any changes in TFV-DP production amongst the *non-Lactobacillus* dominant samples is believed to be due to the relative abundance of *G. vaginalis*. That is, amongst the study samples, *G. vaginalis* was not the dominating taxa while the in-vitro co-cultures strictly used *G. vaginalis.*

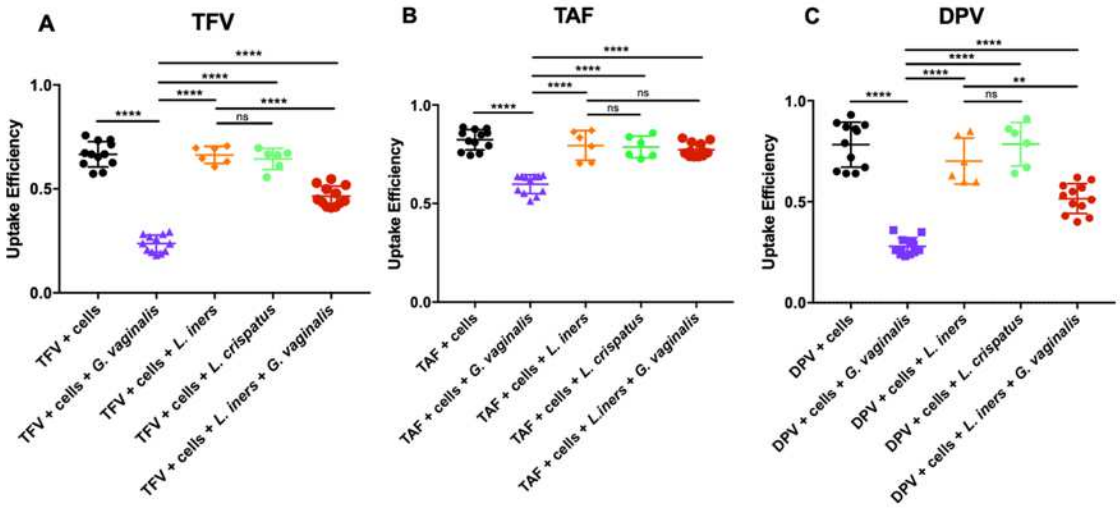


Figure 4. 7: Uptake Efficiency of Jurkat Cells in Converting Drug into Active Metabolite after 1 Hour. Uptake efficiency is defined as the amount of intracellular drug divided by the amount of drug added extracellularly.

(A) Uptake efficiency of Jurkat cells in converting TFV into TFV-DP based on 1) TFV + cells alone (black) 2) TFV + cells + *G. vaginalis* (purple) 3) TFV + cells + *L. iners* (orange) 4) TFV + cells + *L. crispatus* (green) 5) TFV + cells + *L. iners* + *G. vaginalis* (red). Bacteria were added 15 minutes prior to incubation at 37C and added at a ratio of bacteria to cells of 2.5:1. Co-culture experiments (4) were added at a 1:1 ratio of *L. iners*: *G. vaginalis* and an overall ratio of total bacteria to cells at 2.5:1. N=12

(B) Uptake efficiency of Jurkat cells in converting TAF into TFV-DP based on 1) TAF + cells alone (black) 2) TAF + cells + *G. vaginalis* (purple) 3) TAF + cells + *L. iners* (orange) 4) TFV + cells + *L. crispatus* (green) 5) TFV + cells + *L. iners* + *G. vaginalis* (red). Bacteria were added 15 minutes prior to incubation at 37C and added at a ratio of bacteria to cells of 2.5:1. Co-culture experiments (4) were added at a 1:1 ratio of *L. iners*: *G. vaginalis* and an overall ratio of total bacteria to cells at 2.5:1. N=12

(C) Uptake efficiency of Jurkat cells in accumulating intracellular DPV based on 1) DPV + cells alone (black) 2) DPV + cells + *G. vaginalis* (purple) 3) DPV + cells + *L. crispatus* (orange) 4) TFV + cells + *L. crispatus* (green) 5) TFV + cells + *L. iners* + *G. vaginalis* (red). Bacteria were added 15 minutes prior to incubation at 37C and added at a ratio of bacteria to cells of 2.5:1. Co-culture experiments (4) were added at a 1:1 ratio of *L. crispatus*: *G. vaginalis* and an overall ratio of total bacteria to cells at 2.5:1. N=12

HIV infection *in vitro* is increased in the presence of dysbiotic vaginal bacteria

Given our findings that dysbiotic vaginal bacteria such as *G. vaginalis* can differentially impact drug concentrations, we investigated the impact of this phenomena on HIV productive infection. Using CEM-GFP cells and HIV-1_{LAI} virus, we assessed productive infection of cells following

cultures with drug as well as different bacteria. Figure 4.8 depicts representative staining plots of the fluorescence of GFP from infected CEM cells. We monitored the fraction of CEM-GFP cells infected from 24 to 72 hours post inoculation (Figure 4.9A). In order to determine if cells were infected, we used flow cytometry to measure GFP fluorescence. Figure 4.9 demonstrates representative staining plots of cells alone (A), cells + virus (B), cells + virus + TFV (C), cells + virus + TFV + *L. crispatus* (D), and cells + virus + TFV + *G. vaginalis* (E) looking at %GFP versus forward scatter. Gating strategy followed doublet exclusion and live dead analysis.

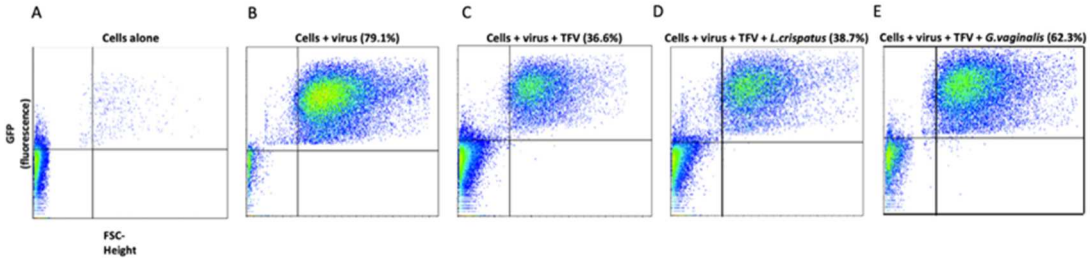


Figure 4. 8: Representative Staining Plots Demonstrating % Fluorescence of CEM-GFP Cells 48 Hours After 37C Incubation

(A) % GFP fluorescence of CEM-GFP cells alone.
(B) % GFP fluorescence of CEM-GFP cells + HIV-1_{LAI}.
(C) % GFP fluorescence of CEM-GFP cells + HIV-1_{LAI} + TFV
(D) % GFP fluorescence of CEM-GFP cells + HIV-1_{LAI} + TFV + *L. crispatus*
(E) % GFP fluorescence of CEM-GFP cells + HIV-1_{LAI} + TFV + *G. vaginalis*

In our co-cultures, we saw a lower fraction of infected cells following TFV administration (5uM) when compared with virus and cells alone (p=0.0379) and observed no difference when compared with TFV and *Lactobacillus* (p=0.6190) as early as 24 hours post incubation (Figure 7A). While we saw no statistically significant difference between TFV administration alone and TFV with *G. vaginalis* (p=0.1909), there was still a slight difference in median values (26.4 vs. 41.5). At 48 hours, we observed a significant difference in infected cells when co-cultured with *G. vaginalis* compared to TFV alone (p=0.0022) and with TFV + *Lactobacillus* (p=0.0022), and these trends continued through 72 hours (Figure 4.9A). In order to elucidate the effects of drug degradation on frequency of infected cells, we evaluated the correlation between rate of TFV degradation (µg/hour) with frequency of infected cells and observed a significant correlation at

48 hours (R^2=0.6604, p<0.0001; Figure 4.9B). Figure 4.9C details the rate of degradation of TFV over all time points. Across all time points we observed statistically significant difference between *G. vaginalis* and *Lactobacillus* or TFV alone. We did not see a significant difference between samples with *Lactobacillus* and abiotic controls.

Following TAF administration (5uM), there was a significantly lower amount of infected cells (p<0.0001) but no observable difference when *Lactobacillus* was added (p=0.7316; Figure 4.9D). We did however see a higher number of infected cells when cultured with *G. vaginalis* when compared with *L. crispatus* (p=0.0087) after 24 hours. The 48 and 72-hour time points exhibited similar trends. Although significantly lower than the effects seen with TFV, we observed a significant correlation between the rate of TAF degradation and % of infected cells (R^2=0.482, p=0.0014; Figure 4.9E). Figure 4.9F summarizes the rate of TAF degradation over time. Across all time points we observed statistically significant difference between *G. vaginalis* and *Lactobacillus* or TAF alone. We did not see a significant difference between samples with *Lactobacillus* and abiotic controls. However, despite this slight difference, overall TAF had very limited degradation.

Finally, we evaluated the effects vaginal bacteria had on the ability for DPV to prevent HIV infection *in vitro*. As early as 24 hours post incubation, we observed a significant difference in infected cells when compared with controls (p<0.0001; Figure 4.9G). DPV was added at 5uM. Similar to TFV and TAF, we saw no difference between DPV alone and DPV with *Lactobacillus* (p=0.8864; Figure 4.9G). We saw a higher frequency of infected cells when cultured with *G. vaginalis* compared with DPV alone (p=0.0001) and when compared with DPV + *Lactobacillus* (p=0.0006). We also found a significant correlation between rate of DPV degradation and frequency of infected cells (R^2=0.8581, p<0.0001; Figure 4.9H). Lastly, Figure 4.9I depicts the rate of DPV degradation over time. Across all time points we observed statistically significant

difference between *G. vaginalis* and *Lactobacillus* or DPV alone. We did not see a significant difference between samples with *Lactobacillus* and abiotic controls.

In samples with TFV concentrations > 15uM, the percentage of infected cells did not significantly change, implying that infected cells did not produce new infectious virions at these drug concentrations. However, in samples <5uM, the number of fluorescent cells increased with time for up to 48 hours indicating viral escape and infection of new target cells. These data match IC50 values for TFV[206]. Of note, at high MOIs (>0.1), a decrease in fluorescence was observed at later time points (post 72 hours), because infected CEM-GFP cells are killed by virus-induced apoptosis[207]. Similar experiments with TAF and DPV were observed.

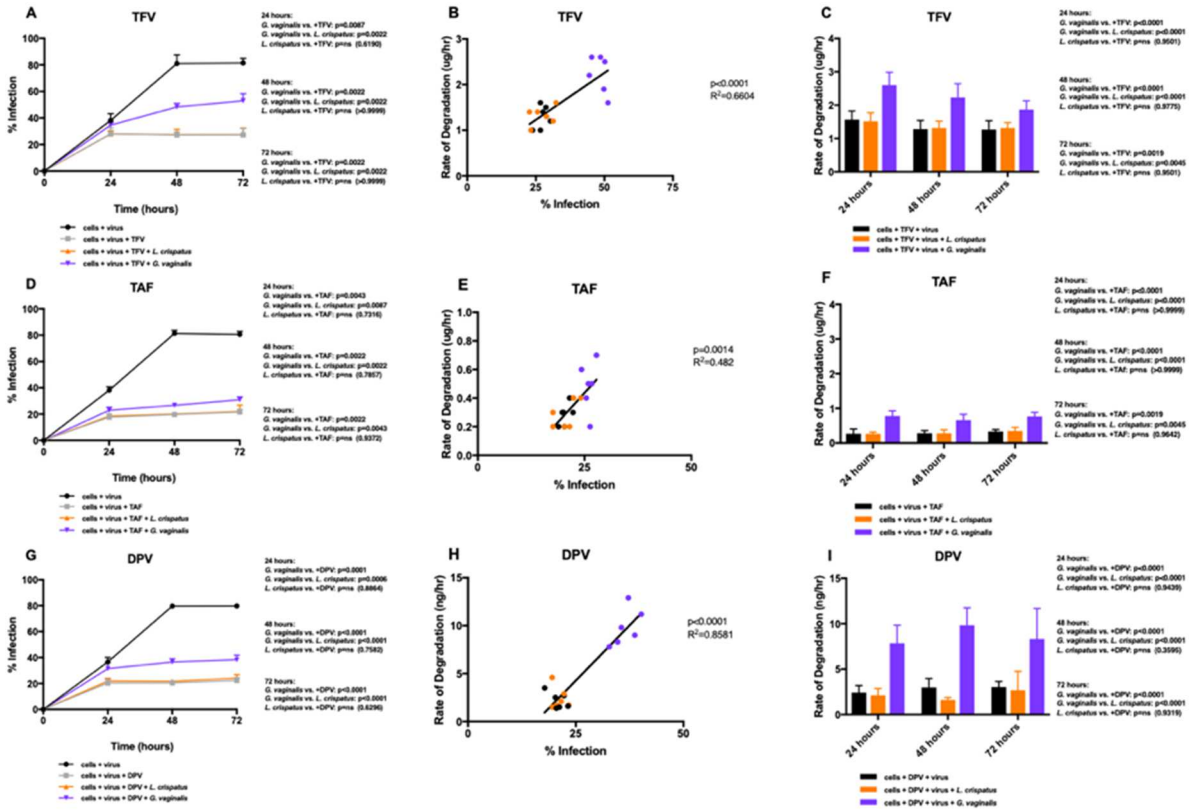


Figure 4. 9: HIV-1_{LAI} Infection of CEM-GFP Cells after Incubation at 37C

(A) % Infection of CEM-GFP cells over time up to 72 hours post incubation with 1) CEM-GFP cells + HIV-1_{LAI} 2) CEM-GFP cells + HIV-1_{LAI} + TFV 3) CEM-GFP cells + HIV-1_{LAI} + TFV + *L. crispatus* 4) CEM-GFP cells + HIV-1_{LAI} + TFV + *G. vaginalis*. CEM-GFP cells were incubated for 30 minutes with bacterial

inoculum. TFV was added and allowed to incubate for 1.5 hours prior to HIV-1_{LAI} addition. Purple *G. vaginalis*; Orange, *L. crispatus;* Black, virus alone; Gray, virus and TFV. N=6

(B) Rate of TFV degradation versus % infection of CEM-GFP cells 48 hours after incubation with virus. Purple *G. vaginalis*; Orange, *L. crispatus;* Black, TFV alone. N=6

(C) Rate of TFV degradation over time up to 72 hours. Purple *G. vaginalis*; Orange, *L. crispatus;* Black, TFV alone. N=6

(D) % Infection of CEM-GFP cells over time up to 72 hours post incubation with 1) CEM-GFP cells + HIV-1_{LAI} 2) CEM-GFP cells + HIV-1_{LAI} + TAF 3) CEM-GFP cells + HIV-1_{LAI} + TAF + *L. crispatus* 4) CEM-GFP cells + HIV-1_{LAI} + TAF + *G. vaginalis*. CEM-GFP cells were incubated for 30 minutes with bacterial inoculum. TAF was added and allowed to incubate for 1.5 hours prior to HIV-1_{LAI} addition. Purple *G. vaginalis*; Orange, *L. crispatus;* Black, virus alone; Gray, virus and TAF. N=6

(E) Rate of TAF degradation versus % infection of CEM-GFP cells 48 hours after incubation with virus. Purple *G. vaginalis*; Orange, *L. crispatus;* Black, TAF alone. N=6

(F) Rate of TAF degradation over time up to 72 hours. Purple *G. vaginalis*; Orange, *L. crispatus;* Black, TAF alone. N=6

(G) % Infection of CEM-GFP cells over time up to 72 hours post incubation with 1) CEM-GFP cells + HIV-1_{LAI} 2) CEM-GFP cells + HIV-1_{LAI} + DPV 3) CEM-GFP cells + HIV-1_{LAI} + DPV + *L. crispatus* 4) CEM-GFP cells + HIV-1_{LAI} + DPV + *G. vaginalis*. CEM-GFP cells were incubated for 30 minutes with bacterial inoculum. DPV was added and allowed to incubate for 1.5 hours prior to HIV-1_{LAI} addition. Purple *G. vaginalis*; Orange, *L. crispatus;* Black, virus alone; Gray, virus and DPV. N=6

(H) Rate of DPV degradation versus % infection of CEM-GFP cells 48 hours after incubation with virus. Purple *G. vaginalis*; Orange, *L. crispatus;* Black, DPV alone. N=6

TFV uptake into Jurkat cells and *G. vaginalis* is inhibited by endocytosis inhibitors

Given our findings, we investigated potential uptake mechanisms for how TFV is metabolized by *G. vaginalis.* Using Jurkat cells, we assessed extracellular TFV levels after 24 hours of co-culture with different bacteria. We monitored TFV levels after co-culture with dimethyl amiloride (DMA), a known NA^+-H^+ exchanger inhibitor, in the presence of Jurkat cells only, *G. vaginalis only,* and Jurkat cells with *G. vaginalis* (Fig. 4.10). Similar experiments were done using *L. iners* (Fig. 4.10).

Following the administration of DMA, we saw an increase in extracellular TFV in the presence of Jurkat cells when compared with Jurkat cells alone ($p=0.0003$, Fig. 4.10). Similarly, the addition of DMA also increased extracellular TFV levels in the presence of *G. vaginalis* when compared with *G. vaginalis* alone ($p<0.0001$, Fig. 4.10. Together, the effects of Jurkat cells and *G. vaginalis* on extracellular TFV levels were negated with the addition of DMA ($p<0.0001$, Fig. 4.10). Conversely, the administration of DMA to *L. iners* co-cultures had no impact on extracellular TFV levels when compared with *L. iners* alone ($p>0.9999$, Fig. 4.10).

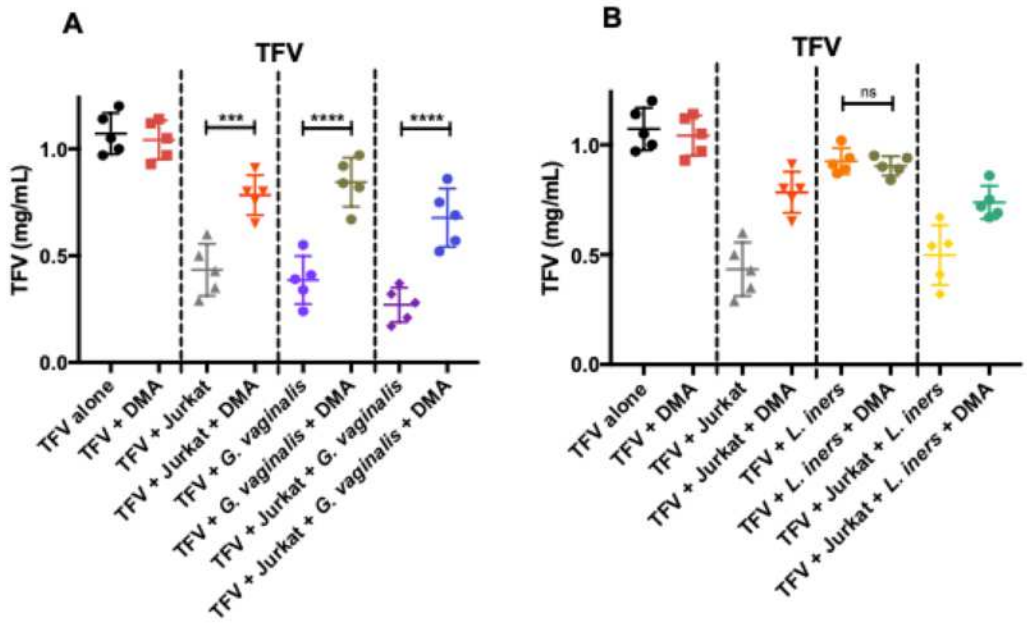


Figure 4. 10: TFV Remaining After 24 Hours of Incubation with Endocytosis Inhibitor, DMA

(A) TFV remaining after 24 hours based on 1) TFV alone (black) 2) TFV + DMA (red) 3) TFV + Jurkat (gray) 4) TFV + Jurkat + DMA (dark orange) 5) TFV + *G. vaginalis* (purple) 6) TFV + *G. vaginalis* + DMA (dark green) 7) TFV + Jurkat + *G. vaginalis* (dark purple) 8) TFV + Jurkat + *G. vaginalis* + DMA (dark blue). Jurkat cells were exposed to 100uM DMA for 30 min prior to treatment with TFV (1mg/mL) and *G. vaginalis*. Co-culture experiments were added at a 2.5:1 ratio of bacteria to cells. N=5
(B) TFV remaining after 24 hours based on 1) TFV alone (black) 2) TFV + DMA (red) 3) TFV + Jurkat (gray) 4) TFV + Jurkat + DMA (dark orange) 5) TFV + *L. iners* (orange) 6) TFV + *L. iners* + DMA (dark green) 7) TFV + Jurkat + *L. iners* (yellow) 8) TFV + Jurkat + *L. iners* + DMA (teal). Jurkat cells were exposed to 100uM DMA for 30 min prior to treatment with TFV (1mg/mL) and *L. iners*. Co-culture experiments were added at a 2.5:1 ratio of bacteria to cells. N=5

Discussion

This study provides the first detailed characterization of the impact of the vaginal microbiome on anti-retrovirals drug PK/PD using both *ex vivo* and *in vitro* assays. Using primary CVL samples, we confirmed that TFV is metabolized by vaginal bacteria from women with dysbiosis and demonstrated for the first time a decrease of DPV in the presence of vaginal dysbiosis. In addition, we describe that TAF does not exhibit prominent bacteria-mediated degradation.

As previously demonstrated, TFV exhibits poor permeability into cell membranes and relies on an energy-dependent uptake mechanism mediated by uptake transporters or endocytosis[208]. Previous reports have identified organic anion transporters (OAT) 1 and 3 playing a key role in TFV uptake[208]. TAF is a newer TFV based compound, approved for treatment of HIV and

undergoing evaluation as a PrEP agent, characterized for rapid cell uptake. TAF is much more lipophilic than TFV due to the amidate group and relies primarily on passive diffusion, and is characterized by a rapid cell uptake[208]. DPV has been used in two major trials, ASPIRE and the Ring Study, where DPV vaginal rings were used for HIV prevention, with efficacy of 37% and 31%, respectively[209,210].

Here we calculated the degradation efficiency as rate of degradation as amount lost per hour. The inverse relationship of the relative abundance of *Lactobacillus* and the lower the rate of TFV and DPV degradation emphasizes the importance of *Lactobacillus* in preventing host-microbe drug degradation. While drug levels do highlight the potential for these interactions, in order to elucidate the effect on pharmacodynamically active metabolites, we assessed intracellular active drug levels. *Lactobacillus* dominance impacts extracellular and intracellular TFV and DPV concentrations. As a result, the amount of *Lactobacillus* also impacts formation of intracellular TFV-DP and accumulation of intracellular DPV respectively. Rates of intracellular formation also correlate with how much *Lactobacillus* is present, further emphasizing the importance of *Lactobacillus* dominance. Our data also support that TAF conversion to TFV-DP is mostly unaffected by vaginal microbiota, potentially due to more rapid uptake mechanisms than TFV or DPV.

Taken in this context, our results indicate an additional explanation for low efficacy in PrEP trials in women. While high adherence is certainly critical to protection, our studies suggest that lack of effectiveness and lower drug levels may be driven by vaginal microbial communities. Despite a recent post-hoc analysis of the Partners PrEP study demonstrating no difference in oral PrEP efficacy in BV versus non-BV women defined by Nugent score[84], our study identifies a gap in accurate diagnosis and how specific bacteria, identified using 16S rRNA, are better predictors of bacteria-mediated metabolism than by using Nugent score only (Figure 4.11). Despite a recent

study highlighting production of TFV-DP from *L. crispatus*, we saw no TFV-DP formation when cultured with TFV alone and attribute this difference to different culturing media used[211].

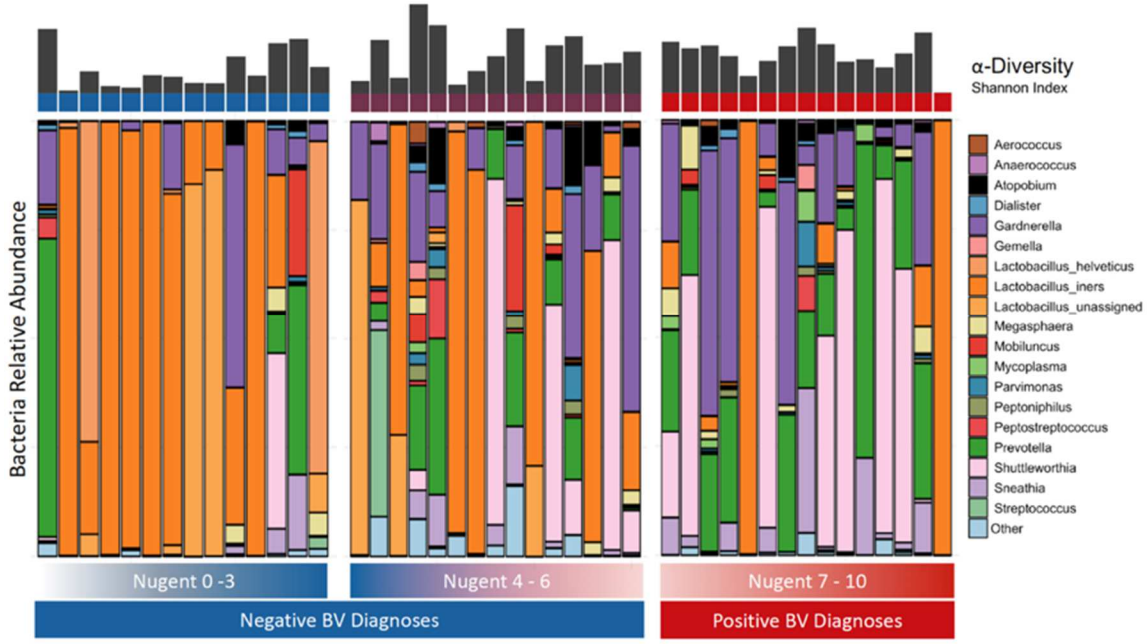


Figure 4. 11: Bacterial Relative Abundance Ranked by Nugent Score

(A) Relative abundance of CVLs from 44 women with and without diagnosed BV grouped by Nugent score. Nugent score cutoffs were 0-3, 4-6, and 7-10. The 19 most abundant phyla are shown. Shannon diversity plots showing alpha diversity in CVLs. Blue, BV negative by Nugent score at time of collection; Red, BV positive by Nugent score at time of collection.

Further studies are needed to elucidate the discrepancies between Nugent score and 16S rRNA sequencing, and to improve diagnostic tools of BV in clinical practice and to predict efficacy of PrEP. Furthermore, better understanding the role of both vaginal and gastrointestinal microbiota on metabolism of oral PrEP is urgently needed.

While the prevalence of *Lactobacillus* dominance seems to be the major driving factor in bacteria-mediate metabolism, our *in vitro* cultures also elucidated specific bacterial species with efficiency of conversion to active drug compounds. *G. vaginalis* has been previously shown to cause biodegradation in the CAPRISA 004 samples[3] and here, significantly alters the efficiency in TFV, TAF, and DPV. Of note, TAF uptake efficiency in the presence of *G. vaginalis* was not

significantly different than the uptake efficiency of TFV with cells alone, again emphasizing the difference in uptake mechanisms exhibited by these two compounds. The DMA studies highlight potential mechanisms into how TFV is uptaken into Jurkat and *G. vaginalis* cells. The use of an endocytosis inhibitor, DMA, prevented intracellular uptake into *G. vaginalis* highlighting a potential preferential targeting of TFV by *G. vaginalis*. This is a phenomena not seen with *L. iners*. Further studies are needed to determine whether *G. vaginalis* uptake can be inhibited specifically, thus negating the effects caused by these dysbiotic microbes. In doing so, women with *G. vaginalis* may be protected against bacteria-mediated metabolism of TFV. Future studies should also include the use of other *Lactobacillus spp.* as well as other PrEP therapies.

Our *in vitro* studies also allowed us to explore potential therapeutic benefits as well as mimic samples from women who are transitioning from non-BV to BV. Our co-cultures with combinations of *Lactobacillus* spp. and *G. vaginalis* highlight the potential for a therapeutic benefit of administering *Lactobacillus* to women with BV to prevent these negative interactions with drugs. Of note, when doing a mixed culture with TAF, we completely negate even the minor effects caused by *G. vaginalis*. It appears both healthy vaginal microbial communities and drug compound structure and uptake play a significant role in varying efficacy.

Our study not only has demonstrated *Lactobacillus* association with altering drug levels and impacting formation of intracellular active metabolites, but also affecting productive infection of cells *in vitro*. Our infection assays further validated the impact *G. vaginalis* can have on productive cell infection. As soon as 24 hours and as long as 72 hours post inoculation, we see how the presence of *G. vaginalis* enhances cell productive infection through drug metabolism. We observed similar trends using DPV and with TAF supporting the notion that some BV-associated bacteria linked to bacterial-mediated drug metabolism have no preference of NRTIs over NNRTIs.

Limitations of this study include lack of longitudinal samples where shifts in bacterial communities could be monitored and assessed for corresponding drug level changes. Furthermore, lack of samples available from clinical trials with DPV or TAF as a PrEP drug in women is a limitation. Additional studies quantifying bacterial concentrations via qPCR would also provide more information regarding these host-microbe drug interactions. Future studies should also include evaluating other BV-associated bacteria such as *Prevotella, Mobiluncus, Shuttleworthia,* and *Sneathia.* Our preliminary studies have demonstrated that multiple bacteria can biodegrade TFV and DPV *in vitro*, thus future studies using mixed cultures is an important follow up.

Beyond our group, other investigators are beginning to identify more microbe-TFV interactions. In the Partners PrEP study, the efficacy of oral PrEP was compared among women with abnormal vs normal vaginal microbiota. Their study did not identify any significant differences in efficacy regardless of BV status. Instead of using a molecular diagnosis such as 16S rRNA analysis, this group used a Nugent score system[84]. As detailed above, using Nugent scoring is potential not as effective in identifying the correct microbes, the ones that can play a role in altering drug concentrations. Similarly, another group identified tenofovir uptake into human cells being reduced as vaginal pH increased. This supports our findings in that a women with a loss of *Lactobacillus* loses the main source of lactic acid production, which keeps the vaginal microbiome at a low pH. Additionally, this group identified inhibition of endocytosis of TFV due to adenine, which was increased in samples cultured with *G. vaginalis*[211]. They did however, identify a few differences from our study. While we observed effects of *G. vaginalis* on DPV, they attributed the impact as a result of drug binding[211] to bacteria rather than bacteria induced metabolism. Similarly, they identified *Lactobacillus crispatus* as being able to actively transport tenofovir[211]. This study, however, uses 2 major differences that could be attributed to the

observed discrepancies. To measure their TFV-DP levels from Jurkat cells, this group does not use LC-MS/MS but rather liquid scintillation counting which only measures radioactivity. If a metabolite was being broken down extracellularly or transported out of the bacteria cell upon bacteria mediated metabolism, the radioactivity would measure the same. The second major difference is the strain difference of *L. crispatus*. While exploratory studies are currently underway, it is not well known how the strain to strain variability alters these bacteria induced metabolic events. The strain of *L. crispatus* (ATCC vs. clinical isolate) could explain the differences seen in TFV interactions with the species.

Chapter V
Discussion, Conclusions, and Future Directions

Summary of Major Findings

The broad purpose of this thesis was to address several unanswered questions relating to mechanisms for increased HIV transmission within an altered microbiome within the FRT and investigating better potential therapies for HIV prevention, summarized in Chapter I. In Chapter II, we evaluated the microbial communities from cervico-vaginal cytobrushes and performed in-vitro co-culture assays to assess the impact of dysbiosis on neutrophil accumulation and their contribution to tissue damage. We sought to answer several specific questions: 1) How well does Nugent score identify microbial communities? 2) How does a dysbiotic community, consisting of non-*Lactobacillus* dominance, impact neutrophil lifespan and accumulation? 3) Are these neutrophils activated? 4) What specific taxa within BV communities can impact neutrophil accumulation and activation? 5) Do these bacteria cause neutrophils to increase tissue damage?

We hypothesized that a shift from a *Lactobacillus* dominant to a diverse community consisting of BV-associated bacteria would increase neutrophil lifespan and lead to accumulation within the FRT as neutrophils are often the first responders to pathogens due to their anti-microbial properties. Similarly, we hypothesized that this change in neutrophil lifespan and this activation of neutrophils would be driven by specific BV-associated bacteria such as *G. vaginalis*. Due to the release of reactive oxygen species and potential to cause epithelial barrier damage, we hypothesized that these pathogenic bacteria would indeed cause barrier damage via neutrophils. We found that 16S rRNA analysis is a far better evaluation tool in identifying community structure versus Nugent scoring. Additionally, we found that the community shift to a non-*Lactobacillus* dominant environment led to increased activated neutrophils and increased neutrophil and accumulation within the FRT. Furthermore, we identified three specific taxa that

cause these changes in-vitro in *G. vaginalis, P. bivia,* and *A. vaginae*. We found that these three bacteria also cause increased barrier damage from neutrophils. Our data demonstrate that neutrophil accumulation within the FRT during BV has the propensity to exacerbate mucosal tissue damage and dysfunction. Neutrophils exhibited reduced homeostatic apoptosis and increase lifespan during inflammatory conditions such as IBD, yet no prior studies had examined neutrophil apoptosis as a potential mechanism for neutrophil accumulation in the FRT during BV or the potential connection between neutrophil lifespan and BV-associated bacteria. These pathogenic bacteria drive neutrophil accumulation and increased lifespan due to the first response and anti-microbial function of neutrophils. Future interventions need to eradicate BV bacteria to prevent unwanted damage cause by these neutrophils that may be contributing to increased HIV transmission risk through inflammation and barrier damage cause by neutrophil proteases. The ability of *Lactobacillus* spp. to drive neutrophil apoptosis has broader implications for inflammatory conditions other than HIV. The use of vaginal suppositories or probiotics can reduce excessive inflammation in several disease states. Further studies are needed to determine the mechanisms involved in the ability of *Lactobacillus* to increase neutrophil apoptosis in order to elucidate how *Lactobacillus* may be used as a therapy for neutrophil-driven inflammation. Overall, this study provides increased understanding of relationships between neutrophils, vaginal dysbiosis, and the mucosal barrier and elucidates potential mechanisms of increased HIV transmission in women with BV.

In Chapter III, we evaluated the impact microbial communities can have on HIV prevention strategies, notably PrEP. We sought to answer several questions: 1) Does BV affect trial efficacy? 2) Are specific bacteria involved in lower PrEP drug levels? 3) By what mechanism do these bacteria alter PrEP drug levels? 4) Do these interactions happen at a relevant physiological rate?

We hypothesized that BV status would impact HIV acquisition and that women with BV would have decreased PrEP drug levels. Similarly, we hypothesized that bacteria such as *G. vaginalis*, would be directly involved with lower drug levels due to bacteria-mediated drug metabolism, notably the scavenging for the base nucleotide adenine. Lastly, we hypothesized that this metabolism would occur faster than target cell uptake thus providing a potential mechanism for how BV bacteria decrease PrEP drug levels and impact HIV transmission. We found that women with a *Lactobacillus* dominant vaginal microbiome had significantly higher efficacy when compared to women with a non-*Lactobacillus* dominant community. We identified *G. vaginalis* as bacterium that metabolized tenofovir via cleaving the oxy-phosphonic acid resulting in a metabolite formation of adenine. Additionally, we found that his metabolism would impact uptake efficiency of tenofovir and result in decreased intracellular active metabolites of tenofovir. In Chapter IV, we built upon this bacteria-mediated metabolism and focuses on the specific questions: 1) Would these interactions occur with bacteria from ex-vivo samples? 2) Do other bacteria besides *G. vaginalis* also metabolize tenofovir? 3) What about other drugs? 4) Would this metabolism impact formation of the active metabolite TFV-DP into target cells? 5) Can we alleviate this bacteria metabolism? 6) Does this metabolism directly impact HIV infection?

We hypothesized that using bacteria from clinical samples would also exhibit tenofovir metabolism and that this would not be exclusive to just *G. vaginalis* nor tenofovir. We also hypothesized that this metabolism would impact PD relevant metabolites from all PrEP drugs and that this metabolism would not only impact drug levels, but cell productive infection as well. We found that bacteria from ex-vivo samples did indeed metabolize tenofovir but also DPV. While we did not see metabolism from TAF, we do not rule out the possibility that metabolism can occur. Additionally, we identified other potential bacteria that may decrease drug levels such as BVAB-1 and *Prevotella* spp. Of note, the bacteria-mediated drug metabolism did decrease intracellular active metabolite formation within target cells and our HIV infection

studies highlighted how this metabolism can alter HIV productive infection. Additionally, our study has demonstrated a potentially therapeutic intervention for at-risk women with BV through our combination co-cultures using both *Lactobacillus* and *G. vaginalis* where the addition of both bacteria did increase drug levels versus just *Gardnerella* alone.

Taken together, the data presented in this thesis improves our understanding of the mechanisms contributing to HIV transmission and BV and specifically the role of the vaginal microbiome in barrier damage, inflammation and drug metabolism.

Future Directions and ongoing Studies

Several important unanswered questions remain that should be investigated in future studies. First, how these BV-associated bacteria alter neutrophil phenotypes and the mechanism by which they increase neutrophil lifespan. Additionally, while the propensity for neutrophils to cause barrier and tissue damage is apparent, the extent to how much they contribute and the impact on HIV transmission is unknown. Future studies could identify HIV infection rates in the presence of BV-associated and healthy commensal bacteria and their breaching of barriers due to neutrophil-induced damage.

Similarly, identifying the specific enzymes by which bacteria such as *G. vaginalis* metabolize PrEP drugs should be included in future studies. Identifying enzymes involved would be useful in potential future therapeutic interventions where high expression of these enzymes would result in either antibiotic use to eradicate the bacteria or an enzyme inhibitor to prevent increased HIV transmission risk. Additionally, identifying specific taxa that can impact drug levels to the point where HIV infection rates are increased would also be valuable future studies.

Broader Implications

Women are a highly underserved community when it comes to HIV prevention and treatment research. As a unique microenvironment, the FRT of reproductive age women is modulated by hormones and protected by mucosal cells and vaginal secretions. The likelihood that HIV infects women via vaginal intercourse can be increased by behavioral or biological factors that affect the integrity of the FRT mucosa. Examination of factors that impact FRT vulnerability of reproductive age women can provide important guidance towards strategies to prevent HIV infection in this highly vulnerable population.

The work presented in this thesis has important implications for HIV transmission. These data can be applied to investigate new HIV prevention strategies and enhance our current ones. Here, we begin the investigation into fully elucidating the ongoing neutrophil accumulation and association with HIV risk and this transient neutrophil recruitment within the FRT. Reducing neutrophil recruitment, accumulation, and inflammation within the FRT is a promising HIV prevention strategy. These studies also elucidate potential interactions between drugs and microbes, providing mechanisms for the variability seen in translating pharmacokinetics and pharmacodynamics in clinical trials, as well as highlighting the significant role the microbiome plays in drug uptake to target sites and systemic availability, and how this may affect virus transmission and treatment. Women are among the most vulnerable and understudied in HIV prevention and treatment research. Understanding the interactions between vaginal bacterial and PrEP drugs is essential to better design and conducting studies assessing HIV prevention among women and ameliorating the effect of the HIV epidemic in this highly vulnerable population.

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