EXAMINING ABIOTIC AND BIOTIC FACTORS INFLUENCING BACTERIAL AND HOST INTERACTIONS IN THE FEMALE REPRODUCTIVE TRACT

EXAMINING ABIOTIC AND BIOTIC FACTORS INFLUENCING BACTERIAL AND HOST INTERACTIONS IN THE FEMALE REPRODUCTIVE TRACT

By Haley A. Dupont, B. Sc

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AUTHOR: Haley A. Dupont, B.Sc. (Mount Allison University)

SUPERVISOR: Dr. Charu Kaushic

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ABSTRACT

Background: Currently, the leading route of new HIV-1 infection is via heterosexual transmission, in which women are disproportionately burdened. As such, understanding the mechanisms that regulate susceptibility in the female reproductive tract is imperative. One key factor associated with a fourfold increased risk of HIV-1 acquisition is a dysbiotic vaginal microbiota (VMB). A dysbiotic VMB is characterized by a diverse mix of anaerobic species without any appreciable amounts of beneficial *Lactobacillus* species. Our understanding of the species-specific manner by which vaginal bacteria interact with one another and with the host to induce susceptibility remains incompletely understood. Moreover, without an effective strategy to select for an optimal *Lactobacillus*-dominant VMB, characterization of vaginal bacteria is required to develop more rational preventative and therapeutic options. With this, this study was designed to elucidate the interactions between common vaginal bacteria and host vaginal epithelial cells. The phenotypic and metabolic characteristics of these bacteria were also examined to provide a deeper understanding about the conditions in which each species may be able to survive and thrive.

Method of Study: An *in vitro* cell culture system was used to grow vaginal epithelial cells in an air-liquid interface. Cells were subsequently cocultured with one or two vaginal bacterial species for 24 hours. Factors that may lead to increased susceptibility to HIV-1 infection, including cell viability, epithelial barrier integrity, cytokine production and bacterial adherence were assessed. Vaginal bacteria were also phenotypically analyzed for hemolysis, hydrogen peroxide production, ability to alter pH, inhibition activity, and their metabolic ability to grow on various nutritional resources. Common vaginal bacteria analyzed included

dysbiosis associated species *Gardnerella vaginalis* and *Prevotella bivia*, as well as *Lactobacillus* species *L. crispatus* and *L. iners*.

Results: The presence of *P. bivia, G. vaginalis* and *L. iners* resulted in reduced viability of vaginal epithelial cells, reduced barrier integrity and the production of pro-inflammatory cytokines. Conversely, the presence of *L. crispatus* did not, and was able to negate these adverse effects when placed in a dual species coculture with either of the other species. Additionally, we found that *L. crispatus* was the only one of these four species to produce hydrogen peroxide, and its supernatant was capable of inhibiting the growth of *G. vaginalis* and *P. bivia*. While we found that all four vaginal species could use glycogen for their growth, *L. crispatus* was able to use the widest range of carbohydrates tested. This translated to *L. crispatus* significantly outcompeting the other three bacterial species when cocultured in bacterial broth media with various carbohydrates tested.

Conclusions: Our data provides insight into the species-specific nature by which common vaginal bacteria may interact with vaginal epithelial cells to increase host susceptibility to infection through cytotoxicity, decreased barrier function, and inflammation. While this was found for *P. bivia, G. vaginalis* and *L. iners*, we importantly observed the ability of *L. crispatus* to largely mitigate these effects. With our phenotypic characterization highlighting various defense mechanisms as *L. crispatus'* disposal and its ability to outcompete the other vaginal bacteria, our findings place *L. crispatus* as the species most adept to provide protection in the FRT. Together, this work contributes to a better understanding of the interactions that govern the dynamics of the VMB and can be built upon to develop more rationale therapeutic or prophylactic interventions to improve the reproductive health of many vulnerable women.

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All experiments were conceived and designed by Haley Dupont and Dr. Charu Kaushic. Dr. Nuch Tanphaichitr provided bacterial strain *L. crispatus* (SJ-3C-US). Haley Dupont performed all experiments. Haley Dupont wrote this dissertation with contributions from Dr. Charu Kaushic.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

AIDS: Acquired Immunodeficiency Syndrome

ALI: Air-Liquid Interface

ATCC: American Type Culture Collection

BV: Bacterial Vaginosis

CFU: Colony Forming Units

CST: Community State Type

CVL: Cervicovaginal Lavage

FRT: Female Reproductive Tract

FITC: Fluorescein Isothiocyanate

H_2O_2: Hydrogen Peroxide

HESN: Human Immunodeficiency Virus Exposed Seronegative

HIV: Human Immunodeficiency Virus

IL-1RA: Interleukin-1 Receptor Antagonist

IL-1α: Interleukin 1 alpha

IL-1β: Interleukin 1 beta

IL-6: Interleukin 6

IL-8: Interleukin 8

kDa: Kilodalton

KSFM: Keratinocyte Serum Free Media

LDH: Lactate Dehydrogenase

LGT: Lower Genital Tract

MIP-1α: Macrophage Inflammatory Protein 1 alpha

MPA: Medroxyprogesterone Acetate

MRS: De Man, Rogosa and Sharpe

N-9: Nonoxynol 9

OD: Optical Density

RANTES: Regulated on activation, normal T cell expressed and secreted

ROS: Reactive Oxygen Species

STI: Sexually Transmitted Infection

TER: Transepithelial Resistance

TLR: Toll Like Receptor

TNF-α: Tumor Necrosis Factor alpha

UGT: Upper Genital tract

VMB: Vaginal Microbiota

CHAPTER 1: INTRODUCTION

1.1 HIV-1 Epidemiology in Women:

Despite the considerable advances made to reduce the morbidity and mortality of human immunodeficiency virus (HIV-1) infection since the onset of the epidemic, approximately 1.8 million new HIV-1 infections still occur annually[1]. Sub-Saharan Africa is the epicentre of the global HIV epidemic, in which they bear nearly two thirds of all new infections and 75% of AIDS-related mortality[1]. With this, significant heterogeneity exists in terms of certain populations being consistently more vulnerable to infection than others. Focusing HIV-1 prevention efforts on such high-incidence populations is likely to enable the greatest gains to be made in altering current epidemiological trajectories towards control of the HIV epidemic. An important key population in sub-Saharan Africa is young women and adolescent girls aged 15-24 years old, who comprise nearly 25% of all new HIV-1 infections in the region despite representing just 10% of the population[1,2]. This percentage translates to more than four-times of their male counterparts. On the population level, the high incidence in young women is sustaining intergenerational transmission of HIV-1 and contributes to the overall disproportionate burden of HIV-1 in women compared to men[1]. Indeed, approximately 60% of all people living with HIV-1 globally are women. As such, preventing HIV-1 infection in adolescent girls and young women who are at a distinctly high risk of acquisition is crucial to achieve the challenging goal of accomplishing an AIDS-free generation and/or epidemic control. However, despite this imperative, evidence-based prevention options available to adolescent girls and young women remain limited, and thus immediate action is needed to mediate the risk of this key population.

1.1.2 HIV-1 Infection in Women:

Worldwide, the most common mode of transmission of HIV-1 is across the genital mucosa[3]. In order for HIV-1 to establish productive infection in the female reproductive tract (FRT), infectious virions from the donor must cross the epithelial barrier to infect target cells[4]. HIV-1 infects cells expressing the CD4 receptor and either a CXCR4 or CCR5 co-receptor. HIV-1 that utilizes the CXCR4 co-receptor for entry is defined as an X4 tropic viruses, while those that use CCR5 are R5 tropic viruses[5]. While dendritic cells and macrophages are susceptible targets, HIV-1 preferentially infects CD4+ T cells which are generally considered to be the initial subset of cells infected with HIV-1. Moreover, the majority of productive HIV-1 infections are caused by R5 tropic viruses[6]. The cells co-expressing CCR5 can spread to the associated iliac lymph nodes and later disseminate systemically with associated viremia within 4 to 11 days[4,7]. Within 30 days of infection, massive viral replication with the death of many CD4 T cells occurs, especially those in the gut-associated lymphoid tissue[8]. The immune response eventually stabilizes the viral replication leading to a viral load set point within approximately 2 months, in which subsequently neutralizing antibodies are produced[9]. Symptoms of acute HIV-1 infection may reflect the immune response to the virus, particularly the cytokine storm that follows infection, with resolution upon the control of viral replication[3,10]. Higher viral load and lower CD4 count at seroconversion as well as a high viral load set point have all been associated with a higher likelihood of disease progression[11]. This combined with the fact that there is not a complete cure for HIV-1, efforts for preventing initial infection is imperative to protect those most vulnerable[3].

1.2 Female Reproductive Tract:

With the current leading route for transmission of HIV-1 being via heterosexual transmission, in which young women are disproportionately burdened, it is important to analyze the protective mechanisms of the FRT[1]. The FRT contains a number of structural, environmental, and immunological defense mechanisms that protect the host from a diverse array of potentially pathogenic organisms, including HIV-1. A key physical defense mechanism of the FRT is the epithelial barrier[12]. In the upper FRT, consisting of the endocervix, uterus and fallopian tubes, is a single layer of columnar epithelial cells joined by tight junction proteins. Conversely, the lower FRT, consisting of the vagina and ectocervix, is lined by a multilayered squamous epithelium. Although HIV-1 virions can transmit through both the upper and lower FRT, the lower FRT has the greatest surface area and exposure to semen carrying HIV-1 and is therefore the more likely site for infection to occur. The multilayered squamous epithelium includes a mitotically active basal layer and a terminally differentiated superficial layer of flattened cornified cells which serve to form a barrier against invasive pathogens[13]. Epithelial integrity is mediated by protein structures acting to adhere cells to one another (i.e. tight and adherence junctions) or to the extracellular matrix. Additionally, epithelial cells in the FRT secrete a dynamic fluid composed of glycosylated mucins and a diverse array of defense molecules[14]. This hydrophobic layer of mucus contains hundreds to thousands of soluble proteins, including immune factors and antimicrobial agents, mucins and antiproteases, which all serve to provide further protection against invading organisms and epithelial damage[15]. Importantly, the mucus itself provides a substantial physical barrier against HIV-1 migration and penetration and has been shown to effectivity trap HIV-1 virions *ex vivo*[16]. Importantly, these barriers are in direct contact with

the indigenous vaginal microbiota (VMB), which has long been recognized to influence protection and susceptibility in the FRT[17]. The exact nature of the VMBs influence on the intricate network of interactions between host cells and mucosal, endocrine, and immunological factors that work in concert to influence susceptibility in the FRT is current area of intense research. Underlying the epithelium is a dense layer of stromal fibroblasts which contains a diverse population of leukocytes, including $CD4^+$ HIV-1 target cells[18]. As such, HIV virions must transverse the epithelial barrier to reach their target cells in order to establish productive infection.

1.2.1 Female Susceptibility to Sexually Transmitted Infections:

Despite the considerable resources devoted to understanding the pathogenesis of HIV-1, the mechanisms regulating susceptibility in the FRT remain incompletely understood. With this, elucidating the conditions that increase susceptibility to an otherwise rare transmission event is essential to understand before effective prophylactic measures can be taken. Per coital frequency of HIV-1 transmission is quite low, being approximately 0.1% for unprotected vaginal intercourse, demonstrating the effectiveness of the inherent FRT barriers[19]. As such, understanding the factors that are contributing to the disproportionally high rates of new HIV-1 infection in women is imperative.

The act of sexual intercourse itself can result in micro-abrasions in the mucosal surface, resulting in wound healing processes and increased vascularity, infiltration and recruitment of immune cells, and increased inflammatory cytokines and proteins[21]. Semen itself is highly basic and increases vaginal pH, which may in turn alter the protective mucus layer in the FRT[20]. Semen exposure also increases pro-inflammatory cytokines in the FRT

compared with protected intercourse. HIV-1 can penetrate as much as 10 μm into the squamous epithelium in the FRT where target cells reside, and this invasion is significantly increased upon tight or adhesive junction disruption[21]. While micro-abrasions in the FRT occur even during consensual intercourse, any factors that serve to further disrupt the barrier or to increase the number of available target cells, their activation status and/or dissemination could affect the likelihood of productive infection.

1.2.2 Mucosal Inflammation and Barrier Disruption:

Initial inflammatory responses in the FRT are largely initiated by epithelial cells through activation of pattern recognition receptors, which induces the secretion of soluble defense factors (i.e. antimicrobial peptides, AMPs) and cytokines to stimulate a response from immune cells. An inflammatory cascade at the mucosal level, despite having factors that can inhibit HIV-1 virions, can increase risk of HIV-1 infection[22]. Indeed, increased levels of α-defensins and/or pro-inflammatory cytokines/chemokines are associated with increased rates of HIV-1 acquisition. Recently, in the CAPRISA-004 trial, it was found that women who had elevated levels of pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-8, TNF-α, MIP-1α and IP-10, had a threefold increased risk of HIV-1 infection[23]. An increased pro-inflammatory cytokine profile is associated with increased protease levels, barrier disruption and increased frequency of CD4+ T cells[24].

Conversely, reduced inflammation and immune activation in the FRT is associated with HIV-1 protection[25]. This has largely been demonstrated from HIV exposed seronegative (HESN) women, where reduced immune activation of HIV-1 target cells, along with lowered mucosal cytokine/chemokine profiles may limit target cell abundance and activation and

therefore reduce susceptibility to infection[26]. Additionally, elevated levels of protective proteins, such as serpins and antiproteases, are important for direct inhibition of proteases which are important for immune cell migration, activation and barrier disruption[27]. As such, it has long been recognized that soluble factors within the mucosa are important factors in affecting inflammation status and the likelihood of HIV-1 transmission.

1.3 Vaginal Microbiota:

Understanding that factors that can modulate inflammation and barrier function in the FRT may be critical in the quest to control the high prevalence of new HIV-1 infections in the women at highest risk of infection. One key factor associated with a fourfold increased risk of HIV-1 acquisition is bacterial vaginosis (BV)[28,29,30]. BV is defined as a shift from a protective VMB with low diversity and dominated by *Lactobacillus* species, to an unfavourable microbiota comprised of a diverse mix of anaerobes with low levels of lactobacilli[31,32]. While BV has been used as an umbrella term to define diversity of the VMB, not all cases are associated with the symptomatic condition. As such, there is a push to more inclusively define such compositions as dysbiotic. Importantly, a diverse anaerobic microbiota, whether symptomatic or asymptomatic, is prevalent in the young women in sub-Saharan Africa who are at the greatest risk of infection[33]. As the etiology of increased diversity remains elusive, the mechanisms by which more protective or dysbiotic bacterial communities are able to colonize and persist in the FRT are critical to understand. Elucidating the interactions between the host and vaginal bacteria that ultimately determine the composition and maintenance of the vaginal microbiota can identify novel interventions

to elicit stable changes in bacterial community composition in the FRT to reduce HIV-1 acquisition in the women most susceptible.

1.3.1 Vaginal Microbiota Composition:

The bilateral interaction between the host and its VMB plays a crucial role in reproductive health and susceptibility to infections in the FRT. Recently, culture-independent analysis of the VMB has identified five distinct microbial communities with notable differences in species composition and frequency[34]. These defined microbial communities are either dominated by one of four *Lactobacillus* species or are composed of a wide range of mixed anaerobes without any appreciable quantities of lactobacilli. Importantly, these communities are associated with significantly different levels of protection against STIs, including HIV-1[28,30,35].

1.3.2 *Lactobacillus*-dominated VMB:

A VMB of low diversity and dominated by *Lactobacillus* species is associated with greater protection in the FRT. These communities are dominated by either *L. crispatus, L. gasseri, L. jensenii* or *L. iners*, in which the three former are associated with the greatest levels of protection, while *L. iners* is linked to variable protection[36]. *Lactobacillus* species are thought to be the keystone vaginal species that contribute to protective immunity in the FRT by providing non-specific defense against a variety of non-indigenous and pathogenic organisms. In a recent prospective study of 236 HIV-uninfected South African women, none of the women who had a vaginal community dominated by *L. crispatus* acquired HIV-1[35]. Conversely, diverse communities with low *Lactobacillus* abundance were associated with a fourfold increased risk of HIV-1 acquisition, even in the absence of symptomatic BV.

1.3.2.1 Protection by Lactobacilli

Lactobacilli are a genus of Gram positive, rod shaped bacteria. The protection observed in women with a *Lactobacillus*-dominated community is thought to be primarily achieved through their production of lactic acid, lowering the vaginal pH to 3.5-4.5. Lactic acid alone has been shown to inactivate or kill numerous known vaginal pathogens, and the ability for cervicovaginal mucus collected from women to trap HIV-1 virions *ex vivo* was found to be dependent on lactic acid composition[37,38,39,40,41]. Hydrogen peroxide production has also been widely cited as a key mechanism by which lactobacilli confer protection in the FRT[42]. While the mean oxygen levels in the FRT have been estimated to be much lower than atmospheric levels of oxygen (2% vs 21%, respectively), these concentrations may be sufficient enough to have antimicrobial effects[43]. Moreover, transient increases in oxygen levels have been reported upon or after tampon use, sexual arousal, and likely sexual intercourse, in which hydrogen peroxide production may be especially important for contributing to the stability of the VMB[44,45].

The VMB may also influence barrier function, in which disruption can facilitate productive HIV-1 infection by increasing contact between virions and target cells, or by enabling the transversion of non-indigenous organisms across the epithelium that may illicit an immune response and thus increase target cell infiltration. The ability of *Lactobacillus* species to form epithelial-adherent microcolonies and stimulate co-aggregation between species provides an addition physical barrier in the FRT to prevent nonindigenous organisms from colonizing or transversing the FRT epithelium. Several vaginal *Lactobacillus* isolates have been shown to block adhesion of pathogens to vaginal epithelial cells *in vitro*,

including species associated with BV such as *G. vaginalis*[46,47,48]. Additionally, Zevin et al., found that *Lactobacillus*-dominant communities were associated with high levels of proteins involved in keratinization and epidermis development, which may lead to an enhanced barrier function[49].

Lastly, several studies have analyzed inflammatory markers in association with VMB composition and have revealed that *L. crispatus*-dominated communities are associated with the lowest levels of inflammation[50,51,52]. *In vitro* co-cultures of bacteria with cervical and vaginal epithelial cell lines support these findings, where *L. crispatus* induced minimal inflammatory responses in these models, whereas vaginal anaerobes induced high levels of pro-inflammatory cytokine secretion[53]. Moreover, women with a *Lactobacillus* dominant VMB have lower levels of inflammatory cytokines and decreased activated CD4+ T cells when compared to women with a diverse VMB[32]. *In vitro* studies with clinical isolates of *Lactobacillus* showed that the bacteria were able to reduce the production of pro-inflammatory cytokines IL-6, IL-8 and TNF-α in vaginal epithelial cell cultures stimulation with TLR agonists[54,55,56]. Additionally, treatment of vaginal and cervical epithelial cell lines and primary cervicovaginal cells with lactic acid induced significant production of the anti-inflammatory cytokine IL-1RA and inhibited production of pro-inflammatory cytokines induced by TLR-agonists[57].

Importantly, not all vaginal lactobacilli are associated with robust protection in the VMB. Namely, high levels of *L. iners* can be detected in both healthy women and women with BV, which is not observed for the other vaginal *Lactobacillus* species[36,58]. Moreover, communities dominated by *L. iners* have been associated with a higher vaginal pH than other *Lactobacillus*-dominated communities, and *in vitro* studies show higher levels of

inflammation in epithelial cells lines treated with *L. iners*-dominant VMB cervicovaginal mucus than *L. crispatus*-dominated[59]. Additionally, *L. iners* usually becomes the dominant species post-BV treatment, and is not associated with protection against later relapse. As such, it has been questioned whether *L. iners* can play an active role in dysbiotic onset as well as recovery from BV[58,60].

1.3.3 Dysbiotic VMB:

In opposition to a *Lactobacillus*-dominated VMB, a dysbiotic VMB is often associated with adverse reproductive outcomes and increased susceptibility to STIs and is a hallmark of the symptomatic dysbiosis characterizing BV[61]. These communities are composed of diverse anaerobes, including species of *Gardnerella, Prevotella, Atopobium, Megasphaera,* and *Dialister*[34]. With this, recent high-throughput sequencing studies have revealed previously unappreciated diversity in the VMB of asymptomatic women[62,63]. This heterogeneity has challenged conventional associations between individual species, vaginal discomfort, and reproductive health and has led to a more focused, in-depth approach to understanding how bacterial communities as a whole can modulate health and protection in the FRT. This is particularly important, as women with diverse anaerobic communities devoid of appreciable amounts of lactobacilli are still at a heightened risk of HIV-1 acquisition, even in the absence of symptomatic BV[35,64.]

Despite considerable attempts focused on elucidating the pathogenesis of BV, the etiology remains unknown. Multiple factors have been reported as risk-factors for the development of BV, including heterosexual intercourse, new or multiple sexual partners, Black and Hispanic ethnicity, smoking, douching, antibiotics, and hormonal changes[65].

However, no one single factor has been definitively linked to the onset of BV, and the development of the condition is likely multifactorial. With this, Swindsikski et al. found the presence of a dense adherent polymicrobial biofilm on the vaginal epithelial surface in biopsies of women with BV, whereas only thin loose biofilms are found in women with *Lactobacillus*-dominated communities[66]. It is speculated that the biofilm is initiated by *G. vaginalis*, in which it then becomes a scaffold for other species to adhere to, such as *Prevotella bivia*. However, it is still unclear if these species are active in the initiation of BV, or if they are passive and adapting to changes in the vaginal environment. Moreover, the relative contribution that these biofilms play in the pathology of BV is unknown but are proposed to contribute to the antibiotic resistance and recurrence that are observed in many women with BV[67].

1.3.3.1 Susceptibility and a dysbiotic VMB:

While a dysbiotic microbiome may passively enhance susceptibility due to a lack of lactic acid and thus a higher pH, there is also evidence of active mucin degradation in cervicovaginal mucus. Mucin-degrading enzymes, including sialidase, α-fucosidase, α- and β-galactosidase, N-acetyl-glucosaminidase, and glycine and arginine aminopeptidases, are shown to be elevated in women with BV, and are associated with thinner cervicovaginal mucus[68,69]. By thinning the mucus, this active degradation by bacteria associated with BV may attenuate the trapping ability of normal mucus and thus facilitate the mobility of HIV-1 virions to the underlying epithelium. VMB diversity has also been associated with markers of cytoskeletal alterations and cell death, and decreases in wound healing proteins, indicative of decreased barrier function[70]. Importantly, even in the absence of clinical BV

diagnosis, communities with high levels of *G. vaginalis* are still associated with barrier dysfunction[71]. These findings suggest that anaerobic, non-*Lactobacillus*-dominated communities may decrease the barrier function of the vaginal epithelium to increase susceptibility to a number of invasive pathogens, including HIV-1.

Several studies have analyzed inflammatory markers in association with VMB composition and have revealed a direct correlation between microbial diversity and elevated pro-inflammatory cytokines[72,73,74]. Compared with lactobacilli-dominated microbiotas, dysbiotic VMBs are marked by increased levels of several proinflammatory cytokines. While variation exists between studies, the most consistently observed increases are with IL-1β, IL-6, IL-8 and TNF-α. Moreover, longitudinal studies have revealed that community shifts characterized by increased diversity and anaerobe abundance are associated with a paralleled increase in pro-inflammatory cytokine levels, suggesting that the bacteria may be a strong driver of inflammation in the FRT[75,76]. Importantly, diverse VMBs lacking appreciable numbers of lactobacilli associated with a fourfold increased risk of HIV acquisition also had significantly higher levels of pro-inflammatory cytokines[35]. Strikingly, these communities also had a 17-fold increase in activated $CCR5^+$ $CD4^+$ T cells (HIV target cells) in the FRT.

Unfortunately, an effective treatment for BV, or a means to select for a *Lactobacillus*-dominated VMB is presently not available. The currently available treatments with metronidazole or clindamycin are associated with moderate short-term cure rates, however the majority of women experience relapse within two to four weeks[77]. Recent studies examining the potential of *Lactobacillus* probiotics in the treatment of BV show promising

short-term results, however longitudinal studies are required to determine if these probiotics can induce lasting shifts in the VMB[78,79]. As these distinct bacterial communities can influence and modulate protection and health in the FRT, understanding the mechanisms allowing for shifts and persistent colonization of certain vaginal bacteria is essential to develop more effective methods to prevent the adverse outcomes associated with a dysbiotic VMB.

1.3.4 VMB Community Composition Stability:

Despite the tendency for *Lactobacillus* to dominate the VMB in the majority of asymptomatic reproductive-age women, the community compositions have recently been shown to be rather personalized. This has been revealed by high-throughput sequencing studies and longitudinal analyses, in which stark variances in species structure and temporal dynamics are observed even between individuals with the same dominating species[80,81,82]. Gajer et al. found that communities that were non-*iners*-*Lactobacillus* dominant tended to be relatively stable, and when transitions occurred they typically were just dominated by another *Lactobacillus* species[83]. Conversely, *L. iners*-dominated communities experienced more shifts, and tended to shift to diverse anaerobic communities. Moreover, *L. iners* has consistently been found in women with symptomatic and asymptomatic BV[47]. Points of instability were correlated with hormonal fluctuations over the menstrual cycle, VMB community composition, and sexual activity.

Large scale studies evaluating the VMB community composition have also revealed significant differences in species composition between women of different ethnic groups. In North America, approximately 90% and 80% of healthy White and Asian women, respectively, have communities dominated by *Lactobacillus*. However, this is only true for

60% of Black and Hispanic women in North America, and 37-62% of Black women in sub-Saharan Africa[34,84]. These findings are in line with earlier studies using culture-dependent techniques demonstrating a higher prevalence of BV in Black and Hispanic women[85]. Likewise, in a recent study conducted in South African women, only 37% of women had *Lactobacillus* dominated communities, with *L. iners* dominating the majority of these communities.

Taken together, the dynamic differences that are observed within and between women's VMB composition suggests that host factors may play a significant role in determining VMB community composition. With such low levels of protective *lactobacillus*-dominance in regions such as South Africa where 70% of new HIV-1 occur in women, it is imperative to understand the factors responsible for VMB composition and how we can manipulate these.

1.4 Factors Shaping the Vaginal Microbiome

1.4.1 Hormones:

Endogenous female sex hormones influence various aspects of the FRT including the VMB, where these influences are most glaring during the major hormonal shifts at puberty and menopause. Before the increase in estrogen levels and the initiation of cyclic menstruation, the VMB of young girls has relatively low or absent levels of lactobacilli and is instead composed mainly of anaerobic bacteria[86,87]. Later, the levels of lactobacilli have been shown to correlate with stage in puberty, with pre- and perimenarcheal girls containing vaginal communities with prominent lactobacilli members that increase into reproductive years[88]. Likewise, the onset of menopause is characterized by a decrease in estrogen and a

coinciding decrease in lactobacilli levels[89]. Notably, estradiol-based hormone replacement therapy in post-menopausal has been shown to maintain *Lactobacillus* dominance[90]. The analyses of fluctuations of community composition as a function of the menstrual cycle found that community composition stability was greatest when estrogen levels were highest[55,91]. Studies employing 16S rRNA gene sequencing have also revealed that women on oral contraceptives had enhanced *L. crispatus* and decreased BV-associated bacteria, supporting previous findings that hormonal contraceptives can reduce the risk of BV by up to 30%[92,93,94]. Our lab has recently shown that DMPA use in a cohort of Kenyan sex-workers was significantly associated with diverse VMB composition, and this was in the absence of clinical BV diagnosis. As DMPA is a progestin-based contraceptive, these findings may be due to its hypo-estrogenic effect[95].

Together, these studies highlight the stark association between estradiol and a stable *Lactobacillus*-dominated VMB. The *Lactobacillus*-promoting effects of estradiol are thought to be mainly achieved due to its ability to enhance glycogen deposition in vaginal epithelial cells[96]. This is supported by animal models, in which estradiol treatment was shown to induce glycogen deposition in the vaginal tissue of hamsters and non-human primates[97,98]. This glycogen can then be secreted by vaginal epithelial cells and serve as a central resource for lactobacilli. While the association between estradiol and a stable *Lactobacillus*-dominated VMB is evident, understanding the multifactorial interactions between the VMB, hormones, nutrients and immune factors in the FRT requires more in-depth investigations.

1.4.2 Resource Availability:

The nutrients available to the indigenous vaginal bacteria are exclusively host derived. It follows that variances and alterations in host physiology that determine what nutrients are available could thus influence the capability and success of bacterial colonization by certain species. Resources available for bacterial consumption exist in the mucus that contains a rich mix of carbohydrates, proteins, fatty acids, and trace elements, and also epithelial cells, which are rich in glycogen in reproductive-age women[99]. Glycogen, a polymer of glucose, has long been deemed a key nutrient that directly enriches for vaginal lactobacilli, and has been cited as the reason for estradiol's *Lactobacillus*-promoting effects. However, this interpretation is largely based on correlative data showing an association between vaginal cell-free glycogen levels and lactobacilli abundance[100,101].

Importantly, the paradigm that vaginal lactobacilli cannot directly metabolize glycogen has only recently been put into question. Early studies of isolation of *Lactobacillus* strains from the FRT and metagenomics studies have all claimed that *Lactobacillus* strains were unable to metabolize glycogen and lacked the genetic machinery to do so[102]. *In vitro* studies have tested strains of *L. crispatus*, *L. gasseri*, and *L. jensenii* and found they were unable to directly use glycogen as a resource for growth[103,74]. Much of the attention was thus focused on α-amylase as an enzyme that can cleave α-(1,4) glycosidic bonds in glycogen to produce maltose, maltotriose, and α-dextrins in the FRT [73,104,105,106]. It was identified that women with BV had lower α-amylase levels, and that women on DMPA, and thus a hypo-estrogenic state, had lower levels of both glycogen and α-amylase[107]. While these studies reinforce a model in which estradiol promotes glycogen and α-amylase levels which positively selects for a *Lactobacillus*-dominated VMB, the exact nature of these associations may not be as simplified as previously thought. A recent report has identified the presence

of the enzyme putative pullulanase type I in several *L. crispatus* isolates, which correlated with their ability to grow on glycogen supplemented media[108].

These findings highlight the extensive gaps in our understanding of the metabolic behaviours of vaginal bacteria that need to be answered before conclusive assertions can be made. The recent finding that *L. crispatus* strains were able to utilize glycogen denounced prior claims that this was not possible, and thus underscores the need to functionally characterize vaginal bacteria's metabolic proficiencies rather than solely attempting to infer such capabilities from metagenomics studies. As such, it is critical to determine whether this is the case for other vaginal *Lactobacillus* species, non-lactobacilli vaginal bacteria, and the extent to which these species can effectively utilize such resources. Additionally, understanding how the presence of these abiotic factors may influence competitive or symbiotic interactions is required to enrich our understanding of VMB community composition selection.

1.4.3 Competitive Interactions:

At present, it is unclear why only four of the more than 130 species of *Lactobacillus* known commonly dominate the VMB. Moreover, it is unclear why vaginal bacterial communities differ between women, especially of different ethnicity, and what drives communities to change in composition over time. While a wide gap in our knowledge of these factors exists, recent efforts to elucidate possible influences have shed light on this subject. To gain a deeper understanding of the complicated interpersonal and temporal dynamics of the vaginal microbiome, attempts to identify conditions under which particular vaginal bacteria thrive have been made through comparative genomic analyses.

Mendes-Soares et el. compared the genome of 25 *Lactobacillus* species in an attempt to identify specific traits common to vaginal *Lactobacillus* species allowing for their dominating colonization[109]. However, the comparison between vaginal lactobacilli revealed species-specific traits that may allow each species to uniquely interact with the environment to effectively dominate the vaginal community. These were proteins involved in resistance to phage infections, bacteriocins, and metabolism. While the functional importance of the differing protein-encoding sequences detected remain incompletely understood, this suggests that each vaginal *Lactobacillus* species may have unique mechanisms of interacting with the host and competitively excluding other microbes. France et al. comprehensively compared the genomes of *L. crispatus* and *L. iners*[110]. While they shared many overlapping traits, their genomes differed in many regards that may provide them with distinct competitive abilities in differing environments. *L. iners* has a much smaller genome and does not appear to be capable of producing hydrogen peroxide. Thus, during conditions when oxygen increases in the FRT, such as with tampon use and heterosexual intercourse, *L. iners* may have decreased defences and allow for the outgrowth of anaerobic species. This therefore could be a factor contributing to the relative instability observed in *L. iners*-dominated communities. While both species shared key fermentation-pathways needed to metabolize certain glycogen breakdown products, there were key differences in their genetic potential for carbon metabolism. *L. crispatus* had a significantly greater number of genes devoted to carbohydrate metabolism and possessed the genetic capability to ferment several sugars that *L. iners* could not. In addition to the lack of genes related to the biosynthesis of essential amino acids in *L. iners*, these findings suggest it may rely more heavily on exogenous resources and be more susceptible to environmental fluctuations. This notion is

in line with temporal dynamic studies that found vaginal communities dominated by *L. iners* were less stable than those dominated by other vaginal lactobacilli. Moreover, *L. crispatus* uniquely possessed genes coding for an iron transport system, which may give a competitive advantage over other species to effectively sequester iron. This may provide an additional explanation for the enhanced stability of *L. crispatus*-dominated communities during menses. While these findings suggest that *L. iners* has reduced metabolic capabilities and thus less of a competitive advantage, *L. iners* possess unique traits that may contribute to its ability to flourish under differing environments. Notably, *L. iners* has the ability to produce inerolysin, a pore-forming cytolysin. This, in combination with its ability to bind human fibronectin and maintain close contact with host tissue, may allow *L. iners* to obtain nutrients from host cells when exogenous resources are scarce. This may provide an explanation for the observed shift to *L. iners* during menses, and the increase in *L. iners* abundance and dominance in women on hormonal contraceptives that induce a hypo-estrogenic state, such as DMPA, that may reduce local levels of glycogen.

1.4.4 Species variability:

While this comparative genomic analysis of the genetic variation between dominating species provides clues as to the conditions that each species may flourish in, it is important to highlight the limitations of these studies. Importantly, these studies did not take into account the genetic diversity and varied metabolic potential that may exist within the same species. For example, the genome of *Escherichia coli* can vary by as much as 25% between strains[111]. Moreover, there may be conditional expression of certain genes under specific abiotic and biotic environments. With this, recent comparisons of strains and expression

diversity of vaginal bacteria under certain conditions have highlighted how important these differences may be.

Gardnerella vaginalis is highly associated with BV but can also be found in asymptomatic women where it has been revealed that particular strains possess greater virulence potential than others[112]. Glycosidases present in *G. vaginalis* capable of thinning the protective layer of vaginal mucus have not been shown to be encoded by all strains of *G. vaginalis*[117]. Additionally, strains have been found to differ in expression of vaginolysin, the pore-forming cytolysin[113]. In functional comparisons between strains isolated from women with differing vaginal conditions, strains isolated from women with BV were more virulent than strains from women without BV in terms of adhesion, cytotoxicity and displacement of pre-adhered *L. crispatus*[114]. These differences may serve to explain the presence of *G. vaginalis* in both healthy and symptomatic women, and thus caution should be taken when exclusively incriminating this species in the initiation of BV.

To date, the only genomic comparison of different vaginal *Lactobacillus* isolates have been conducted for *L. iners*, as this species can also be found in both healthy and dysbiotic conditions. Macklaim et al. analyzed the expression profiles of *L. iners* isolated from two women with BV and two without[115]. The comparison revealed that *L. iners* differentially expressed 10% of its gene complement between the two conditions. They found that inerolysin, the pore forming cytolysin, was upregulated at least 6-fold in BV, in which it was active at a higher pH. As this elevated pH range corresponds to that observed in women with BV, this supports the notion that this unique trait of *L. iners* contributes to its pathogenic potential and adaptability under BV conditions. Moreover, they found genes involved in mucin and glycerol transport and metabolism to be highly upregulated under BV conditions,

suggesting that this species may be adapting to altered resource availability. Additionally, genes related to bacteriophage defense were also upregulated, which may be a mechanism by which *L. iners* can survive under BV conditions with heightened bacteriophage load. This analysis suggests that the persistence of *L. iners* under considerably altered conditions may be due to its ability to respond and regulate is genomic functions to differing environments.

Taken together, these few studies highlight the stark influence that strain diversity and altered expression can have on functional outcomes. As such, future studies should seek to sample and sequence a greater variety and number of vaginal isolates. Additionally, expression patterns under altered conditions highlight that inferring function from genetic potential should be made with caution. Cultivation of these isolates would allow for critical information regarding bacterial phenotypes under specific environmental conditions, and also allow for the investigation of bacterial interactions among themselves and with the human host.

1.5 Experimental Models of the VMB:

Model systems are created to reflect biologically relevant factors that can be manipulated to illuminate important mechanisms and pathways that give us a better understanding of the biological system in question. In the study of the VMB, the components relevant to the FRT are the microbes, their host and the vaginal environment. While no model system perfectly represents all the complex features of the FRT, innovative simplistic models can further our understanding of the host-microbe and microbe-microbe interactions that underlie VMB community composition and can lead to a dysbiotic state. Current model systems to study the VMB include cell-free biofilm studies, epithelial cell line cultures, 3D-multilayer cultures, and inoculation of gnotobiotic mice[54,55,56,116]. Each model system can

provide important answers and insights, depending on the question or hypothesis being tested. While typically a robust animal model would provide the best assessment of factors determining composition, susceptibility to infections, and perturbations that can influence community dynamics and interactions with the host, such a model does not presently exist. The major obstacle to the establishment of an animal model is that the VMB colonizing typical laboratory animals, including nonhuman primates, is naturally diverse in nature[117,118]. As such, with an optimal human VMB characterized by low-diversity and *Lactobacillus*-dominance, this poses significant limitations to investigate bacterial dysbiosis of the VMB. With this, Teixeria et el have used gnotobiotic mice inoculated with *G. vaginalis* to recapitulate several features of bacterial dysbiosis, providing an intriguing future model system[118]. To advance such a model to better reflect the VMB and address questions concerning dysbiosis etiology and treatment, the system would have to consistently support the development of reproducible bacterial communities. While these advancements have not been made to date, *in vitro* systems can aid in answering more refined and nuanced questions. The majority of current *in vitro* systems employ the use of epithelial cells lines, either primary or immortalized, and the addition of a single bacterial species[54,55,56]. Assessments include evaluations of immune responses, adherence, and ability to mitigate responses to TLR-ligands or other infectious microorganisms. While these studies have all provided important information and insights concerning common species inhabiting the VMB, these systems could be improved upon to tackle more complex questions. Such advancements include adding additional cell types, more complex bacterial combinations, and important environmental factors. While the current *in vitro* systems lack other cell types and underlying structures, such as the lamina propria and vasculature, the more complex a

system becomes, the harder it becomes to delineate the exact mechanisms or cell types contributing to the observations made. As such, while the addition of these factors would better reflect *in vivo* conditions, there is still value in defining and understanding interactions in more simplistic models before more complex features are added. By this framework, in the context of defining interactions with the epithelial barrier then is a priority to make the simplified system more reflective of *in vivo* conditions. With this, many previous studies have utilized epithelial monolayers in liquid-liquid interfaces which do not accurately reflect the multilayered squamous epithelium *in vivo*[119]. Moreover, these studies have been conducted at atmospheric levels of oxygen, whereby the contributions of bacterial by-products such as hydrogen peroxide would be inflated and thus could distort overall interactions and results[44,45,86]. Perhaps the most important advancement of VMB studies is the establishment of multispecies communities. While single-species analyses have provided important information into the host-bacterial interactions that may occur in the FRT, there is increasing awareness that these interactions can be fundamentally altered in more complex multispecies communities[120]. Several features of multispecies communities are not measurable in single-species settings, such as diversity, competition, stability and fluctuations in abundances[121]. Moreover, with dysbiosis of the VMB being defined as a multispecies condition whereby the dynamic interactions between vaginal species ultimately determine community composition and interactions with the host, a mechanistic understanding of these interactions is required. While the current model systems are far from perfect, advancing and refining existing models can better mimic host responses in the FRT. Moreover, the incorporation of microbial communities to these simplified systems are needed to better understand the interspecies and bacterial-host interactions that ultimately

lead to dysbiosis of the VMB. These characterizations will advance our ability to interrogate vaginal bacterial communities to develop more rational preventative and therapeutic interventions.

1.6 Probiotics and the VMB:

Currently, the available antibiotic treatments for BV are largely ineffective, with a low cure frequency and high recurrence rates[122]. This, in combination with the increasing concern of antibiotic resistance has made alternative curative or prevention interventions of vaginal dysbiosis an active area of research[123]. With this, the increasing awareness of the beneficial features of *Lactobacillus* species has put a growing emphasis on the possibility of using exogenous lactobacilli in the form of probiotics or live biotherapeutic products to optimize the VMB[124]. Probiotics are defined as "live microorganisms that, when consumed in appropriate amounts, confer a health benefit on the host"[125]. Probiotic lactobacilli have the potential to spatially occupy the vaginal niche and thus preclude the colonization of other bacteria and their biofilms, to produce antimicrobial compounds, to induce an anti-inflammatory environment, and to inhibit common infectious pathogens[126]. With this, there have been several clinical studies evaluating the effectiveness of lactobacilli probiotics at colonizing the VMB, curing BV and preventing relapse. These studies all have varied method of administration (orally or vaginally), various trial methodologies, distinct study populations and varying endpoints and evaluations[127,128,129,130,131]. To date, most probiotic strains have originated from the gut or from traditional fermented food, and are not common inhabitants of the FRT. This is with the exception of LACTIN-V, comprising a vaginally isolated stain of *L. crispatus* (strain CTV-05)[132]. Importantly, no major safety concerns were

reported in any of the probiotic trails, making the continuation of future trails promising. While vaginal detection differed in timepoints assessed, the study evaluating strain concentration most frequently showed rapid increases and decreases after each administration[133]. Moreover, consistent amongst all studies was the evidence that detection of the probiotic strains after cessation of dosing does not last long, suggesting colonization of the vaginal tract is poor[128-133]. With this, there is significant heterogeneity between studies, suggesting that more controlled and standardized methods be used between trials. In terms of acting as a treatment for BV, many studies showed cure of BV and lower rates of recurrence and/or time between relapses[127,129,131,133]. Vaginally applied probiotics appear to be most efficacious, as well as when used in combination with antibiotics. However, with many evaluations after cessation being relatively short term for most studies, and the rapidly decreasing vaginal detection of most probiotic strains, the long-term results of this require future investigation. Moreover, amongst the diverse array of *Lactobacillus* species known to inhabit that human body, only four *Lactobacillus* species are found to dominate the VMB. While the reason for this is unknown, it suggests that these species have specially adapted to survive and persist in the FRT. As the majority of these studies are using *Lactobacillus* strains not naturally found to colonize the FRT, they may not contain the specificities and qualifications required to persistently colonize the FRT. With this, the studies evaluating *L. crispatus* did not show superior performance over other lactobacilli probiotics in terms of colonization, however this may be due to several reasons[132]. Of note, it was shown that while 90% of women who lacked *L. crispatus* colonization at enrollment were successfully colonized by the *L. crispatus* probiotic strain, whereas only 51% of those with an already *L. crispatus*-dominant VMB were. This may be due to the indigenous species outcompeting the

probiotic strain. With more successful colonization in women with BV, with the caveat of vaginal intercourse reducing successful colonization, this probiotic may show more promising long-term results. With this, *L. crispatus CTV-05* (Lactin-V) is currently being evaluated for preventing BV recurrence in a phase II-b randomized trial following a five-day course of metronidazole, which may provide more clear indications if vaginal isolates are more efficacious probiotics.

In addition to the use of probiotics to modify and shape the VMB, there has also been increasing interest in the use of prebiotics[134]. Prebiotics are defined as substrates that are selectively used by beneficial microorganisms to increase their numbers and/or activity to improve the health of the host[135]. While the concept of using growth substrates for resident microorganisms to induce compositional and metabolic changes has been relatively unexplored for the VMB, the concept has triggered a vast amount of research in initiatives to transform the gut microbiota. Prebiotics for the gut microbiota are required to be non-digestible foods that are resistant to gastric acid, hydrolysis of gastrointestinal enzymes, and intestinal absorption, however these stipulations may not be as important if vaginally administrated[136]. While the use of prebiotics to modulate the VMB has promising potential, the lack of metabolic characterization of vaginal bacteria significantly hampers the rational design of appropriate and effective substrates to select for an optimal VMB. As such, characterization of the metabolic capabilities and proficiencies of vaginal species will allow for the identification of substrates that may be selectively used by beneficial species, or specifically discourage the growth of unwanted species. Together, while there are promising avenues for a number of new approaches and strategies to harness the VMB to improve women's health, we still lack fundamental knowledge about the bacteria that comprise the

VMB. More in-depth characterizations will certainly increase our understanding of vaginal bacteria and the interactions that may influence community dynamics, and thus allow for the rational design of more effective therapeutics.

CHAPTER 2: RATIONALE & HYPOTHESIS

Recent high-throughput technologies have greatly improved our understanding of the complexity of the VMB; however, extensive gaps in our knowledge exist regarding interspecies interactions, bacterial-host interactions, and host factors that may select for specific community compositions and ultimately influence protection or susceptibility in the FRT[34]. The high variation of community composition within and between women further complicates the quest toward a mechanistic understanding of this dynamic system that is so important to women's reproductive health[34,83]. However, this knowledge is critically needed as women with a dysbiotic VMB are at a fourfold increased risk of HIV-1 acquisition, and this type of VMB composition is highly prevalent in regions where HIV-1 is endemic[62]. To advance the field towards the development of more effective therapeutics and prevention strategies, there is a need to define the species-specific nature by which vaginal bacteria can influence factors relevant to susceptibility in the FRT. Unfortunately, mechanistic research on the VMB has often been hampered by the lack of a robust experimental model that captures physiologic interactions between bacterial species and with the host vaginal microenvironment[137]. An optimal model system of the VMB must support the development of reproducible bacterial communities and allow for the controlled evaluation of host, microbe and environmental factors that may impact VMB composition and its effect on susceptibility in the FRT. While the field currently lacks a model that can recapitulate all of the complex biological and structural features of the human FRT, simplified *in vitro* systems can start to dissect factors that may lead to susceptibility in the FRT. Previous *in vitro* analyses have focused on single-species analyses, and while these studies have provided important information regarding interactions with host cells, there is a critical need to define

these interactions in more complex community configurations. Elucidating the interspecies interactions that could provide insights on virulence expression, synergism, or antagonism will deepen our understanding of the biotic factors governing bacterial community composition and inform new targets for intervention strategies. Moreover, while it is widely accepted that certain *Lactobacillus*-dominant VMBs are associated with greater protection against a number of adverse reproductive outcomes and STI acquisition, there is increasing awareness that specific species of *Lactobacillus* may be more optimal than others[28,29,35,36]. As such, it is important to delineate the defining characteristics of each species that may determine their pathogenic or protective tendencies and the arsenal of defense mechanisms at their disposal.

In addition to these important analyses, we still know surprisingly little about *how* vaginal dysbiosis develops or why women with a diverse VMB are more likely to harbor such potentially pathogenic bacteria[32]. Knowledge of the abiotic factors that can select for or discourage the growth of specific species could have important implications for prophylactic and therapeutic interventions to select for an optimal VMB composition[134]. As such, characterization of the metabolic capabilities and proficiencies of vaginal species will allow for the identification of substrates that may be selectively used by beneficial species, or specifically discourage the growth of less-favorable species. Together, being able to interrogate the conditions under which certain species dominate, virulence phenotypes are expressed, and bacterial-induced damage occurs will advance our ability to rationally design more effective interventions.

Within this context, my thesis plan was to delineate how specific vaginal bacteria interact with one another and host epithelial cells to ultimately influence factors that may

lead to susceptibility in the FRT. In the quest to identify appropriate and effective substrates to select for an optimal VMB, I also characterized the metabolic capabilities of vaginal bacteria under various abiotic conditions. Based on previous genomic, proteomic and biologic analyses, **we hypothesized that dysbiosis-associated bacterial species found in the VMB will result in the greatest reduction in barrier function and induction of inflammation**. Moreover, based on correlations between elevated glycogen levels and *Lactobacillus*-dominance in the FRT, **we hypothesized *Lactobacillus* will be able to more efficiently utilize glycogen breakdown products to dominate over dysbiosis-associated bacteria in coculture conditions**. Specifically, we believe that *Lactobacillus* species associated with the greatest protection in the VMB (*L. crispatus*) will enhance barrier function and an induce an anti-inflammatory environment. Conversely, species associated with an increase to HIV-1 acquisition compared to other *Lactobacillus* species, defined as being intermediate (*L. iners*) or those associated with the highest rates of HIV-1 infection, defined as dysbiotic (*Gardnerella vaginalis* and *Prevotella bivia*) will lead to a reduction in epithelial barrier integrity and induce pro-inflammatory cytokines[35]. Moreover, we believe that *L. crispatus*, but not *L. iners,* cocultured with dysbiotic-associated species will be able to reduce the decreased barrier function and inflammation induced by dysbiosis-associated species in isolation. In terms of metabolic capabilities, we believe that *L. crispatus* will be able to most effectively utilize the greatest number of glycogen breakdown products compared to the other vaginal species, and this will correspond to their dominance under conditions where these resources are abundant.

We addressed our hypothesis in two specific aims:

Aim 1: Analyze interactions between host vaginal cells and common vaginal bacterial

Aim 2: Characterize vaginal bacterial phenotypes and create *in vitro* communities representative of both protective and dysbiotic VMB

CHAPTER 3: MATERIALS AND METHODS

3.1 Bacteria Stock Preparation:

Lactobacillus crispatus SJ-3C-US (PTA10138) from ATCC was generously provided by Dr. Nuch Tanphaichitr (University of Ottawa). *Lactobacillus jensenii* (ATCC 25258), *Lactobacillus iners* (ATCC 55195), *Prevotella Bivia* (ATCC 29303) and *Gardnerella vaginalis* (ATCC 14019) were purchased from ATCC. All lactobacilli and *G. vaginalis* were grown in ATCC medium 1685 (NYC III medium) at 37°C in anaerobic conditions using the GasPak EZ Anaerobe Container System (Becton Dickenson, Cat, 260001). *P. bivia* was grown in Modified Reinforced Clostridial (MRC) Medium (ATCC #2107) in a Bactron IV anaerobic chamber (90% N_2, 5% CO_2, and 5% H_2). Frozen stocks of each bacteria were made with 20% glycerol and stored at -80°C until further use. The bacterial stock concentration was determined through serial dilutions plated onto MRS Agar (*L. crispatus* and *L. jensenii*) or Tryptic Soy Agar supplemented with 5% sheep's blood (*L. iners*, *G. vaginalis* and *P. bivia*) to enumerate colonies. Colony forming units from serial dilutions (CFU/mL) were correlated with Optical Density at 600nm and plotted to obtain a linear equation for future analyses.

3.2 Cell Media Preparation:

Keratinocyte serum-free growth medium (KSFM) (Thermofisher, Cat.17005042) was supplemented by the addition of epidermal growth factor (EGF) to a final concertation of 0.1ng/mL, bovine pituitary extract (BPE) to a final concentration of 0.05mg/mL. 200uL of 1M CaCl2 was added for every 500mL of KSFM prepared. All prepared media was stored at 4°C.

3.3 Vk2/E6E7 Air-Liquid Interface Cell Culture:

Vk2 cells (ATCC CRL-2616™) were cultured in antibiotic free keratinocyte serum free media (KSFM) (Thermofisher, Cat.17005042) until 80% confluency. The flask containing the Vk2 cells was washed with phosphate buffered saline (PBS), and trypsinized using 1X trypsin-EDTA. Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12) (Thermofisher, Cat. 12634-010) containing 10% fetal bovine serium (FBS) was added to the trypsinized cells to deactivate the trypsin. The cell containing solution was then centrifuged at 1,500 rpm for 5 minutes. The supernatant was then decanted and the cell pellet was resuspended in PBS, and centrifuged at 1,500 rpm for 5 minutes. The supernatant was decanted, and the cell pellet was resuspended in antibiotic free KSFM. Cells were then enumerated via trypan blue exclusion assay (see below) using a hemocytometer. 60,000 cells were then seeded in the apical side of 0.4um transwells (VWR, Cat. 82050-022) in 24 well plates. The apical side of the cell culture was topped up to 300uL with antibiotic-free KSFM. 700uL of antibiotic-free KSFM was added to the basolateral side. The 24 well plates were shaken briefly to ensure that the cells are distributed evenly in each of the transwells. One day after seeding, the apical media was aspirated to induce air-liquid interface (ALI) conditions. Basolateral media was changed every two days. Vk2 ALI cultures were subsequently incubated in anaerobic conditions using the GasPak EZ Anaerobe Container System (Becton Dickenson, Cat, 260001).

3.4 Vk2/E6E7 and Vaginal Bacteria Coculture System:

Frozen stock of bacteria were inoculated in 15mL polypropylene tubes containing 10mL of NYC III media for lactobacilli and *G. vaginalis* or MRC media for *P. bivia* using the GasPak EZ

Anaerobe Container System. To reach the following initial bacterial concentration of 5.0 +/- 0.2 x 10^6 CFU/mL. Optical density (OD) readings were measured using a spectrophotometer every hour. Once the bacteria were into late exponential phase (OD: 0.8-1.5), it was harvested (amount used was based on previous OD vs CFU/mL linear equations) and added to a 15mL polypropylene tube containing 10mL of PBS, and centrifuged at 4,000 rpm for 5 minutes. PBS was decanted, and antibiotic free KSFM was added to resuspend the bacterial pellet to a final concentration of 6,000,000 CFU/mL for single species cocultures, or to 3,000,000 CFU/mL for dual species cocultures. 100 μL of the KSFM bacterial suspension was added onto the apical side of 7 day old ALI grown Vk2 cells for monocultures, or 50 μL of each suspension for bacterial coculture experiments. 100 μL of antibiotic free KSFM containing no bacteria was added to any experimental conditions containing no bacteria.

3.4.1 Vk2 Cell Viability Via Trypan Blue Exclusion Assay:

On day 8 of culture, 24 hours after the addition of antibiotic free KSFM to the apical side of each cell culture (with or without bacteria), the apical media was removed. 100 μL of 1X trypsin-EDTA was added onto the apical side of each transwell and incubated at 37°C, 5% CO2 until cells were detached (approximately 30 minutes). Subsequently, 200 uL of DMEM media containing 10% FBS was added to neutralize the trypsin. Live/dead counts are determined using the hemocytometer after trypan blue staining.

Data shown was expressed as: number of live cells/total number of cells x 100% = % live cells.

3.4.2 Vk2 Cell Viability Via Lactate Dehydrogenase Assay:

On day 8 of culture, 24 hours after the addition of antibiotic-free KSFM to the apical side of each cell culture (with or without bacteria), the apical media was collected. The apical media collected was utilized to run a lactate dehydrogenase (LDH) assay (Thermofisher, Cat. 88953) according to the manufacturer's instructions. A maximum lysis control was obtained by adding in 50 μL of lysis buffer provided in the kit of three day 8 old ALI Vk2 culture, diluted with antibiotic-free KSFM to a final volume of 100 μL before being assayed for LDH. LDH activity is represented by absorbance at 490 nm subtracted by absorbance at 680 nm (background). Percent cell viability was calculated by dividing the LDH activity of each experimental condition by the LDH activity of the maximum lysis control x 100%.

3.4.3 Transepithelial Resistance Measurements:

ALI grown Vk2 cells were grown as described above. 100uL of antibiotic free KSFM was added onto the apical side of ALI grown Vk2 cells, and incubated in 37°C, 5% CO2 or in the GasPak EZ Anaerobe Container System before transepithelial resistance (TER) measurements were taken using a voltohmmeter (World Precision Instruments). After TER measurements have been taken, the antibiotic free KSFM on the apical was aspirated. To evaluate the effect of *Lactobacillus* species, *G. vaginalis*, *P. bivia*, or co-cultures on TERs of Vk2 cells, day 7 (prior to the addition of bacteria) was utilized as a reference point. TER was shown as Percent Pre-treatment of TER

The calculations are as follows:

TER at Day 8 (with added bacteria) / TER at day 7 (prior to addition of any bacteria) x 100%

3.4.4 FITC-Dextran Leakage Assay:

On day 8 of culture, 24 hours after the addition of antibiotic-free KSFM to the apical side of each cell culture (with or without bacteria), the apical media was removed. 300 μL of antibiotic-free KSFM containing 10 kDa FITC-Dextran (2.5mg/mL) (Sigma Aldrich, Cat. FD10S250MG) was added onto the apical side of each transwell. After 24 hours, the basolateral media (25 μL) FITC-Dextran concentration was measured using a spectrophotometer at an excitation wavelength of 490nm, and emission wavelength of 520nm. A FITC-Dextran standard curve was created and plotted in order to correlate fluorescence and FITC-Dextran concentration. The concentration of FITC-Dextran in the basolateral media was divided by the FITC-Dextran that was added initially in order to calculate percent leakage of each of the Vk2 cultures.

3.5 Bacterial adherence and enumeration in Vk2 Cocultures:

On day 8 of cell culture, after 24 hours of bacterial co-culturing the apical antibiotic free KSFM (100uL) containing bacteria was serially diluted and plated on MRS agar (*L. crispatus* containing cultures) and/or Tryptic Soy Agar supplemented with 5% sheep's blood (*L. iners*, *G. vaginalis* and *P. bivia* containing cultures) to determine the non-adherent bacterial count (Apical). Subsequently, the apical side of each Vk2 cell culture transwell was washed with PBS. 100 μL of 1% Triton-X 100 was added onto the apical side. After 40 minutes of incubation at 37°C, the 100 μL of 1% Triton-X solution in the apical side of the transwells were serially diluted and plated on previously stated agar plates in order to quantify the adhered bacterial count. After 48 hours, the bacterial colonies were counted. For those

plated on the same agar, bacterial morphology was used to differentiate between bacterial species. The total bacterial count is the sum of non-adherent and adhered bacterial count. The ratio of adhered bacteria is calculated by the number of adhered bacteria divided by the total bacterial count.

3.6 pH measurements in Vk2 cocultures:

pH was measured for both single species and dual species cocultures with Vk2 cells. pH was measured for KSFM media before the addition of vaginal bacteria. Apical media (100 μL) was pooled from at least four wells after 24-hour coculture incubation period. pH was measured using an Oakton pH 700 Benchtop Meter (Thermofisher).

3.7 Cytokine Quantification of Vk2 Coculture Supernatants:

On day 8 of culture, 24 hours after the addition of antibiotic-free KSFM to the apical side of each cell culture (with or without bacteria), the apical (100 μL) and basolateral (700 μL) media was collected and stored at -80°C. Apical and basolateral collected Vk2 supernatants will be assayed for TNF-α, IL-6, IL-8, IL-1α, IL-1β, IL-1RA, MIP-1α, and RANTES using a Magpix multiplex kit (Millipore,) according to manufacturer's instructions.

3.8 Bacterial Phenotypic Assays

3.8.1 Hemolysis Assay:

Hemolytic activity was visually determined by streaking bacterial cultures on tryptic soy agar plates supplemented with 5% sheep or human blood. Blood agar plates were examined

for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green halo around colonies), or γ-haemolysis (no zones around colonies).

3.8.2 Hydrogen Peroxide Assay: All bacterial species were tested for hydrogen peroxide production by the Prussian Blue-plate assay described by Saito et al. Briefly, bacteria were streaked onto Prussian Blue agar, and the plates were incubated at 37°C under anaerobic conditions. The plates were checked for the development of the Prussian blue precipitate after 48-72 hours by exposure to aerobic conditions.

3.8.3 pH of bacterial broth media:

pH of liquid broth media was measured before and after the addition of bacterial species. NYC III media (or Reinforced Modified Clostridial for *P. bivia* analysis) starting at a pH of 6.8 was used with glucose as the substrate. pH was measured using an Oakton pH 700 Benchtop Meter (Thermofisher).

3.8.4 Antimicrobial activity assay. Antimicrobial activity of the lactobacilli species was detected by the agar-disk diffusion assay. 20 μL of bacteria-free culture supernatant filtrate of the lactobacilli species at various dilutions was applied to disks (6 mm) placed on agar plates previously streaked with either *G. vaginalis* or *P. bivia,* using bacteria-free media as a control. The plates were incubated in the Bactron IV anaerobic chamber for 72 hours. Antimicrobial activity was defined as giving a zone of inhibition with a diameter of at least 5 mm where no colonies were observed. To then control for pH, NaOH was added to supernatants to adjust to a pH of 7.0 in which inhibition tests were performed again.

3.8.5 Growth of vaginal bacteria at various pH levels:

All species were grown in unbuffered media, or media buffered to pH of 7.0 or 4.0. Media was buffered with hydrochloric acid at 5 mM (pH 7.0) or 40 mM (pH 4.0). pH of 7.0 was adjusted with NaOH. pH levels were measured and adjusted over a 24 hours period. Bacteria were then enumerated after 24 hours by plating on MRS agar plates (*L. crispatus* and *L.* jensenii) or tryptic soy agar supplemented with 5% sheep's blood (*L. iners, P. bivia* and *G. vaginalis*).

3.9 Bacterial Growth on various Carbohydrates, Proteins and Hormones:

All bacterial species were grown in overnight cultures at 37°C in anaerobic conditions using the GasPak EZ Anaerobe Container System in NYC III Media. Cultures were centrifuged for 5 minutes at 1,500 rpm. Supernatants were decanted, and bacteria were resuspended in PBS, and centrifuged again for 5 minutes at 1,500 rpm. PBS was decanted, and bacteria pellets were resuspended in glucose-free NYC III media containing either no added carbohydrates or 1.0 % glycogen type III from oyster (Sigma-Aldrich), glucose (Sigma-Aldrich), maltose (Sigma-Aldrich), sucrose (Sigma-Aldrich) or lactose (Sigma-Aldrich). For the lactobacilli growth cultures, 20 µL of Oxyrase (Sigma-Aldrich Cat. SAE0013) was added to 1 mL of cultures. 200 µL of each culture were added to a 96-well plate, in which three replicates were performed for each species in all media conditions. A layer of 80 µL of mineral oil (Sigma-Aldrich Cat. 330779) was placed on top of each well. *L. crispatus* cultures containing only NYC III media, only added Oxyrase, only added mineral oil, or both Oxyrase and mineral oil were used as controls to compare the influence of Oxyase and mineral oil on growth conditions. Optical density at 600 nm was read every 30 minutes for 72 hours using a BioTek

Synergy Plate Reader. For protein growth curves, experiments were repeated as previous stated, using NYC III media containing 1.0 % glucose with 5 mg, 10 mg or 20 mg of Lactoferrin (Human recombinant) (Sigma-Aldrich Cat. L4040), Albumin (Human recombinant) (Sigma-Aldrich Cat. A9731) and Mucin Type II (Sigma-Aldrich Cat. M2378) were added to 1 mL of culture. For hormone growth curves, experiments were repeated as previously stated, where NYC III media containing 1.0 % glucose was supplemented with 10^{-9} M estradiol, progesterone or medroxyprogesterone acetate. For *G. vaginalis* and *P. bivia* cultures, these experiments were repeated, however Reinforced Modified Clostridial was used for the growth of *P. bivia*. Once resuspended in media, both species were placed back in the GasPak EZ Anaerobe Container System instead of being inoculated into a 96-well plate. OD measurements were taken every 4 hours.

3.10 Liquid broth cocultures of *L. crispatus* with other vaginal bacteria:

NYC III media containing either 1.0% glucose, glycogen, maltose, sucrose or lactose as the carbohydrate source were inoculated with equal parts frozen stocks of *L. crispatus* and *L. iners* washed with phosphate-buffered saline-washed bacteria. To enumerate colonies, cultures were plated on both MRS agar (for enumeration of *L. crispatus*) and tryptic soy agar supplemented with 5% sheep's blood (for enumeration of *L. iners*) at 4, 8, 12 and 24 hours. These experiments were repeated with fresh cultures of bacteria that were harvested during exponential phase as assessed by optical density measurements from established growth curves of both *L. iners* and *L. crispatus*. From this, 10^6 CFU/mL of each species was added to NYC III media containing previously mentioned carbohydrate compositions and plated as previously described. This observation was replicated for NYC III media containing

combinations of carbohydrates at a final concentration of 0.5% each. Mucin type II was added at a final concentration of 10 mg/mL to NYC media containing glucose and bacterial colonies were assessed as previously described. Additional experiments consisted of the previously described methods, with *L. crispatus* inoculated at 10^5 CFU/mL and *L. iners* inoculated at 10^5 CFU/mL.

CHAPTER 4: RESULTS

4.1: Analyze interactions between host vaginal cells and common vaginal bacteria

In order to understand the ways in which VMB community compositions can influence susceptibility to HIV-1 and other STIs, it is critical to analyze host-bacterial interactions. The epithelial cells of the FRT are continually interacting with the indigenous microbiota, and they may respond to each species colonizing the FRT in a separate manner. To date, many *in vitro* studies have provided important clues about the immunomodulatory impacts that vaginal bacteria can have on FRT epithelial cells. However, it is important to recognize the various ways in which many models did not aptly reflect *in vivo* conditions of the FRT and thus allow us to improve upon such models. Many of these models used non-vaginal epithelial cells, or monolayers of vaginal epithelial cells, which does not accurately represent the multilayered squamous epithelium of the vaginal tract[119]. Moreover, many experiments were conducted in liquid-liquid interface systems, which once more is not biologically relevant to the conditions of the vaginal tract[138]. Other limitations of these studies include analyzing the interactions of a single species with host cells, which may mislabel and under- or over-represent how a species behaves in a community setting. As high diversity VMB communities are associated with fourfold increased risk of HIV-1 acquisition, understanding the host-bacterial interactions mediating this risk is critical[62]. Therefore, we aim to understand how bacterial species commonly found in the vagina interact differentially with vaginal epithelial cells either in isolation or with one another. With this, we established a more biologically relevant model to assess functional correlates that may reflect these species ability to induce protection or susceptibility in the FRT.

4.1.1: Assessment of cell viability of Vk2 cells in coculture with common vaginal bacteria

A primary and fundamental characteristic of bacterial and host interactions is their ability to influence viability of one another. As the bacterial species associated with a dysbiotic-VMB have been associated with a number of virulence factors, we wanted to assess whether these features had any impact on the viability of vaginal epithelial cells[139]. To assess viability of vaginal cells in the presence of select vaginal bacterial species, an *in vitro* air-liquid interface (ALI) cell culture system of vaginal epithelial cells (Vk2/E6E7) grown in transwell inserts was utilized[140]. This system more accurately resembles physiological conditions of the vaginal tract than traditional liquid-liquid interface systems as it allows for the VK2 cells to grow and differentiate into a multilayered squamous epithelium. To date, the majority of *in vitro* studies have conducted their analyses at atmospheric levels of oxygen of 21%. With this, the oxygen levels in the vaginal tract are much lower, at only approximately 2%[141]. Therefore, to better represent the *in vivo* conditions of the FRT, and to provide an environment more conducive to the growth of facultative or obligate anaerobic bacteria, Vk2 ALI cultures were placed in microaerophilic conditions. By this method, our *in vitro* system could be more reflective of *in vivo* oxygen levels in the FRT. This was achieved through the use of a GasPak EZ Anaerobe Container System.

To assess whether the microaerophilic conditions would cause any alterations in vaginal cells that could impact future experiments, Vk2 cells were grown in ALI culture conditions with KSFM for 10 days in which the barrier integrity and viability of Vk2 was monitored every 24 hours. Transepithelial resistance (TER) measurements were taken as a means to assess barrier integrity (Figure 1A), while viability was assessed by Trypan Blue

Exclusion assay (Figure 1B). TER measurements and cell viability reached a maximum value on culture day 9, with significant decreases occurring on days 10 and 11 (Figure 1). As such, all subsequent experiments were completed by day 9, prior to this decline. Additionally, as the microaerophilic conditions did not reduce viability and TERs of VK2's compared to atmospheric levels of oxygen, this system was used in subsequent experiments.

To understand the interactions between vaginal bacteria and host epithelial cells, we cocultured Vk2 cells grown in ALI cultures with common vaginal bacteria. To assess the factors that may lead to the variable levels of susceptibility observed by various VMB community compositions, we assessed vaginal bacteria representative of a protective (*L. crispatus*), intermediate (*L. iners*) and dysbiotic (*P. bivia* and *G. vaginalis*) VMB. In our analyses of species-host interactions, we cocultured each species singularly with Vk2 cells (single species coculture) or combined two species together (dual species coculture) to compare and contrast how these combinations may alter the host response.

To establish the conditions of this coculture system, we chose to analyze interactions using a 1:10 ratio of vaginal epithelial cells to bacteria, as previous studies have found this ratio to be most biologically relevant in terms of bacterial load and adherence measurements[76,119]. Moreover, *in vitro* studies analyzing bacterial-epithelial interactions have ranged from 6 to 48 hours of exposure[142,143]. With this, we chose to analyze interactions after 24 hours as previous studies have shown that significant changes did not occur between 24 to 48 hours, while all readouts were not observable at shorter time points[76,144]. Additionally, to reflect *in vivo* conditions, all bacteria were added to Vk2 cells during their log phase of growth, in which bacterial counts were determined through correlative optical density measurements.

Our first assessment of bacterial-host interactions was to assess cell viability of Vk2 cells cocultured with vaginal bacteria. Bacterial species were added to the apical side of Vk2 cultures in KSFM media on day 7 of Vk2 growth. To establish a multiplicity of infection (MOI) of 10, 6 x 10^6 CFU/mL of each species was added to cocultures. In order to keep this ratio and bacterial load consistent, when analyzing two vaginal species together with epithelial cells, 3 x 10^6 CFU/mL of each species was added to Vk2 cultures simultaneously. After 24 hours of exposure to vaginal bacteria, Vk2 cell viability of cocultures was assessed by Trypan Blue Exclusion cell viability assay (Figure 2) and cytotoxicity measurements were assessed by a Lactate Dehydrogenase (LDH) assay (Figure 3). In our coculture system, the addition of *L. crispatus* alone did not decrease cell viability of Vk2 cells (Figure 2A) or induce cytotoxicity (Figure 3A). Conversely, *L. iners* significantly reduced cell viability ($p < 0.01$) and induced cytotoxicity of Vk2 cells ($p < 0.001$). However, this reduction in cell viability and induction of cytotoxicity was no longer observed when *L. iners* and *L. crispatus* were co-cultured together (Figures 2A & 3A).

Similarly, bacteria associated with dysbiosis were cocultured with Vk2 cells. The presence of *P. bivia* reduced cell viability ($p < 0.01$) (Figure 2B) and induced increases in cytotoxicity ($p < 0.01$) (Figure 3B) by roughly 10%. When *G. vaginalis* was added, cell viability of Vk2 cells dropped by over 30% ($p < 0.001$) and cytotoxicity measurements were similarly significantly increased ($p < 0.001$). Moreover, when *P. bivia* and *G. vaginalis* were added together, cell viability and cytotoxicity measures were not significantly different than that of *G. vaginalis* added alone (Figure 2B & 3B).

To understand how the *Lactobacillus* species may interact with the dysbiosis-associated species, *L. iners* and *L. crispatus* were added to cultures with *P. bivia* or *G. vaginalis*.

Compared to *P. bivia* alone, the addition of *L. iners* enhanced the decreased cell viability ($p < 0.001$), while in contrast the addition of *L. crispatus* negated the reduction in viability back to levels of control (Figure 2C). Similar trends were observed in cytotoxicity measurements ($p < 0.001$) (Figure 3C). Likewise, while the addition of *L. iners* to *G. vaginalis* served to further decrease Vk2 cell viability as compared to G. vaginalis alone ($p < 0.001$), the addition of *L. crispatus* rescued this decrease (Figure 2D). These results were likewise reflected in cytotoxicity measurements ($p < 0.001$) (Figure 3D).

Together, in line with observations of clinical studies and other *in vitro* analyses, our results indicated that *G. vaginalis,* the species most associated with virulence in a dysbiotic VMB, induced the greatest decrease in Vk2 viability[145,146]. While *P. bivia's* role as a pathogenic member of a dysbiotic VMB is less clear, our results indicate any virulent tendencies are largely independent of cytotoxic functions. Moreover, while *L. iners* pathogenic nature is less obvious, it induced levels of cytotoxicity comparable to *G. vaginalis*, and thus is capable of expressing virulence factors at least under these conditions. Conversely, *L. crispatus*, the species most associated with protection in the VMB, did not induce any cytotoxicity or barrier damage, reflecting findings in other *in vitro* studies[147]. Moreover, all dual species cocultures not containing *L. crispatus* induced increased levels of cytotoxicity, while the combination of *L. crispatus* with all other species negated the reduction in cell viability and increases in cytotoxicity measures, bringing levels back up to that of control. As such, our findings support the role of *L. crispatus* as a beneficial species of the VMB.

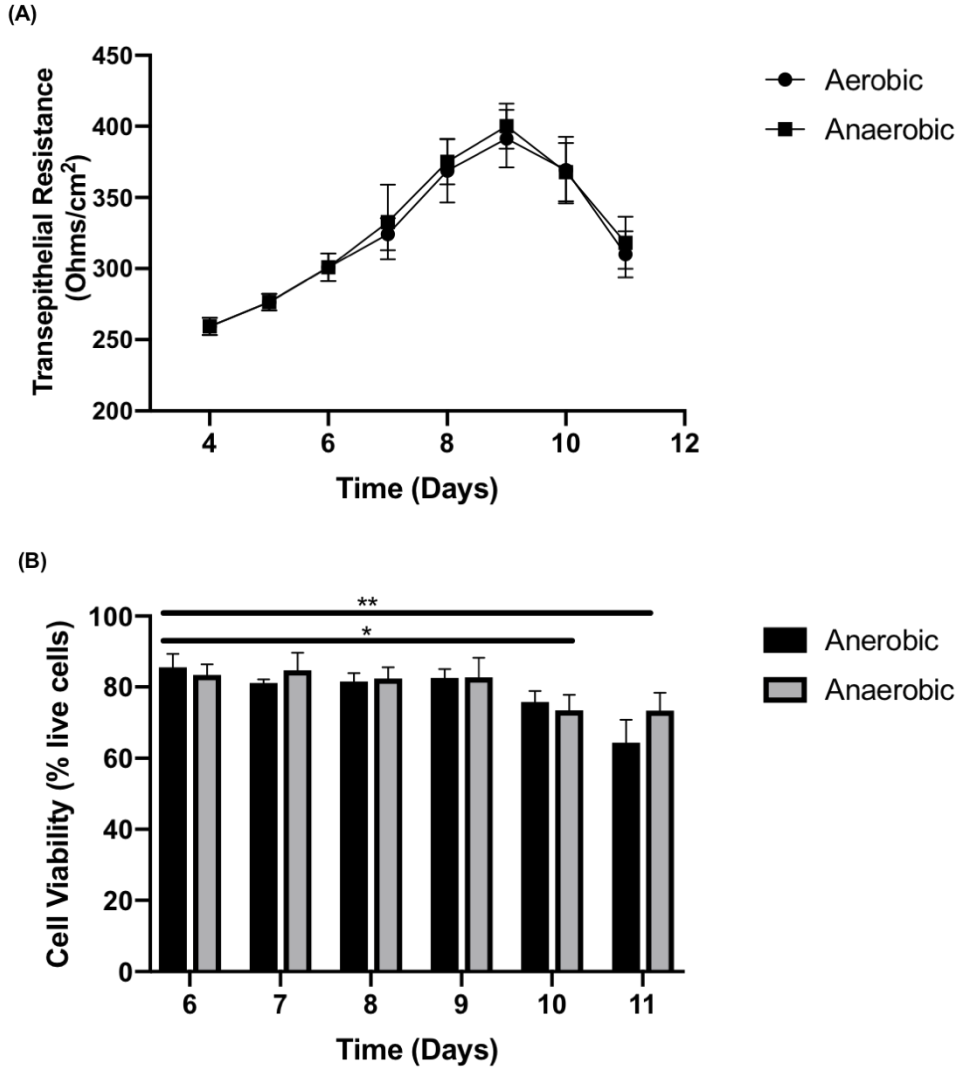

Figure 1: Maximal barrier function of Vk2 cells grown in ALI Culture is reached at culture day 9 in both aerobic and anaerobic conditions. Vk2 cells were grown in ALI culture conditions as described in Materials and Methods in both aerobic and anaerobic conditions. **(A)** TER measurements were taken for three separate well inserts from three experiments on different culture days 4 through 11. **(B)** Cell viability was assessed using trypan blue viability assay on days of 7 through 10. Viability data is representative of three experiments performed with three replicates on each day. Data was analyzed using one-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. *$p<0.05$, **$p<0.01$. Error bars represent SEM.

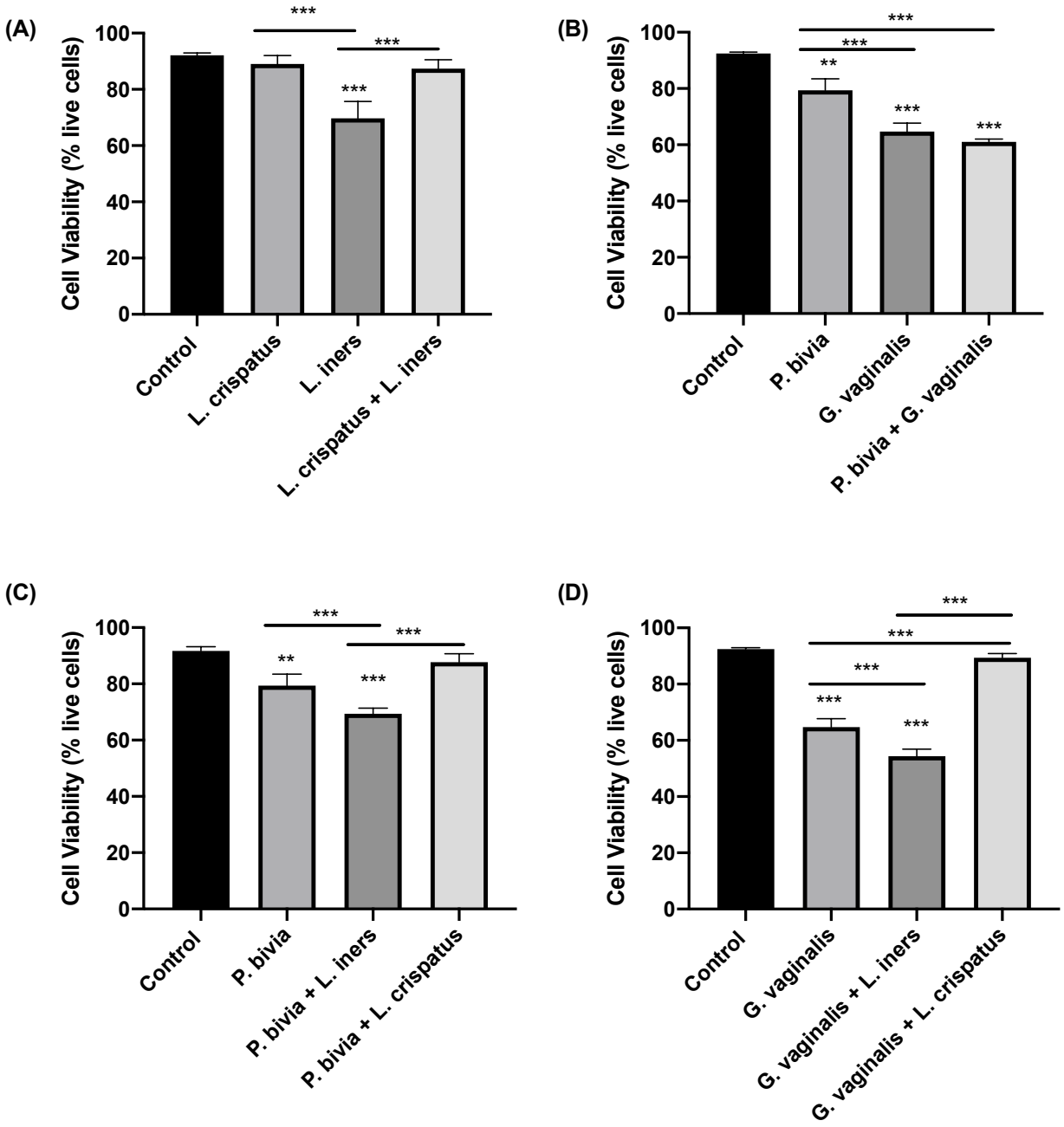

Figure 2: Cell viability of Vk2 cells in co-cultures with vaginal bacteria for 24 hours as assessed by Tryphan Blue Exclusion assay. **(A)** Co-cultured with lactobacilli species in isolation or combination **(B)** Co-cultured with dysbiosis-associated species in isolation or combination **(C)** Co-cultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Co-cultured with *G. vaginalis* in isolation or combination with lactobacilli

species. Cell viability is expressed as percent of live cells. Data shown represents four experiments done in triplicates. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

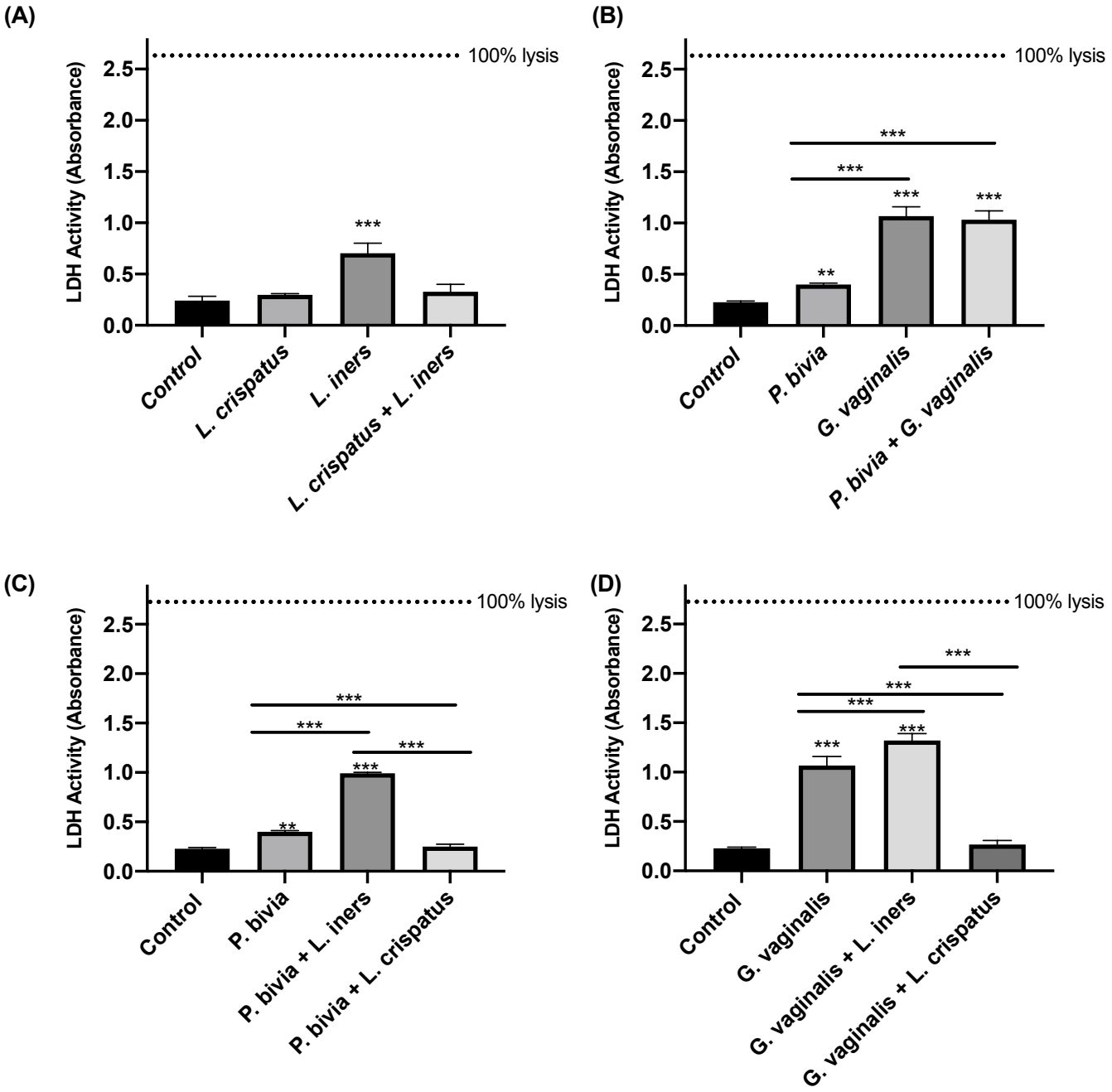

Figure 3: Cytotoxicity of Vk2 cells in co-cultures with vaginal bacteria for 24 hours as assessed by lactate dehydrogenase (LDH) assay. **(A)** Cocultured with lactobacilli species in

isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. The dotted line represents LDH activity of 100% lysis control of three Vk2-only transwells. Data shown represents five experiments done in triplicates. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

4.1.2 Determine the effect of common vaginal bacteria on epithelial barrier function

As an intact and robust vaginal epithelial barrier is imperative to prevent pathogenic organisms from translocating the epithelium, the influence of vaginal bacteria on the barrier function of ALI cultured Vk2 cells was assessed[148]. To measure barrier function, both TER measurements and Fluorescein isothiocyanate (FITC)-dextran leakage assays were performed. The FITC-dextran leakage assay is a paracellular leakage assay that can effectively be used to assess potential HIV-1 leakage across the epithelium. Vaginal bacteria, either as a single species or dual species were added to Vk2 ALI cultures as previously stated for 24 hours, after which FITC-Dextran was added to the apical side of each transwell. After 24 hours, the basolateral media FITC-dextran concentration was measured using a spectrophotometer to determine leakage. The addition of *L. crispatus* did not significantly decrease barrier integrity as measured by both TER measurements (Figure 4A) or FITC-dextran leakage (Figure 5A) as compared to control. Conversely, the addition of *L. iners* led to a significant decrease in TER measurements ($p < 0.001$) and increase in FITC-dextran leakage ($p < 0.001$). Dual species cocultures of *L. iners* with *L. crispatus* were able to rescue much of this decrease in barrier integrity; however, TER measurements were still lower than that of control, and FITC leakage greater ($p < 0.05$)(Figure 4A & 5A).

A compromised epithelial barrier has been speculated as a mechanism by which BV induces increased susceptibility to HIV-1, therefore we assessed the addition of dysbiosis-associated bacteria to Vk2 cultures as previously described for the *Lactobacillus* species[149]. The addition of *P. bivia* alone induced a reduction in TER measurements by nearly 30% ($p < 0.001$) (Figure 4B) and almost doubled FITC-leakage compared to control ($p < 0.001$) (Figure 5B). The addition of *G. vaginalis* induced even greater decreases in TERs ($p < 0.001$) and further increased FITC-leakage ($p < 0.001$). Dual species cocultures of *P. bivia* and *G. vaginalis* had TER measurements that were nearly 10% lower than *P. bivia* single species cocultures ($p < 0.001$), and FITC-leakage significantly greater than both single species cocultures ($p < 0.001$).

To understand if the addition of either *Lactobacillus* species could influence the decrease in barrier integrity observed in cocultures of dysbiosis-associated species, individual lactobacilli were added with either *P. bivia* or *G. vaginalis*. Dual species cocultures of *P. bivia* and *L. iners* induced a nearly 30% decrease in TER measurements ($p < 0.001$) (Figure 4C) and similarly a greater increase in FITC-leakage than *P. bivia* alone ($p < 0.001$) (Figure 5C). Conversely, dual species cocultures of *L. crispatus* and *P. bivia* displayed barrier integrity measurements that were not significantly different from controls. Similar results were observed in *G. vaginalis* cocultures, where the addition of *L. iners* further decreased TER measurements (Figure 4D), and increased FITC-leakage as compared to *G. vaginalis* alone ($p < 0.001$) (Figure 5D). Once more, dual species cocultures of *L. crispatus* and *G. vaginalis* returned barrier integrity measurements back to levels of control (Figures 4D & 5D).

Similar to Vk2 cell viability measurements, the addition of *G. vaginalis*, *P. bivia* and *L.*

iners, and their combinations induced the greatest reduction in barrier integrity measurements. However, the combination of *L. crispatus* to any of these cultures negated these effects and brought barrier integrity measurements back to levels of control. As such, these findings coincide with the association of *L. crispatus* with protection in the FRT, whereby it can negate the harmful effects of dysbiosis-associated species and *L. iners*[150]. Moreover, our findings support the notion that vaginal bacteria can influence the vaginal epithelial barrier, and thus may be a mechanism by which certain species can increase susceptibility to HIV-1 infection.

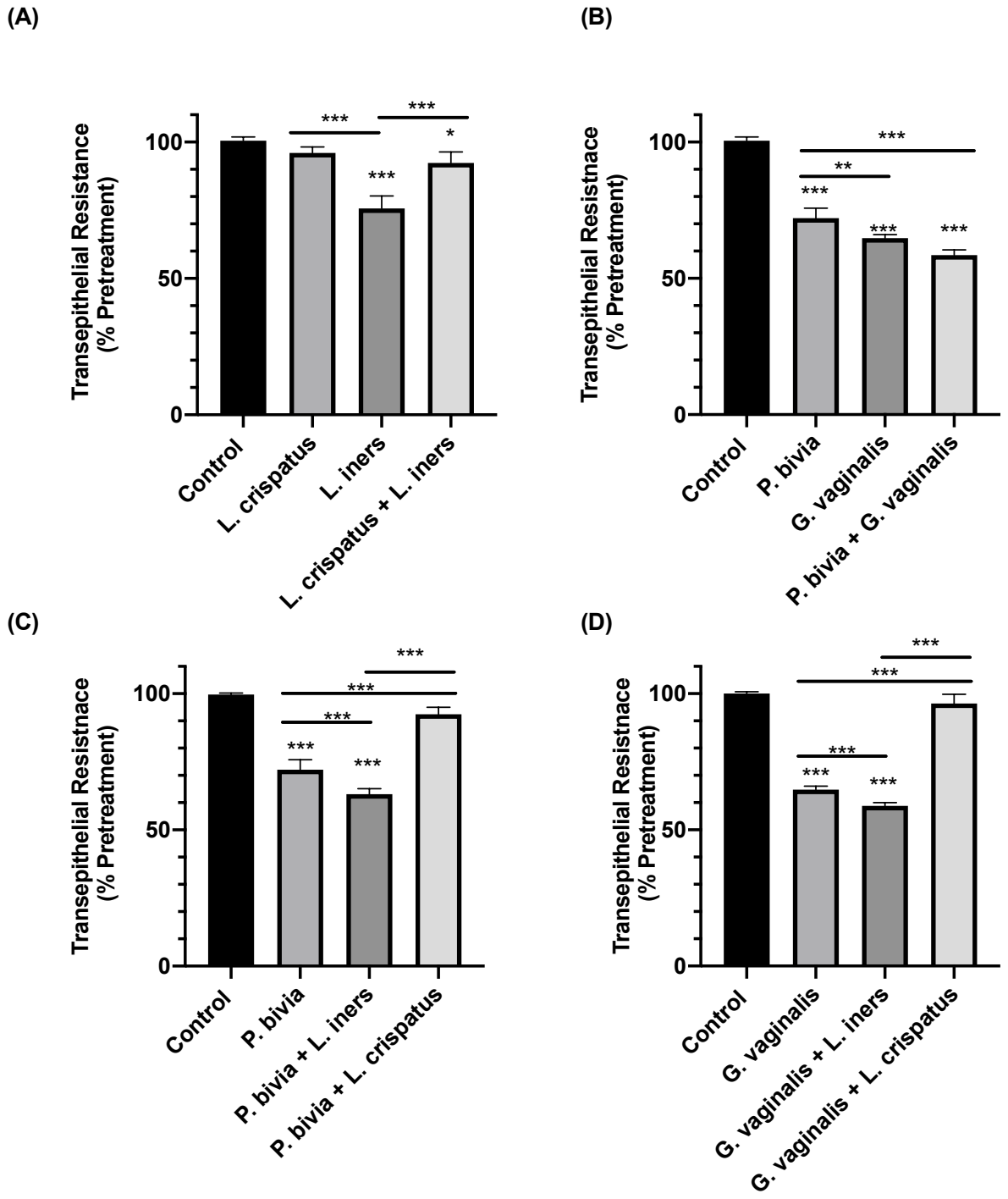

Figure 4: Barrier function of Vk2 cells when co-cultured with vaginal bacteria for 24 hours as assessed by transepithelial resistance (TER) measurements. **A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated

species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Control is Vk2 cells without added bacteria. TER of Vk2 cells is expressed as percent of pre-treatment TER. Data shown represents four separate experiments, each done in triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Error bars shown represent SEM.

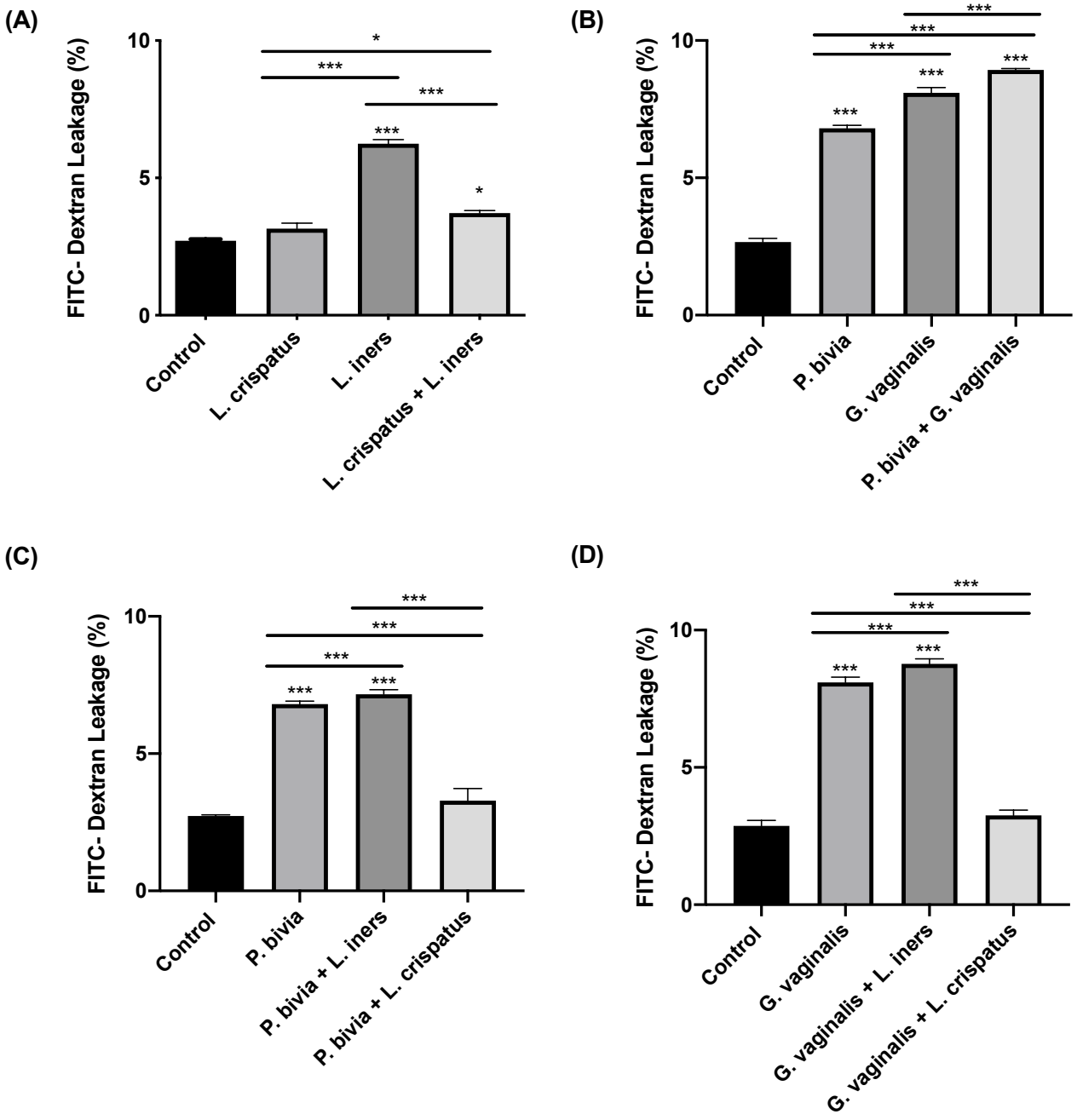

Figure 5: Barrier function of Vk2 cells when co-cultured with vaginal bacteria for 24 hours as assessed by FITC-dextran leakage assay. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Control is Vk2 cells without added bacteria. Data shown represents four separate experiments, each

done in triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

4.1.3 Determine growth and adherence of common vaginal bacteria in Vk2 cocultures

In order to appropriately interpret the results of the interactions between vaginal bacteria and Vk2 cells, we wanted to understand if the viability of bacteria in single or dual species cocultures were altered in our culture system. Moreover, as adherence to vaginal epithelial cells and the ability of lactobacilli to co-aggregate on the epithelium has been postulated as a mechanism by which lactobacilli may defend against invading pathogens and enhance the physical barriers in the FRT, we wanted to evaluate this property as well[151]. To assess bacterial growth in cocultures with Vk2 cells, CFU count was enumerated after 24-hour addition in Vk2 cocultures. The apical media was collected and plated to quantify non-adherent vaginal bacteria; subsequently 1% Triton-X was added to cocultures as a method to detach adhered bacteria. This 1% triton-x suspension was then plated in order to quantify the bacteria that had been adherent. Total bacterial count was calculated as the sum of both non-adherent and adherent fractions.

With an initial inoculum of 6 x 10^6 CFU/mL, we found that both *L. crispatus* and *L. iners* were able to maintain their bacterial load after 24 hours in coculture with Vk2 cells (Figure 6A). Moreover, we found that relatively equal amounts of both species were observed after 24 hours in dual species cocultures of both *L. crispatus* and *L. iners*. These ratios were also reflected in adhered bacterial counts (Figure 7A). While it appeared that *L.*

iners may have adhered in greater proportions in dual species cocultures with *L. crispatus*, this observation was not significantly different.

As biofilm formation has been suggested as a mechanism by which BV-associated bacteria, in particular *G. vaginalis*, are able to resist antibiotic treatment and cause relapses, we wanted to assess bacterial growth and adherence measurements for the dysbiosis-associated species as well[146]. Bacterial loads of both *P. bivia* and *G. vaginalis* did not significantly differ after 24 hours of coculture (Figure 6B), however in *P. bivia* and *G. vaginalis* cocultures, *G. vaginalis* and *P. bivia* appeared to grow significantly ($p < 0.001$). In analyzing their adherence properties, *P. bivia* adhered significantly less in single species cocultures than *G. vaginalis*; however, in dual species cocultures with one another, they adhered in similar proportions (Figure 7B). These results suggest that the combination of *G. vaginalis* and *P. bivia* had a synergistic effect that allowed further growth of both bacteria, in which in the case of *P. bivia* may largely be due to increased adherence.

To understand how *Lactobacillus* species may be able to compete with dysbiosis-associated species, or disrupt their adherence to vaginal epithelial cells, we tested these bacteria in dual species cocultures. The dual species coculture of *L. iners* with *P. bivia* did not significantly alter bacterial loads of either species after 24 hours, while *L. iners* had slightly higher adhered proportions than *P. bivia* ($p < 0.05$) (Figures 6C and 7C). Conversely, in dual species cocultures of *L. crispatus* and *P. bivia*, *L. crispatus* had higher bacterial counts after 24 hours ($p < 0.001$), while *P. bivia* counts experienced a significant drop ($p < 0.001$) (Figure 6C). With this, in the *P. bivia* and *L. crispatus* dual species cocultures, the majority of the surviving *P. bivia* bacteria after 24 hours were adhered to Vk2 cells, resulting in a higher ratio of adherence than *P. bivia* alone ($p < 0.001$) (Figure 7C). Similarly, in the *L. iners* and *G.*

vaginalis dual species cocultures, difference in bacterial counts after 24 hours were not observed for either *L. iners* or *G. vaginalis* (Figure 6D). However, in these cocultures, *G. vaginalis* appeared to adhere in higher proportions compared to *G. vaginalis* alone ($p < 0.001$) (Figure 7D). Conversely, in dual species cocultures of *L. crispatus* and *G. vaginalis,* bacterial counts were significantly lowered for *G. vaginalis* ($p < 0.001$), while *L. crispatus* counts were increased ($p < 0.01$) (Figure 6D). The majority of *G. vaginalis* that were viable after 24 hours with *L. crispatus* were found to be largely adhered bacteria, which accounted for nearly 90% of bacteria enumerated (Figure 7D).

These results indicate that while all bacteria remained viable in their single species cocultures with Vk2 cells, differences in viability and adherence proportions were observed in dual species cocultures. Notably, while most species were able to maintain their bacterial load, if not increase, when cultured with another species, this was not true for either *P. bivia* or *G. vaginalis* in their dual species coculture with *L. crispatus*. As such, it appears that *L. crispatus* has significant antimicrobial activity against both dysbiosis-associated species, and thus outcompetes them under these conditions. These findings are in line with the detection of dysbiosis-associated species in women with an *L. iners*-dominant VMB, which is not the case for other *Lactobacillus*-dominated VMBs[36]. Moreover, the lower rates of BV incidence in women harbouring a *L. crispatus*-dominated VMB as compared to other lactobacilli species are supported by our findings of significantly reduced viability of dysbiosis-associated species in dual species cocultures with *L. crispatus*[152].

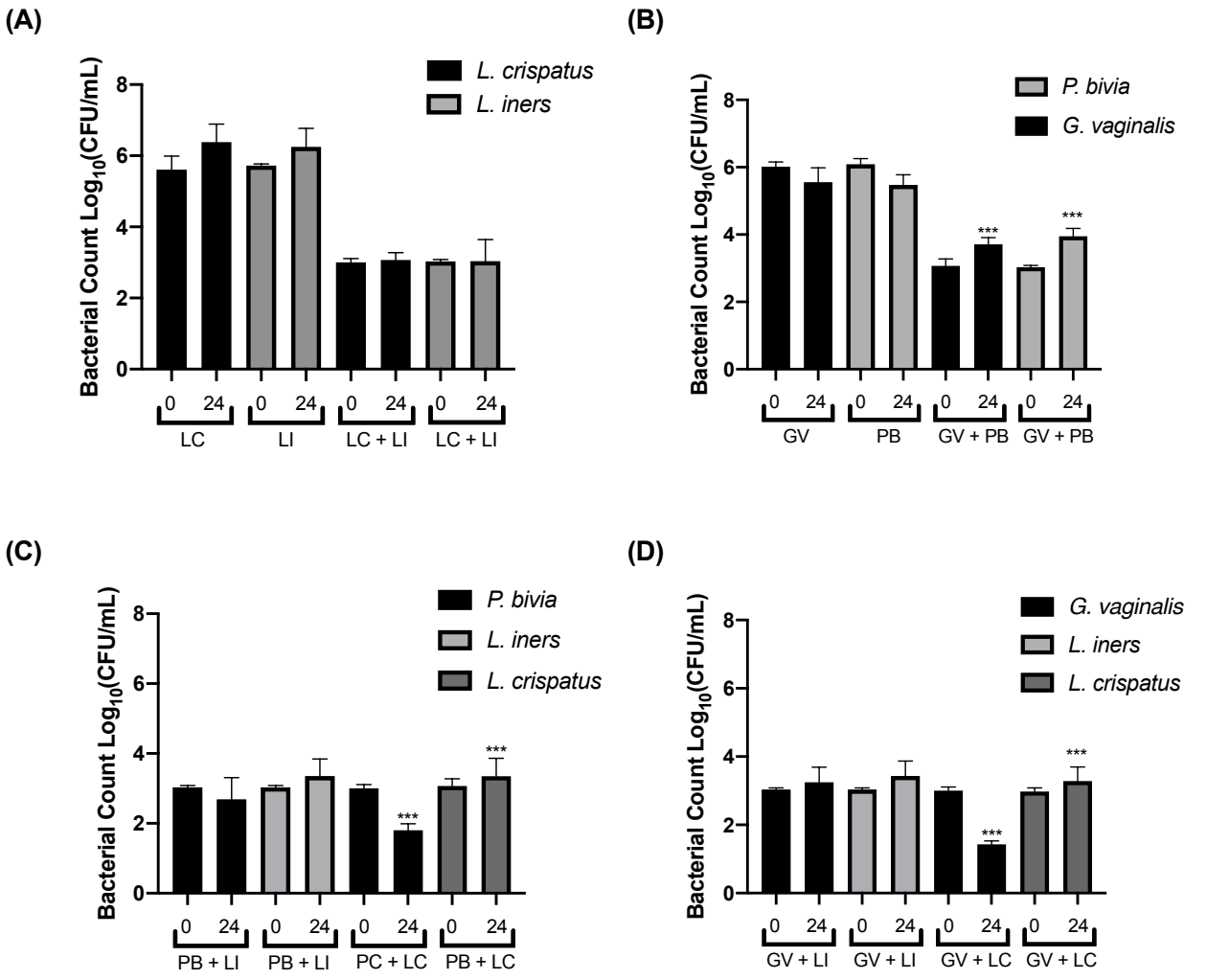

Figure 6: Bacterial count of vaginal bacteria after 24 hours of co-culture with Vk2 cells. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Total bacteria added for single species co-cultures was 6 x 10^6 CFU/mL. For co-cultures, 3 x 10^6 CFU/mL of each bacteria was added. Bacterial count at 0 hours represents bacterial count prior to addition Data shown represents six separate experiments, each done in triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

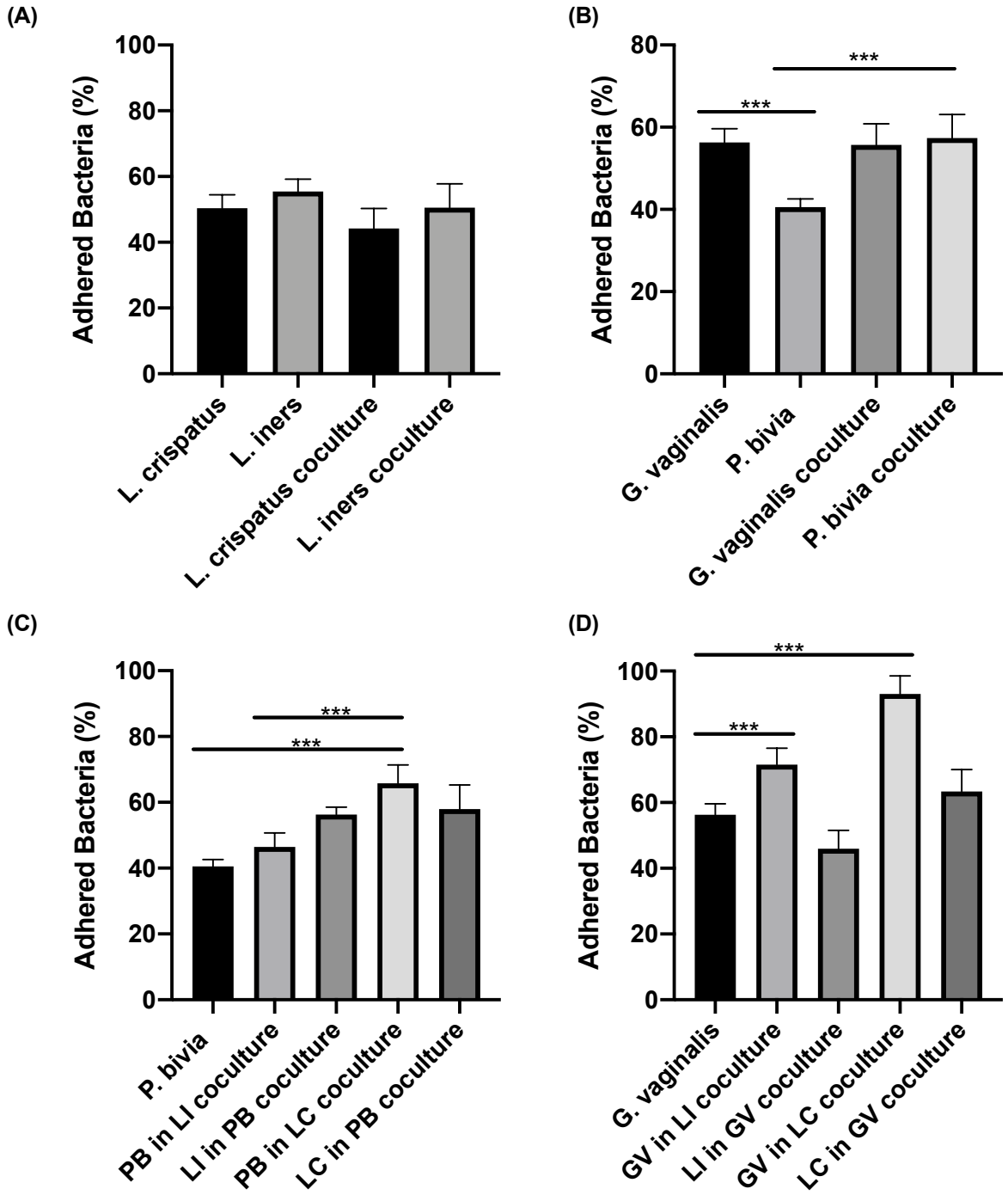

Figure 7: Bacterial adherence of vaginal bacterial after 24 hours of co-culture with Vk2 cells. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Data is shown as percentage of total

bacteria counts adhered to Vk2 cells after 24 hours. Data shown represents six separate experiments, each done in triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

4.1.4 Evaluate the microenvironment of Vk2 epithelial cells in the presence of common vaginal bacteria

The ability for *Lactobacillus* species to lower the pH of the vaginal tract has long been cited as a potent defence mechanism of lactobacilli against invading pathogens and dysbiosis-associated bacteria[153]. As such, we measured if the pH of the cocultures would reflect this difference *in vitro*. Additionally, inflammation in the FRT has long been recognized as an independent risk factor for HIV-1 and a number of other adverse reproductive outcomes, and elevated levels of inflammatory cytokines are often found in women harbouring a dysbiotic VMB[154,62]. With this in mind, we analyzed how the presence of common vaginal bacteria, either alone or in combination, can influence cytokine production by Vk2 cells.

The pH of Vk2 cells grown in KSFM was measured before the addition of bacterial species in both single and dual species cocultures, and 24 hours following. In all coculture conditions, the pH did not significantly alter and remained at a pH of approximately 7.0 (Figure 8). While this does not reflect observations observed *in vivo*, this is likely owing to the buffering capabilities of KSFM media. As such, the protective properties previously observed by the addition of *L. crispatus* to cocultures of species that induced decreased

viability of Vk2 cells and reduced barrier integrity, does not appear to be due to its ability to lower the pH in this co-culture system.

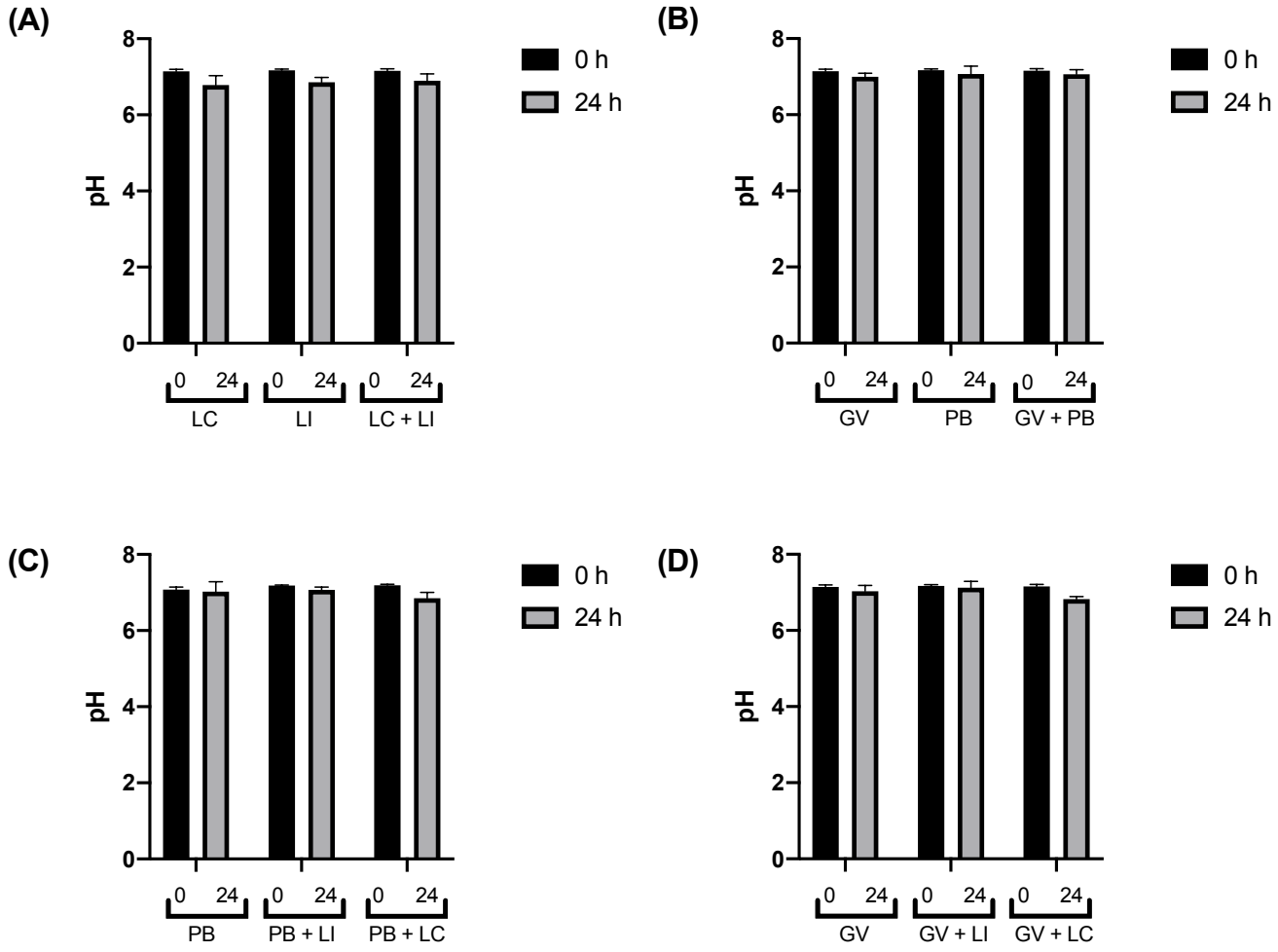

Figure 8: pH of Vk2-vaginal bacteria co-cultures after 24 hours. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Data represents pooled apical media from five transwells, done in triplicates from three separate experiments. Data was analyzed by two-way ANOVA with

Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

To analyze the influence of vaginal bacteria on cytokine production, the apical and basolateral supernatants of Vk2 cells grown in ALI cultures from all culture conditions were collected. A dysbiotic VMB has long been associated with elevated levels of a number of proinflammatory cytokines, however the results are not always consistent[62,155]. While several factors may account for these inconsistencies, a key reason may be due to the variability in composition of a dysbiotic VMB, in which differing abundances of specific species may alter the cytokine milieu. As such, *in vitro* studies can allow for a species-specific analysis of cytokine induction. While many of these cytokines have been analyzed in previous *in vitro* studies of vaginal bacterial, differences in species assessed, cytokine panel used, and model systems makes comparisons between studies and generalizations difficult[156]. As such, we wanted to analyze the most relevant cytokines that are produced by vaginal epithelial cells all under the same conditions for both our single and dual species cocultures. With this, based on our literature search of altered cytokine levels from previous *in vivo* and *in vitro* studies in association with vaginal bacteria, we chose to quantify TNF-α, IL-8, IL-6, IL-β, IL-1α, IL-1RA, RANTES and MIP-1α using a Luminex-MagPix Cytokine Assay[62,157,158,159].

Several cross-sectional and longitudinal studies have reported an increase in TNF-α in the cervicovaginal secretions of women with BV[156,160]. TNF-α is a proinflammatory cytokine and is also thought to be a pivotal cytokine in inducing the proinflammatory milieu observed in women with BV. Together with IL-1β, it is particularly effective in initiating a cascade of other inflammatory mediators by way of the NF-kB signal transduction

pathway[161]. This has been shown for vaginal epithelial cells where TNF-α stimulates the production of IL-8[162]. While TNF-α levels in all coculture conditions were relatively low (below 10 pg/mL), this is not unexpected as this is within the range found in other *in vitro* models[163]. While vaginal epithelial cells don't induce large amounts of TNF-α, differences across the bacterial species cocultures were observed that could have important implications. While the presence of all species induced an increase in TNF-α levels above baseline, lowest levels of induction were observed by *L. crispatus* alone or with other species (Figure 9). Conversely, *P. bivia* induced the greatest induction of TNF-α, followed by *G. vaginalis* and the two combined (Figure 9B). Additionally, the presence of both *L. iners* or *L. crispatus* in dual species cocultures with dysbiosis-associated species showed lowered levels of TNF-α than induced by *P. bivia* or *G. vaginalis* alone, however the effect from *L. crispatus* was more pronounced ($p < 0.001$) (Figure 9C & 9D). As such, unlike our measurements of cell viability and barrier integrity, *L. iners* appears to have a beneficial feature in lowering levels of TNF-α of dysbiosis-associated species. While these results reflect *in vivo* findings where *L. crispatus*-dominated VMB are associated with lower levels of TNF-α, while dysbiotic VMBs are associated with the highest, with such low levels of TNF-α quantified in our system, the biological relevance of these observations require further investigation[62,160].

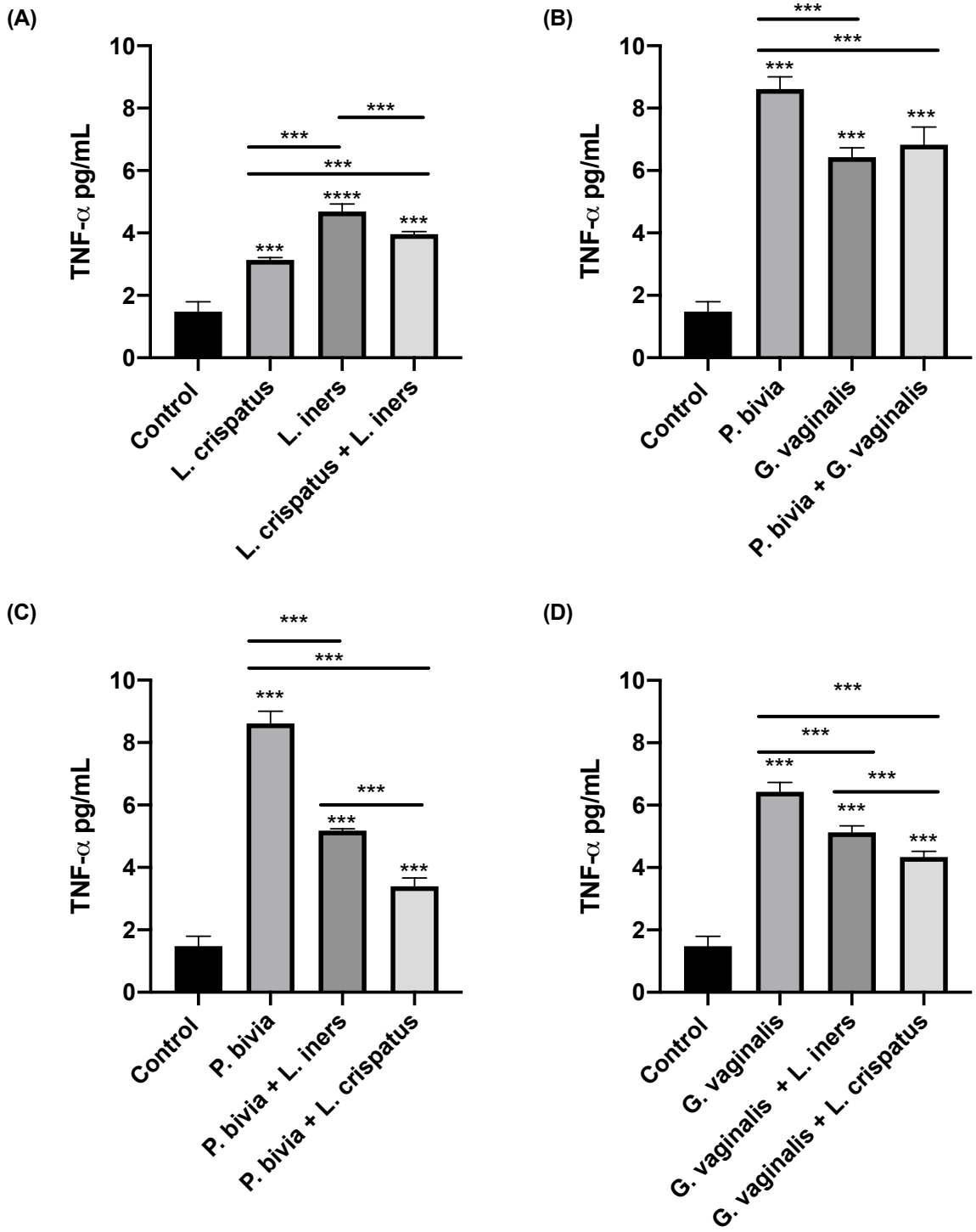

Figure 9: TNF-α levels in the apical media of Vk2 cells after 24 hours of coculture with vaginal bacteria. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis*

in isolation or combination with lactobacilli species. Data shown represents three separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

Another inflammatory marker we chose was IL-8 (or CXCL8), which is a powerful chemokine that can be rapidly induced in epithelial cells and has been shown to be important in neutrophil infiltration in various types of mucosal inflammation[164]. IL-8 has also been shown to be elevated in cervicovaginal lavage samples from women with BV or a dysbiotic VMB[156]. In our coculture system, addition of all bacteria induced IL-8 production from Vk2 cells above baseline, however the greatest induction was observed with *G. vaginalis* and its dual species coculture with *P. bivia* (Figure 10B). *L. iners* also significantly induced IL-8 production, in which high levels were also observed when it was combined in dual species cocultures with either *P. bivia* or *G. vaginalis,* greater than the species on their own. In contrast, the presence of *L. crispatus* in cocultures with either *P. bivia* or *G. vaginals* reduced the levels of IL-8 by more than half ($p < 0.001$) (Figure 10C & 10D). These findings support *in vivo* observations that dysbiotic VMBs with a relatively high abundance of *G. vaginalis* have higher levels of IL-8 as compared to *L. crispatus*-dominated VMB[62,156].

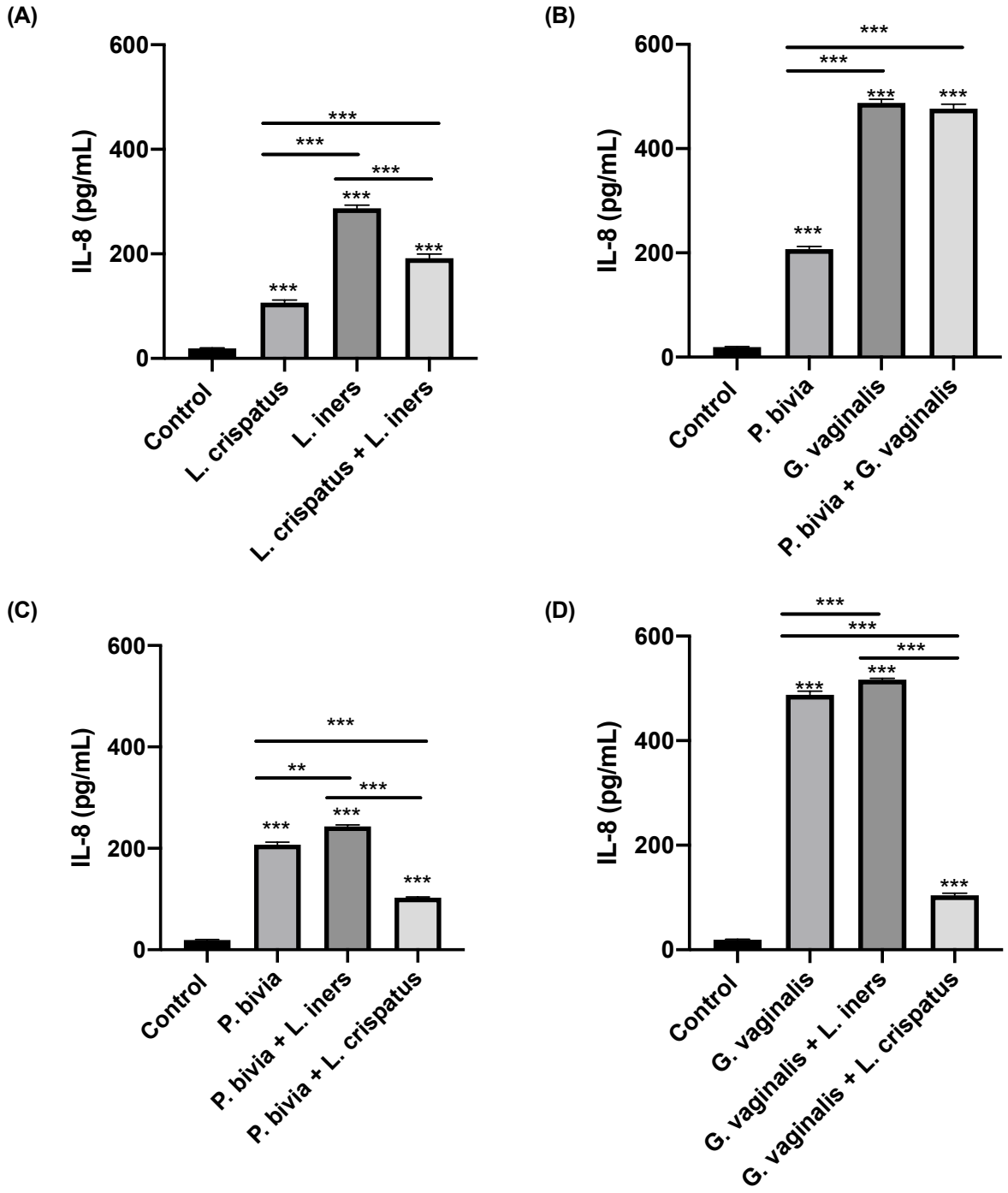

Figure 10: IL-8 levels in the apical media of Vk2 cells after 24 hours of coculture with vaginal

bacteria. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Data shown represents three separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

Another important marker we chose to analyze was IL-6, which is a multifunctional cytokine and a potent inducer of the acute-phase protein response that regulates inflammation[165]. While less consistently than other cytokines, IL-6 has been shown to be elevated in cervicovaginal lavage samples from women with BV in several studies[166]. In our analyses, single species cocultures of both *L. crispatus* and *L. iners* had IL-6 levels below the level of detection, as was observed for baseline no-bacteria control (Figure 11A). However, a significant increase was seen by *P. bivia* and an even greater increase by *G. vaginalis* and their combination ($p < 0.001$) (Figure 11B). Dual species cocultures of *L. iners* with either *P. bivia* or *G. vaginalis* did not significantly differ from cocultures containing them as a single species, however the addition of *L. crispatus* significantly dropped IL-6 quantities to levels just above detection ($p < 0.001$) (Figure 11C & 11D). While IL-6 levels of cocultures with either lactobacilli species were below detection, our findings of significant induction by *P. bivia* and *G. vaginalis* are in line with the *in vivo* studies that have found higher levels of IL-6 in women with a VMB containing both of these species[166].

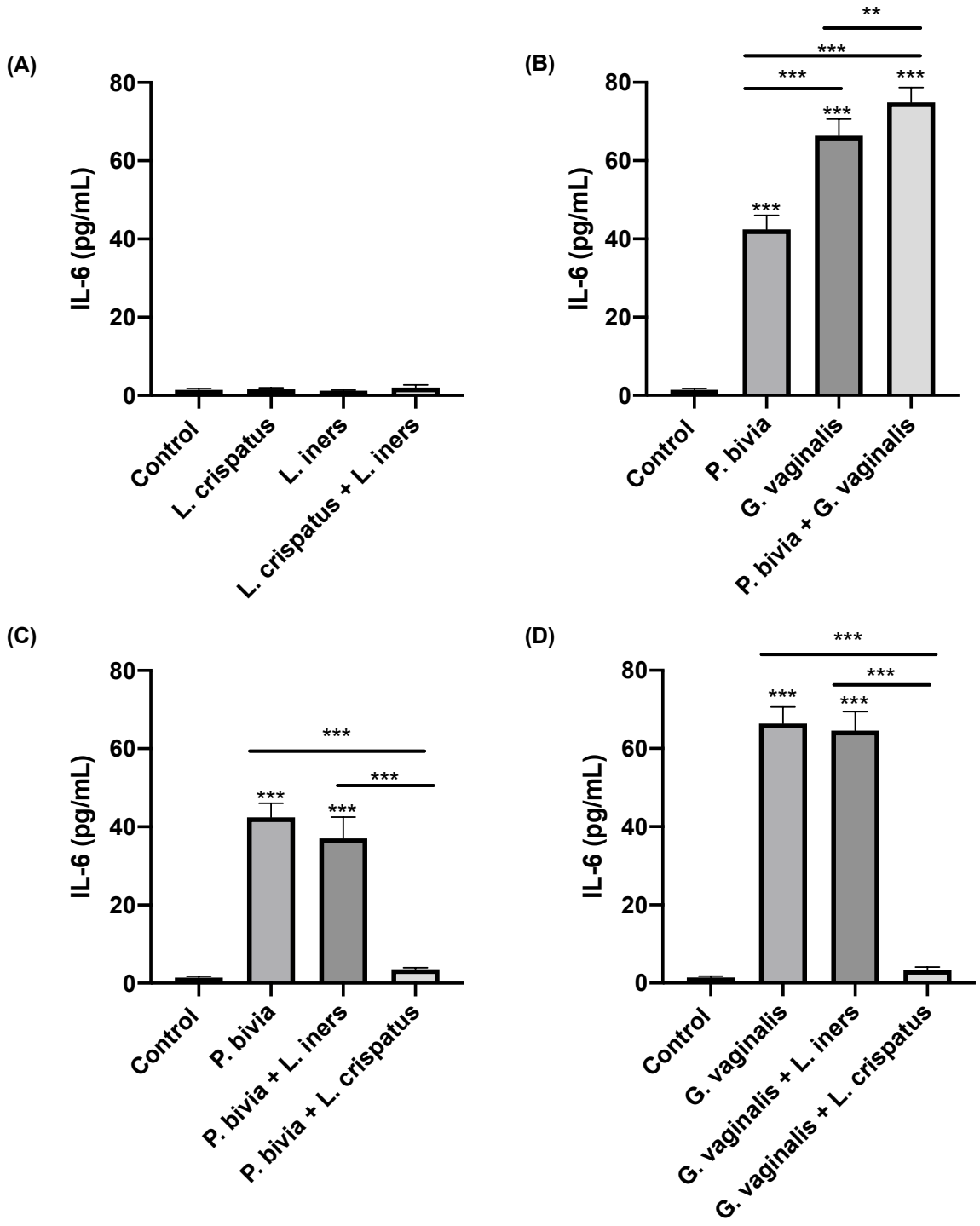
(A)
IL-6 (pg/mL)
0
20
40
60
80
Control
L. crispatus
L. iners
L. crispatus + L. iners
(B)
IL-6 (pg/mL)
0
20
40
60
80
***
**
Control
P. bivia
G. vaginalis
P. bivia + G. vaginalis
(C)
IL-6 (pg/mL)
0
20
40
60
80
***
Control
P. bivia
P. bivia + L. iners
P. bivia + L. crispatus
(D)
IL-6 (pg/mL)
0
20
40
60
80
***
Control
G. vaginalis
G. vaginalis + L. iners
G. vaginalis + L. crispatus

Figure 11: IL-6 levels in the apical media of Vk2 cells after 24 hours of coculture with vaginal bacteria. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Data shown represents three separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

Another important cytokine we chose for our panel was IL-1*β.* IL-1*β* exerts its protective action against infections by activating several responses including the rapid recruitment of neutrophils to inflammatory sites, induction of other cytokines and chemokines, and the stimulation of specific adaptive immunity responses[167]. Moreover, the increase in IL-1β observed in women with BV has been associated with the production of the hydrolytic enzymes sialidase and prolidase that are produced by *G. vaginalis* and thought to contribute to the adverse symptoms of BV[168]. The addition of *L. crispatus* to Vk2 cells did not significantly induce IL-1β production compared to baseline, however the addition of *L. iners* did ($p < 0.001$) (Figure 12A). IL-1β levels were more than 80% higher than *L. iners* in Vk2 cocultures with *P. bivia*, *G. vaginalis* or their combination ($p < 0.001$) (Figure 12B). When either *G. vaginalis* or *P. bivia* were cocultured with *L. iners*, IL-1β levels were significantly higher compared to either species alone by approximately 10% ($p < 0.001$) (Figure 12C & 12D). However, when either species was cocultured with *L. crispatus*, IL-1β levels dropped by more than 80% ($p < 0.001$). These results mirror those found in cervicovaginal lavage samples, where women with a *Lactobacillus*-dominant VMB have low levels of IL-1β compared to those with a dysbiotic VMB[156,169].

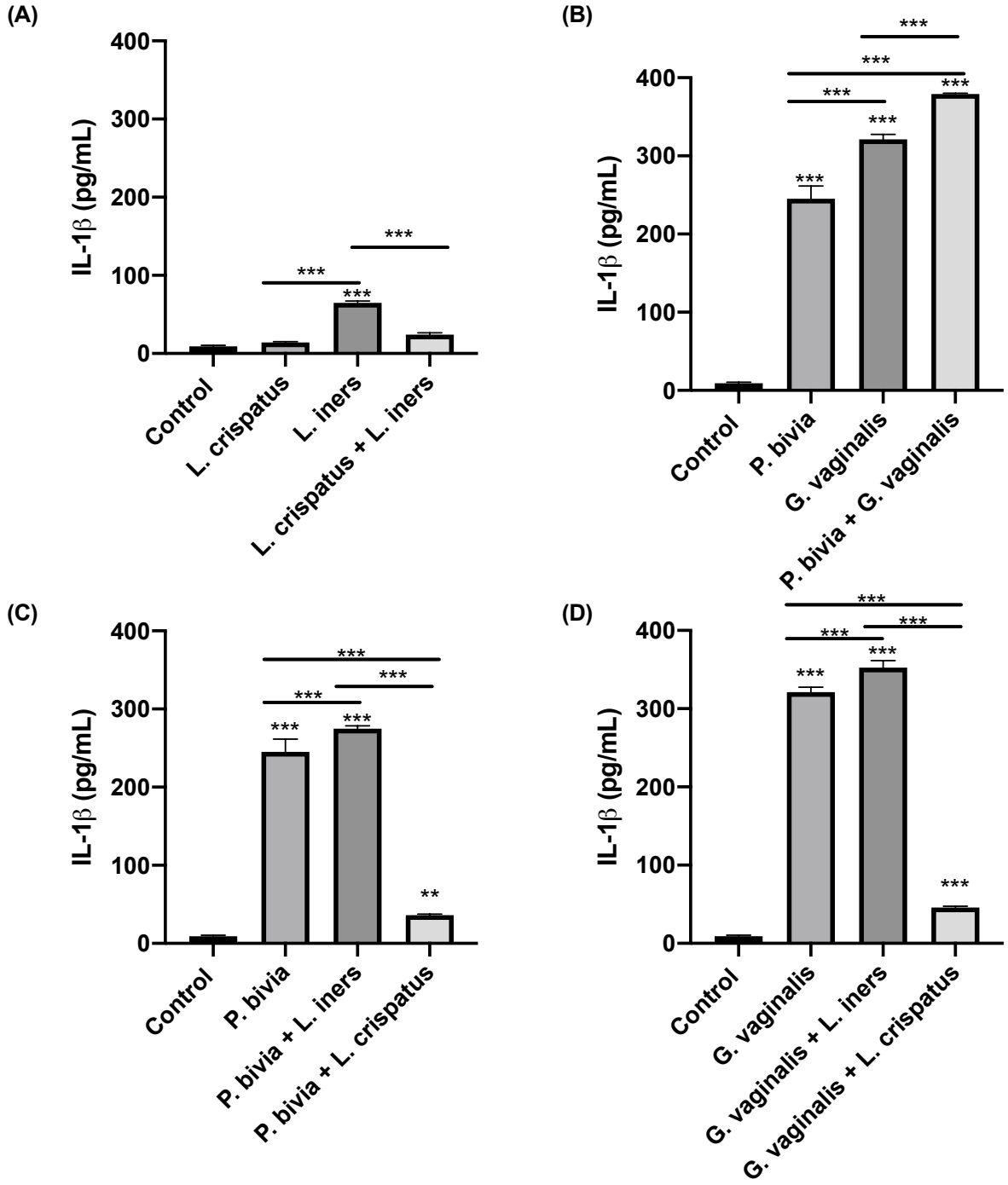

Figure 12: IL-1β levels in the apical media of Vk2 cells after 24 hours of coculture with vaginal bacteria. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G.*

vaginalis in isolation or combination with lactobacilli species. Data shown represents three separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

Another member of the IL-1 family is IL-1α. Both IL-1β and IL-1α can signal through the IL-1R receptor, however IL-1α is more widely and constitutively expressed than IL-1β[170]. IL-1α plays an important role in initiating innate immune responses to many bacterial infections and has been found to be more than 10-fold higher in women with a dysbiosis-associated VMB[171,62]. At baseline there was IL-1α produced, and in our cocultures, all bacterial species significantly increased levels of IL-1α compared to control, however this increase was lowest in cocultures with *L. crispatus*, when either alone or combined with other species (Figure 13). While *L. iners* did induce levels greater than *L. crispatus*, both *P. bivia* and *G. vaginalis* increased IL-1α levels by more than 75%, with the greatest increases observed when they were cocultured together ($p < 0.001$) (Figure 13B). Levels of IL-1α in cocultures of either *P. bivia* or *G. vaginalis* with *L. iners* were lowered by nearly 20% as compared to their cocultures alone ($p < 0.001$) (Figure 13C & 13D). Most notably, when either species was combined with *L. crispatus*, levels of IL-1α were lowered by nearly 70% ($p < 0.001$). These results correspond with findings in cervicovaginal lavage samples where women with a dysbiotic VMB have highly elevated levels of IL-1α[62].

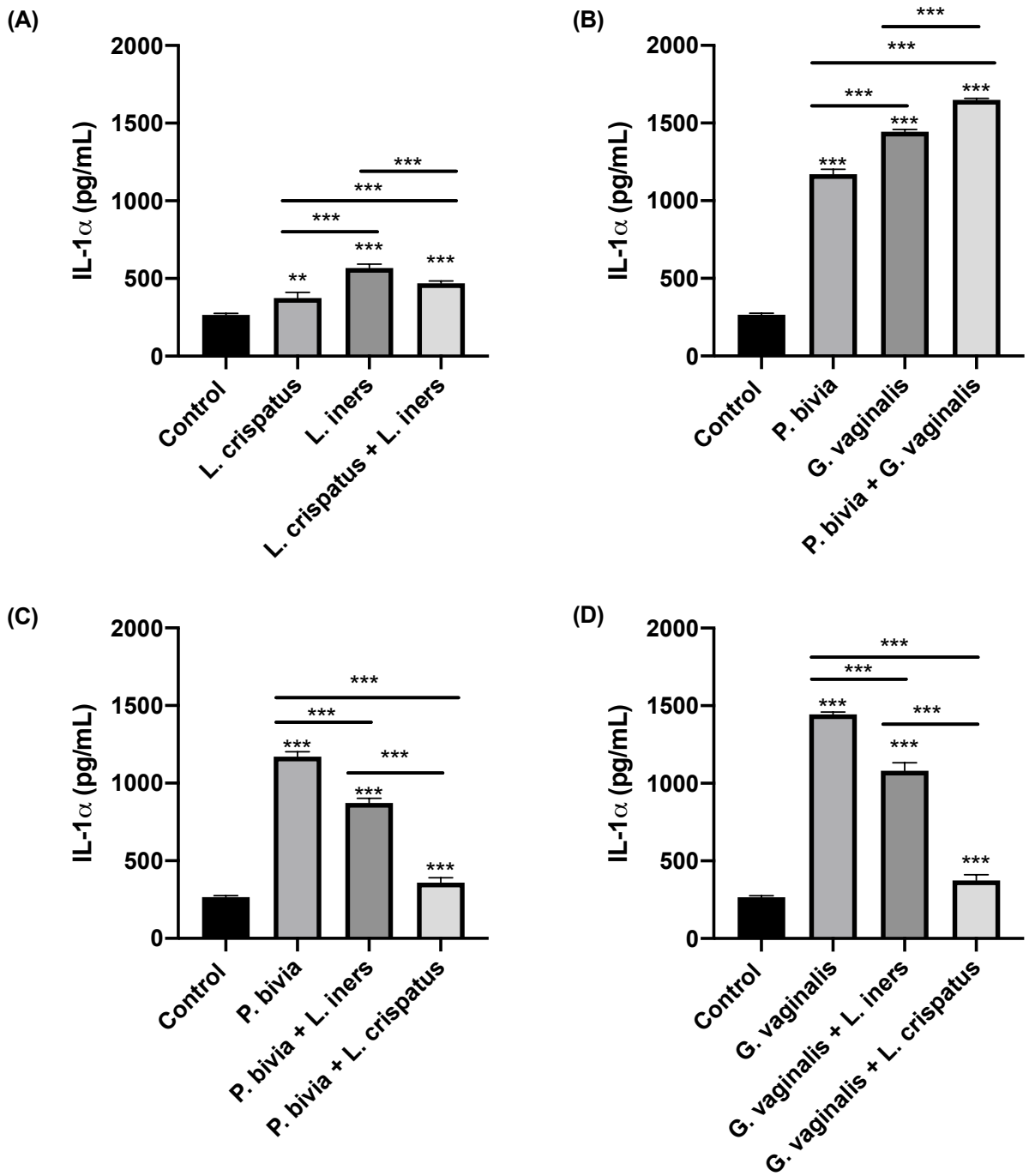

Figure 13: IL-1α levels in the apical media of Vk2 cells after 24 hours of coculture with vaginal bacteria. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** C-

cultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Data shown represents three separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

Both IL1α and IL1β bind to and internalize the membrane receptor IL-1R on virtually any cell type leading to activation of NF-κB and other proinflammatory signal transduction pathways[172]. While this induces the expression of a myriad of inflammatory genes, upon membrane damage epithelial cells also release IL-1 receptor antagonist (IL-1RA). IL-1RA occupies the same receptor but does not activate the cells and thus attenuates the inflammatory response[173]. As both IL1α and IL1β are found to be highly upregulated in women with BV, we chose to assess IL-RA as well. Moreover, several *in vitro* studies with probiotic *Lactobacillus* species, as well as with lactic acid, have cited an increase in this anti-inflammatory cytokine and is thought to be a protective mechanism induced by lactobacilli[174,175]. In all our culture systems tested, IL-1RA levels were above the level of detection that our assay can reliably and accurately detect. Our analysis suggests that cocultures of *L. crispatus* with all other species results in the highest levels of IL-RA, however we caution against interpreting these results as levels are well outside the limits of our assay. To accurately and reliably analyze this cytokine, this assay will need be repeated with diluted samples.

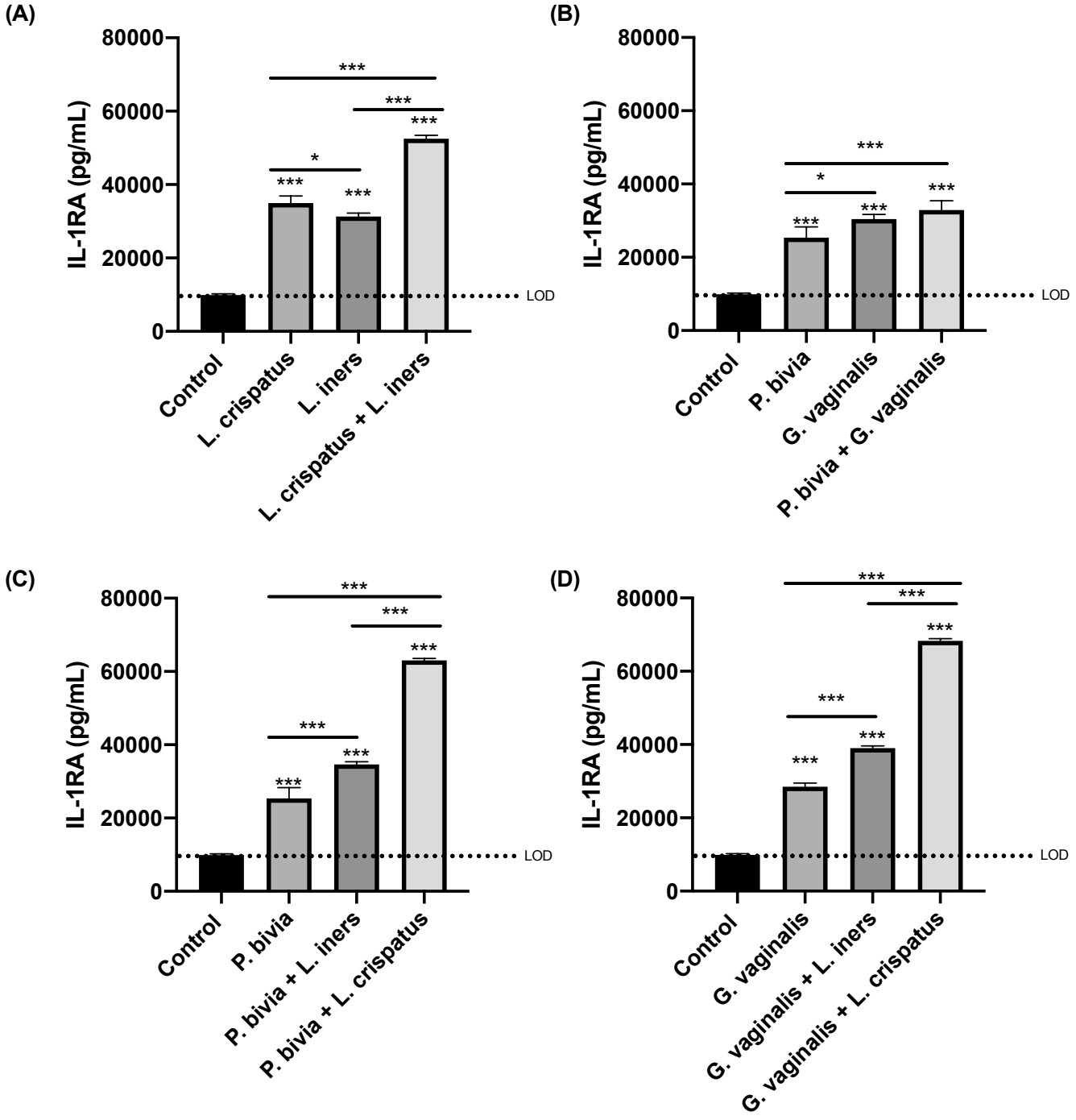

Figure 14: IL-1RA levels in the apical media of Vk2 cells after 24 hours of coculture with vaginal bacteria. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G.*

vaginalis in isolation or combination with lactobacilli species. Data shown represents three separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM. LOD = upper limit of detection of MagPix assay at 10 000 pg/mL.

In our panel we also included MIP-1α as it is a chemokine that is a ligand for HIV-1 coreceptor CCR5 and can specifically recruit CCR5+ target cells into tissues[176]. Additionally, in some studies *Lactobacillus*-dominated VMB were associated with lower levels of MIP-1α which has been cited as a mechanism by which women harbouring these VMB have reduced risk to HIV-1[177]. However, in our analyses of all coculture conditions that we tested, levels of MIP-1α did not significantly differ from that of control (Figure 15). These results may be a reflection of our simplified culture system as it does not reflect how the presence of vaginal bacteria may be able to interact with other cells types, especially in the context of any abrasions to the epithelial barrier.

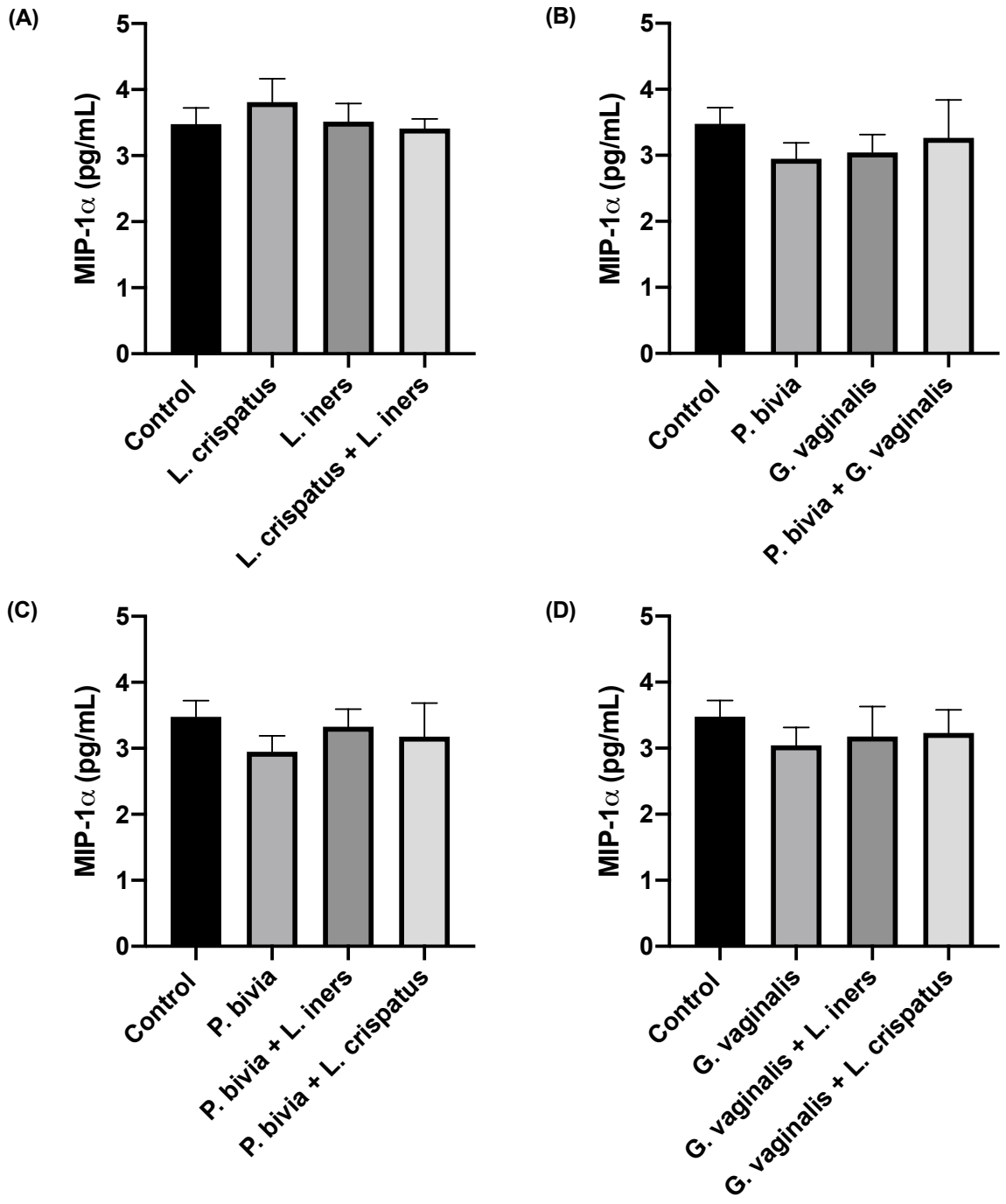

Figure 15: MIP-1α levels in the apical media of Vk2 cells after 24 hours of coculture with vaginal bacteria. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Data shown represents three

separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

Another important chemokine we chose to assess was CCL5, or regulated and normal T cell expressed and secreted protein (RANTES), which is a chemoattractant primarily for T cells and is secreted in response to many viral infections[178]. Elevated levels of RANTES has been cited as a risk factor for HIV-1 and has been found to be upregulated in *in vitro* systems by BV-associated bacteria[179,145]. In our single species cocultures of *L. crispatus* or *L. iners*, or their dual species coculture, levels of RANTES did not significantly differ from that of control (Figure 15A). While increases were observed when *P. bivia* was cocultured with Vk2 cells ($p < 0.01$), levels of RANTES were more than doubled in cocultures with *G. vaginalis* alone or when combined with *P. bivia* ($p < 0.001$) (Figure 15B). When both *P. bivia* or *G. vaginalis* were combined with either *L. iners* or *L. crispatus*, levels of RANTES were significantly dropped, which was by more than half in the case of *G. vaginalis* ($p < 0.001$) (Figure 16C & 16D). While not as pronounced for *P. bivia*, the increase in RANTES seen for *G. vaginalis* in our coculture system is similar to that seen in other *in vitro* studies[145].

Together our analyses assessing the induction of inflammatory cytokines and chemokines mirrors findings from several clinical and *in vitro* studies. Here, we found the dysbiosis-associated species elevated the majority or chemokines and cytokines, reflecting heightened levels observed in cervicovaginal fluids of women with a dysbiotic VMB[62,156]. With this, our analyses allowed us to delineate differences between the two species, as well as their combination. While many of these cytokines have been assessed for *G. vaginalis in vitro*, *P. bivia* has been less-well characterized, in which our study provides a firsthand

account for its induction of many cytokines. In the same regard, *L. iners* is one of the more poorly described *Lactobacillus* species. Here, we showed that while not to the same extent at dysbiosis-associated species for all cytokines, it has a propensity to significantly elevate the levels of many proinflammatory cytokines. Moreover, our results indicated that for several cytokines, dual species cocultures in combinations of *G. vaginalis, P. bivia* or *L. iners* had an additive effect. Conversely, *L. crispatus* either alone or in combination with the other species induced the lowest levels of proinflammatory cytokines. While several cytokines have been assessed in previous *in vitro* studies with *L. crispatus,* our analysis allowed for a side-by-side comparison of the most relevant cytokines, and of novelty its combination with other common vaginal bacteria could be likewise measured.

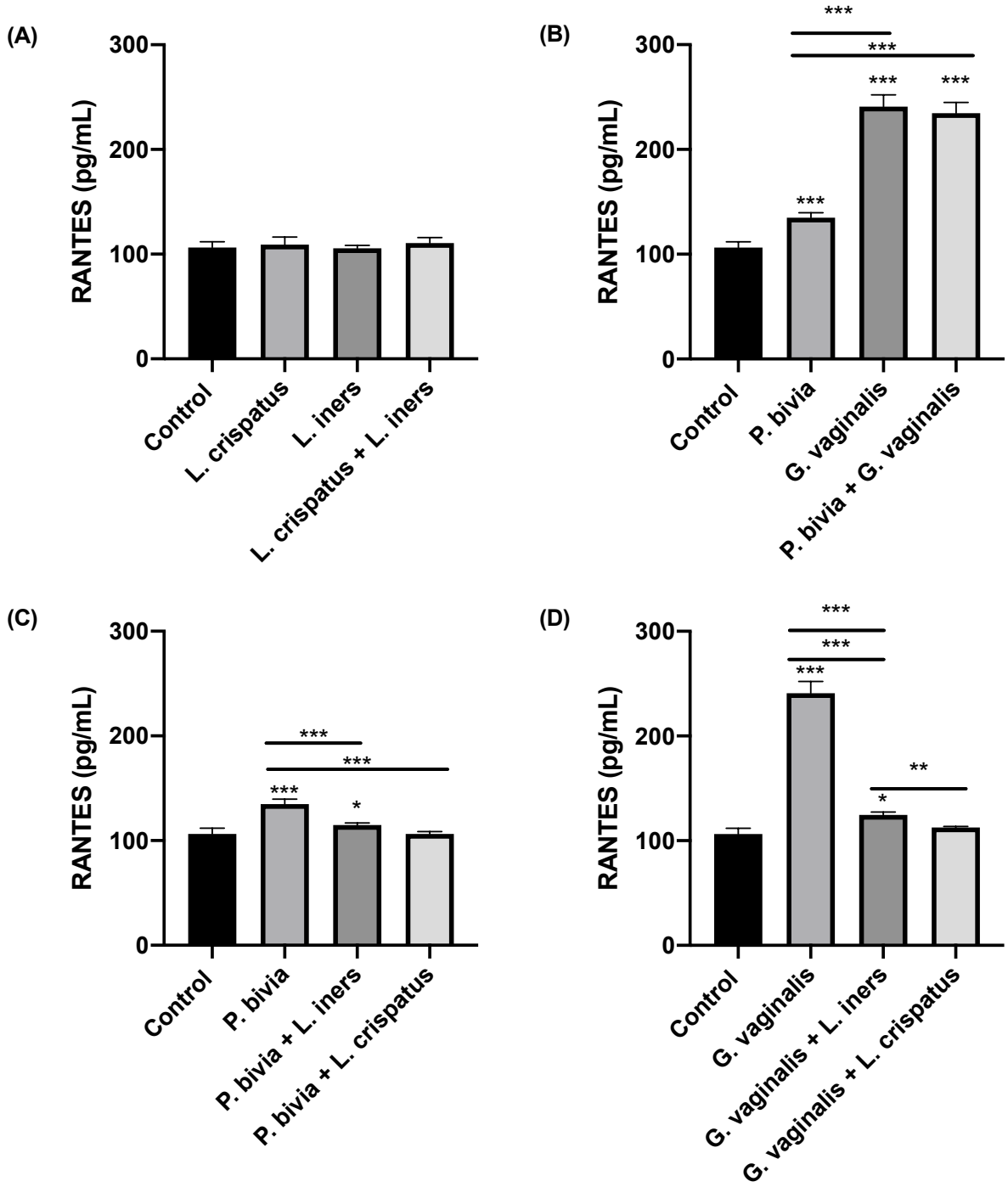

Figure 16: RANTES levels in the apical media of Vk2 cells after 24 hours of coculture with vaginal bacteria. **(A)** Cocultured with lactobacilli species in isolation or combination **(B)** Cocultured with dysbiosis-associated species in isolation or combination **(C)** Cocultured

with *P. bivia* in isolation or combination with lactobacilli species. **(D)** Cocultured with *G. vaginalis* in isolation or combination with lactobacilli species. Data shown represents three separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

4.2: Characterize vaginal bacterial phenotypes and optimize *in vitro* representative bacterial communities correlated with protection and dysbiosis

While metagenomic and metatranscriptomic methods allow for the identification of organisms and microbial co-occurrence relationships in the FRT, we lack a mechanistic understanding of how bacterial interactions shape community assembly, stability, and response to perturbations. Importantly, *in vitro* studies of specific bacteria allows for the investigation of functional phenotypes under the influence of specific abiotic and biotic factors, such as altered carbohydrates, proteins and pH levels that are known to fluctuate in the FRT. Importantly, understanding bacterial phenotypes that may influence the composition, stability and resilience of the VMB has implications for the development of targeted interventions to modulate VMB composition and select for beneficial bacteria in the FRT.

In addition to the previously mentioned vaginal bacteria we have analyzed, these experiments also included *Lactobacillus jensenii*. *L. jensenii*, like *L. crispatus*, is associated with protection against a number of STIs, including HIV-1, as well as optimal reproductive outcomes[180]. However, while *L. crispatus* is found to dominate the VMB in approximately 26% of women, *L. jensenii* is found to be the dominate species in only 5% of women[34]. With this, we wanted to analyze the functional characteristics of these two species to see if we

could identify any common attributes that may connect their protective phenotype, or dissimilarities that may contribute to differences in dominance, in addition to comparisons between all other species.

4.2.1: Analyze the functional characteristics of common vaginal bacteria

To gain mechanistic insights as to how common vaginal bacteria may interact with the host and with one another to ultimately determine the community composition and susceptibility or protection in the FRT, several phenotypic assays were performed. To characterize the phenotypical functions of vaginal bacteria that may be contributing to their virulence or competitive capabilities, hemolytic, hydrogen peroxide, pH, and inhibition assays were performed.

Hemolysis is the premature destruction of red blood cells before the end of their natural life span[181]. This lysis can cause the release of the heme moiety of hemoglobin and its iron. Access to iron within the body can be an intense battle as this is an essential nutrient for certain pathogens and the host. Additionally, accumulation of cell-free heme results in the generation of reactive oxygen species (ROS) and cell damage, which can lead to a cascade of inflammatory responses[182]. As such, the hemolytic capabilities of bacterial species can contribute to their virulence, therefore we wanted to test this amongst the vaginal species. Hemolytic activity was visually determined by streaking bacterial cultures on agar plates supplemented with 5% sheep's blood or human blood. Blood agar plates were examined for signs of β-hemolysis (clear zones around colonies), α-hemolysis (green halo around colonies), or γ-hemolysis (no clearing zone)[183]. While *L. crispatus*, *L. jensenii* and *P. bivia* did not induce the lysis of erythrocytes on sheep blood agar (γ-hemolysis), *L. iners* and *G.*

vaginalis showed α-hemolysis (Table 1). When repeated on human blood, *L. crispatus*, *L. jensenii* and *P. bivia* once more showed γ-hemolysis. However, *L. iners* also showed γ-hemolysis on human blood, while *G. vaginalis* induced β-hemolysis. These findings are validated by the observation of hemolysis on human blood by *G. vaginalis*, which has been used as an identification marker of the species and a potent virulent trait[184]. The identification of inerolysin in *L. iners* has proposed hemolysis by this species, in which our study showed for sheep blood, but not human blood, suggesting this feature may not be as relevant in the FRT for this species[185]. Moreover, to our knowledge no other study has reported hemolysis by either *L. crispatus* or *L. jensenii*.

As the production of hydrogen peroxide has been postulated to be a defense mechanism by which *Lactobacillus* species protect against potentially pathogenic bacteria, hydrogen peroxide production was measured[186]. This was done using Prussian Blue agar plates, in which the reaction with hydrogen peroxide leads to the formation of a blue precipitate that can be visually identified. Genomic studies that have analyzed *L. crispatus* and *L. iners* revealed that while *L. crispatus* contains the metabolic pathways required to produce hydrogen peroxide, these were not present in the genome of *L. iners*[110]. These findings were validated in our assays, where *L. crispatus*, but not *L. iners*, induced the formation of blue halos indicating hydrogen peroxide production (Table 1). Additionally, *L. jensenii* also produced hydrogen peroxide, while both *G. vaginalis* and *P. bivia* did not. These findings are in line with the association between protective *Lactobacillus* species and the ability to produce hydrogen peroxide, thereby precluding *L. iners* from this label.

Another method of protection conferred by non-*iners Lactobacillus* species has been attributed to their association with a low vaginal pH, while dysbiosis-associated VMB are

often characterized with a pH above 5[34]. To understand how each vaginal species may be contributing to the ability to alter pH, this was determined by measuring pH of liquid broth media before and after the addition of bacteria. All vaginal bacteria were capable of fermenting glucose in NYC III media (or Reinforced Modified Clostridial for *P. bivia* analysis) to significantly lower the pH, however *L. crispatus* induced the greatest reduction in pH to below 4, followed by *L. jensenii* which reduced the pH to below 4.5 (Table 1). Though not as drastically, *L. iners* was still capable of lowering the pH to below 5, while both *G. vaginalis* and *P. bivia* were not. These results are consistent with the observation that *L. iners*-dominated VMB and VMBs colonized by dysbiosis-associated bacteria (*P. bivia* and *G. vaginalis*) are found to have a higher vaginal pH than *L. crispatus* or *L. jensenii* dominated VMBs[34].

The *in vitro* cocultures with VK2 cells gave us clues as to how each species may competitively interact or cooperatively coexist in the presence of vaginal epithelial cells. Aside from adherence capabilities and interactions with vaginal cells that may influence these species-species relationships, we wanted to analyze other factors that may also be contributing to the differences in levels of protection observed by *Lactobacillus* species. One of the factors that has been suggested as a key mechanism of defense by *L. crispatus* is the ability to produce antimicrobial products, including bacteriocins[187]. Bacteriocins are proteins synthesized by bacteria that can kill or inhibit the growth of other bacteria, usually by membrane disruption[188]. A method to test contact-independent antimicrobial activity is agar diffusion assays, whereby supernatant-saturated discs from *Lactobacillus* species are placed on agar plates with the bacteria in interest and halos are produced where growth is inhibited. In our analysis, *L. crispatus* was able to inhibit the growth of *P. bivia* and *G.*

vaginalis, while *L. jensenii* only inhibited the growth of *P. bivia* (Table 1). In contrast, *L. iners* was not able to inhibit the growth of either species. To understand if this inhibition was only due to a lowered pH, we used NaOH to neutralize the supernatants of the *Lactobacillus* species. With this, levels of inhibition were comparable to non-neutralized supernatants, suggesting factors other than pH were at play.

Table 1: Phenotypic analysis of vaginal bacteria.

Bacteria	Hemolysis	H_2O_2 Production	pH change (from 6.8)	Inhibition	
				G. vaginalis	*P. bivia*
L. crispatus	γ-hemolysis	Yes	3.87 ***	+	+
L. jensenii	γ-hemolysis	Yes	4.20 ***	+	-
L. iners	α-hemolysis (sheep) γ-hemolysis (human)	No	4.91 ***	-	-
G. vaginalis	α-hemolysis (sheep) β-hemolysis (human)	No	5.74 **		
P. bivia	γ-hemolysis	No	6.11 *		

Hemolytic capabilities of vaginal bacteria were analyzed on sheep blood agar plates where signs of β-haemolysis (clear zones around colonies), α-haemolysis (green halo around colonies), or γ-haemolysis (no zones around colonies) were visualized. Hydrogen peroxide production was measured on Prussian Blue agar plates, in which reaction with hydrogen peroxide leading to the formation of a blue halos surrounding bacterial colonies was visualized. The ability for bacterial by-products to alter pH was determined by measuring pH of NYC III or Reinforced Modified Clostridial media before (pH 6.80) and after the addition of bacteria. Inhibition of growth was measured through the placement of discs with bacterial supernatants placed on agar plates of either *G. vaginalis* or *P. viva*. + indicates zones of clearing of at least 5 mm visualized after 24 hours. Experiments were repeated in triplicates at least three times. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

While likely due to the buffering capacity of KSFM media, we did not see a significant drop in the pH after 24 hours in cocultures of Vk2 cells with vaginal bacteria (Figure 8). However, we did observe differential capabilities of the vaginal bacteria in lowering the pH of the growth medium, which reflected pH levels observed *in vivo* of respective dominating species[34]. As the low pH of non-iners *Lactobacillus*-dominated VMB is thought to create an inhospitable environment for pathogens and dysbiosis-associated bacterial species, we evaluated the ability of the vaginal species to grow in media buffered to different pH levels. All species were capable of growth in unbuffered media as well as media buffered to a pH of 7.0 (Figure 17). Conversely, bacterial counts after 24 hours dropped by more than 1000 fold for both *P. bivia* and *G. vaginalis* when grown in media buffered to a pH of 4.0 ($p < 0.001$). Additionally, counts were lower for *L. iners* when grown in a pH of 4.0 ($p < 0.001$) and for *L. crispatus* when grown at a pH of 7.0 ($p < 0.05$). These findings suggest that the low pH found in *L. crispatus*-dominant VMBs would be enough to preclude the colonization of dysbiosis-associated species.

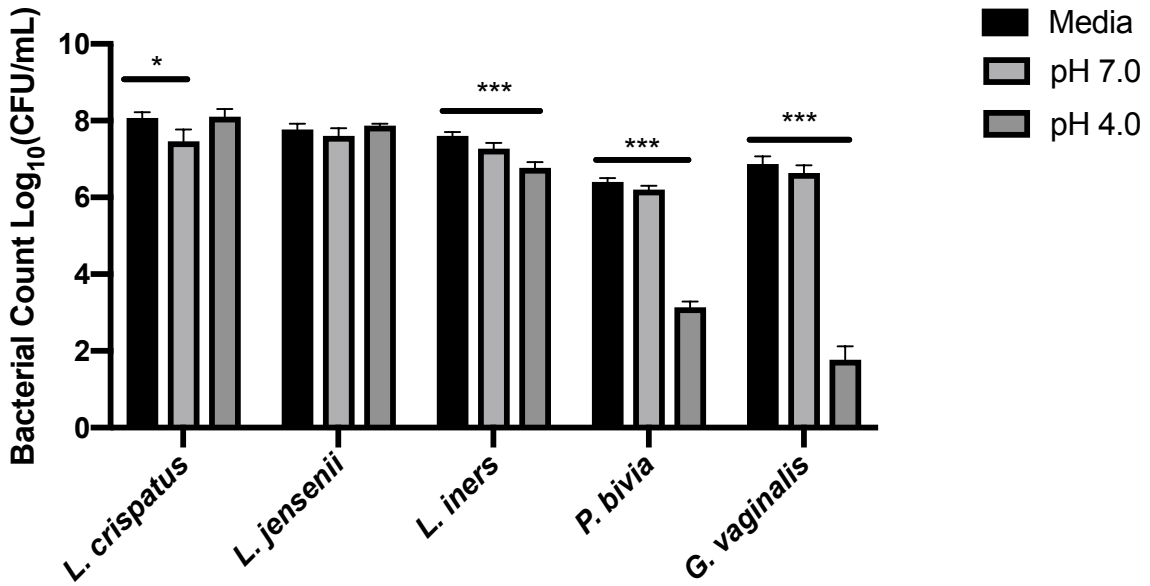

Figure 17: Growth of vaginal bacteria at altered pH levels. *L. crispatus*, *L. Jensenii*, *L. iners, P. bivia* and *G. vaginalis* were grown in unbuffered media, or media buffered to pH of 7.0 or 4.0. Media was buffered with hydrochloric acid at 5 mM (pH 7.0) or 40 mM (pH 4.0). pH of 7.0 was adjusted with NaOH. Bacteria were enumerated after 24 hours. Data shown represents three separate experiments, each done in duplicates or triplicates. Data was analyzed by two-way ANOVA with Holm-Sidak Test to correct for multiple comparisons between media conditions. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represent SEM.

4.2.2: Analyze vaginal bacterial growth in various abiotic conditions

Central to the ability to manage the composition of host-associated microbial communities is the knowledge about the factors determining their dynamics, stability and maintenance. Understanding the underlying governing principals that shape the bacterial community is crucial for designing rational approaches to select for beneficial bacteria or discourage growth of potentially pathogenic bacteria in the FRT. To understand how the diverse conditions common to the FRT may influence specific vaginal bacteria, bacterial growth was analyzed under various abiotic conditions. Recognizing that the nutritional resources available to the indigenous vaginal bacteria are exclusively host derived, we analyzed resources known to be abundant in the FRT. Resources available for bacterial consumption exist in the mucus that contains a rich mix of carbohydrates, proteins, fatty acids, and trace elements, and also epithelial cells, which are rich in glycogen in reproductive-age women[189]. As such, there is a myriad of potential sources of nutrition that bacterial species may use to thrive in the FRT. However, we decided to focus our analysis on carbohydrates and proteins available in the FRT as potential macromolecules that could determine which species are able to flourish.

While various genomic analyses have cited the abilities/inabilities of certain *Lactobacillus* species to metabolize specific carbohydrates, it's important to note that these conclusions have recently been put into question[108]. Early studies of isolated *Lactobacillus* strains from the FRT and metagenomics studies have all claimed that *Lactobacillus* strains were unable to metabolize glycogen and lacked the genetic machinery to do so[101,106,190]. However, a recent analysis has identified the presence of the enzyme putative pullulanase type I in several *L. crispatus* isolates, which correlated with their ability to grow on glycogen supplemented media[108]. These findings underscore the need to functionally characterize vaginal bacteria's metabolic proficiencies rather than solely attempting to infer such capabilities from metagenomics studies. As such, it is critical to investigate prior claims concerning the metabolic capabilities of vaginal bacteria by testing these abilities *in vitro* and analyzing the extent to which these species can effectively utilize various resources.

With glycogen and its breakdown products postulated to select for *Lactobacillus*-dominance, the ability of these carbohydrates to influence bacterial growth were assessed for each vaginal species. Glucose-free NYC III Medium was supplemented with 1.0 % glycogen, glucose, maltose, sucrose or lactose for analysis of *L. crispatus, L. jensenii, L. iners* and *G. vaginalis*, while dextrose-free Modified Reinforced Clostridial Medium supplemented with the previously listed carbohydrates was used for *P. bivia*. For the three *Lactobacillus* species, optical density at 600 nm (OD_{600}) was taken at 30-minute intervals. With the exception of *L. crispatus*, cultures showed limited growth in all conditions. With this, Oxyrase, an enzyme formulation designed to produce anaerobic conditions in bacteriological broth media, was added to cultures and mineral oil was overlaid on top of broth cultures to create a microaerophilic environment. This allowed for the growth of both *L. iners* and *L. jensenii*.

For the dysbiosis-associated bacteria however, even with the addition of Oxyrase and mineral oil, *G. vaginalis* and *P. bivia* showed minimal growth in a 96-well plate. Therefore, both *G. vaginalis* and *P. bivia* were grown in anaerobic conditions and OD measurements were taken every 4 hours.

In comparison of the lactobacilli species, *L. crispatus* displayed the widest range of carbohydrate utilization as it was able to grow on all carbohydrates tested (Figure 18). *L. jesenii* was capable of growth on all carbohydrates tested except glycogen and lactose, while *L. iners* was unable to grow on both sucrose and lactose. Of novelty, *L. crispatus* and *L. iners* showed the ability to grow on glycogen (Figure 18E) despite previous claims that lactobacilli lacked the genetic machinery capable of degrading glycogen[101,106]. Rather, it was postulated that vaginal lactobacilli required the presence of host or bacterial derived amylase to breakdown glycogen into usable carbohydrates. As no enzymes were added to our broth, our results therefore dispute these claims. Of note, both *L. crispatus* and *L. iners* did not grow as efficiently on glycogen as other carbohydrate sources, such as glucose. In analyzing the species associated with a dysbiotic VMB, *P. bivia* and *G. vaginalis* were capable of growing on a number of carbohydrate resources tested (Figure 19). Both species were able to grow on glucose and maltose, while neither could grow on sucrose, and only *P. bivia* could grow on lactose. Interestingly, despite prior claims that glycogen abundance selects for lactobacilli, both *P. bivia* and *G. vaginalis* were capable of growing on glycogen (Figure 19E). Once more, growth on glycogen for both species, and growth on lactose for *P. bivia,* was not as efficient as growth on glucose or maltose.

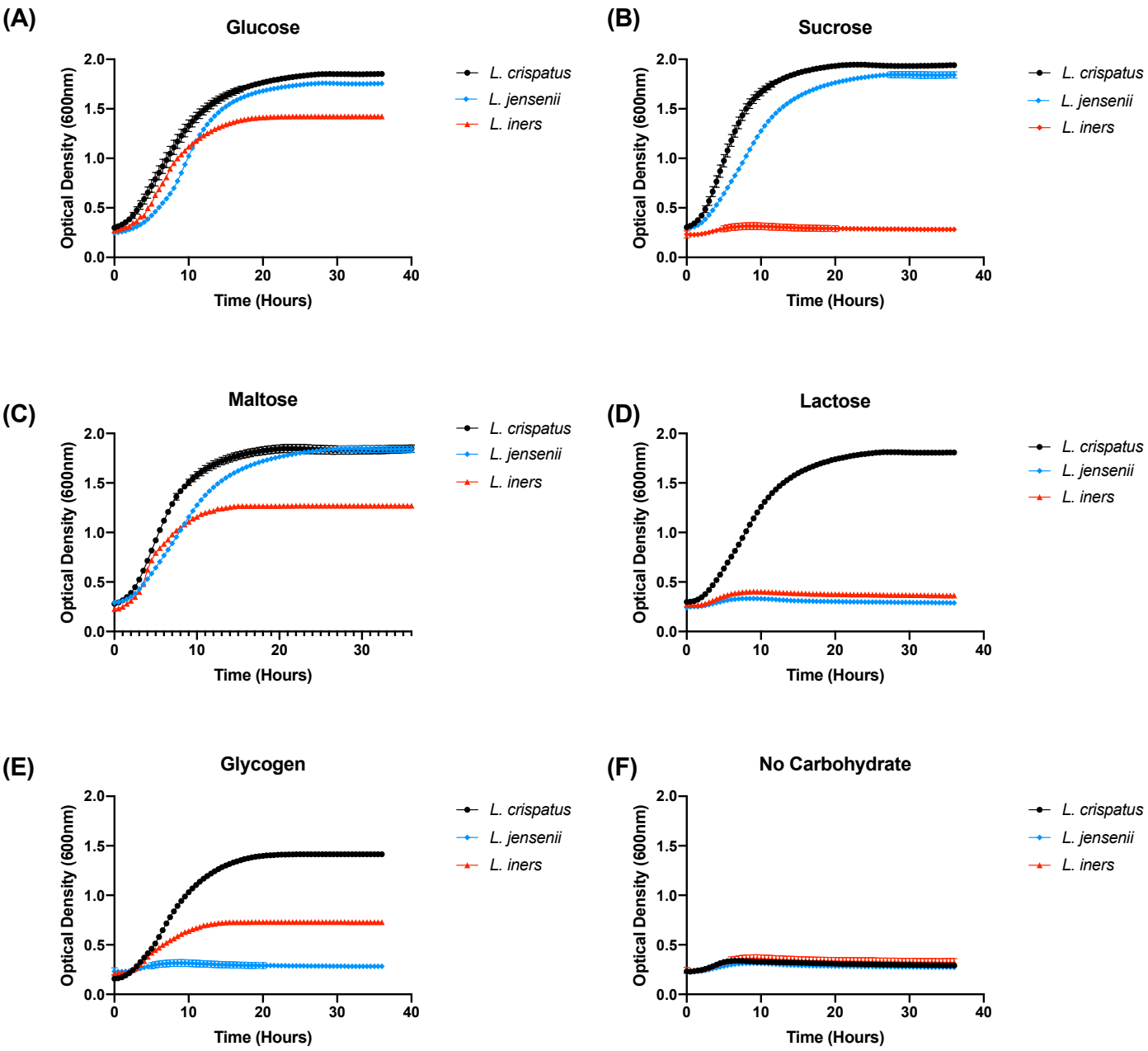

Figure 18: Growth of lactobacilli species on different carbohydrate resources. *L. crispatus, L. jensenii* and *L. iners* were cultured in medium with added Oxyrase Enzyme and mineral oil in a 96 well plate that contained either 1.0 % **(A)** glucose, **(B)** sucrose, **(C)** maltose **(D)** lactose **(E)** glycogen or **(F)** no added carbohydrates for 36 hours. The optical density at 600 nm was read every half hour. Each culture condition was run in triplicate and the triplicates were averaged over six separate experiments. Error bars represent SEM.

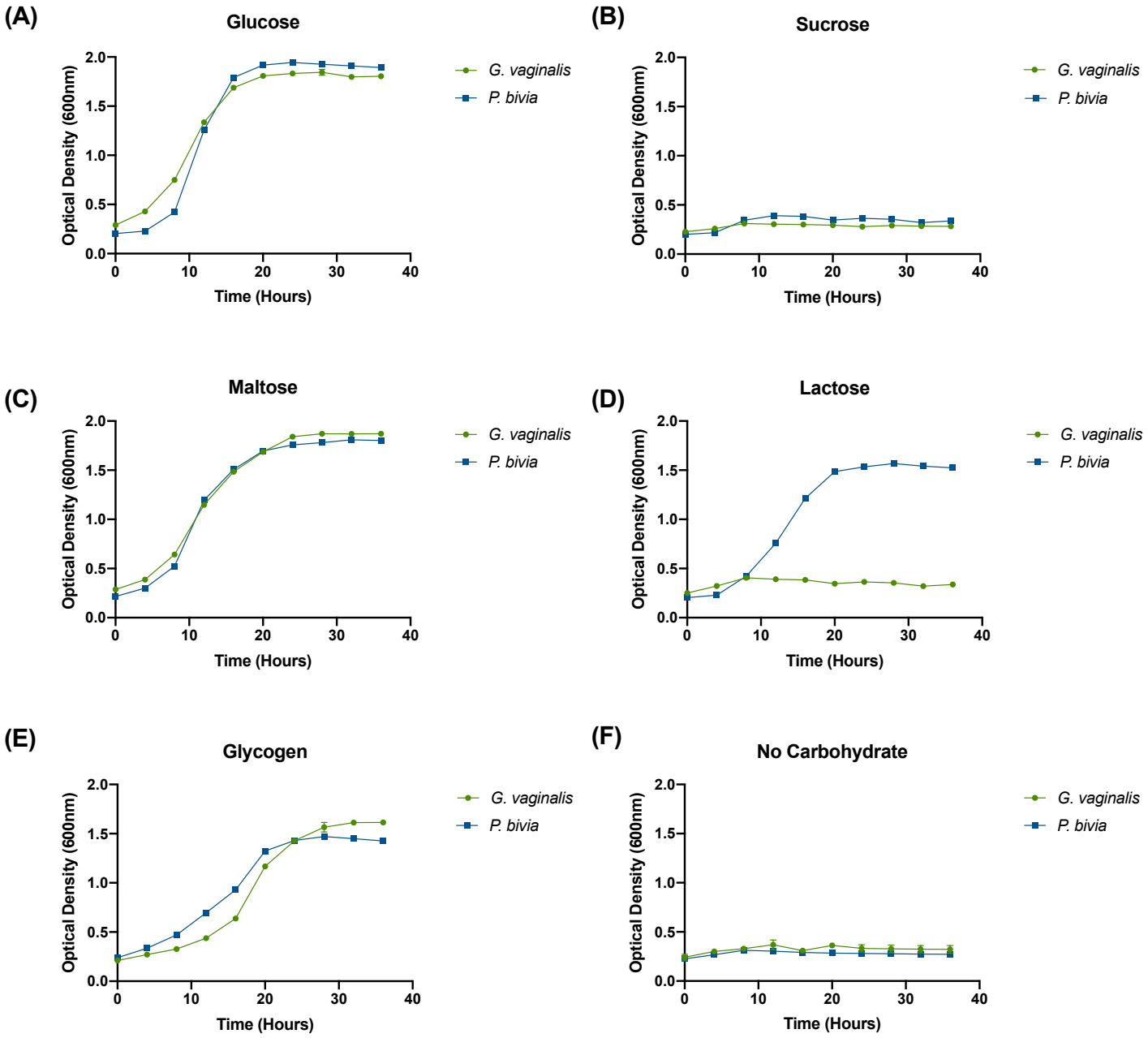

Figure 19: Growth of dysbiosis-associated bacteria on different carbohydrate resources. *G. vaginalis* and *P. bivia* were cultured in medium in anaerobic conditions that contained either 1.0 % **(A)** glucose, **(B)** sucrose, **(C)** maltose **(D)** lactose **(E)** glycogen or **(F)** no added carbohydrates for 36 hours. The optical density at 600 nm was read every four hours. Each

culture condition was run in triplicate and the triplicates were averaged over four separate experiments. Error bars represent SEM.

While these common glycogen-breakdown carbohydrates give important clues into the differences in metabolic capabilities of vaginal bacteria, we wanted to explore this notion further by testing a wider variety of carbohydrate substrates. To do this, we utilized an assays strip that analyzes the ability for bacteria to ferment 50 different carbohydrate substrates. While the vaginal bacteria were unable to ferment a variety of tested substrates, Table 2 includes the notable substrates that at least one bacterial species was able to productively ferment. Of note, *L. crispatus* was able to ferment the greatest number of carbohydrates tested, whereas *L. jensenii* and *L. iners* displayed markedly less capabilities. Interestingly, *L. crispatus*, *L. iners* and *G. vaginalis* were capable of fermenting several substrates that *L. jensenii* was not, such as starch, N-acetyl-glucosamine and glycogen. Moreover, *G. vaginalis* was capable of fermenting glycerol and ribose, while none of the lactobacilli species could. Additionally, both *G. vaginalis* and *L. crispatus* could ferment galactose, while *L. iners* and *L. jensenii* could not. Unfortunately, we were unable to gain reliable information for the fermentation by *P. bivia* on these strips. Current troubleshooting is taking place to hopefully gain insights for this species as well.

Table 2: Growth of vaginal bacteria of various carbohydrate resources

	Glycerol	D-Ribose	D-Galactose	D-Glucose	D-Fructose	D-Manose	N-Acetyl Glucosamine	D-cellobiose
L. crispatus	-	-	+	+	+	+	+	+
L. jensenii	-	-	-	+	+	+	-	+
L. iners	-	-	-	+	+	+	+	-
G. vaginalis	+	+	+	+	+	+	+	-
	D-Maltose	D-Lactose	D-Melibiose	D-Sucrose	D-Trehalose	D-Raffinose	Amidon (starch)	Glycogen
L. crispatus	+	+	+	+	+	+	+	+

L. jensenii	+	-	-	+	+	-	-	-
L. iners	+	-	-	-	-	-	+	+
G. vaginalis	+	-	-	-	-	-	+	+

The growth of *L. crispatus*, *L. Jensenii*, *L. iners* and *G. vaginalis* ability to ferment various carbohydrate resources were tested using an API 50 chip. + indicates a change in colour indicating fermentation, while – represents no change in colour. Data represents growth from 4 separate experiments.

In addition to carbohydrates present in the FRT, an abundance of protein is found in the cervicovaginal fluid. Due to the temporal fluctuations observed in VMB composition across the menstrual cycle in many women, we chose to analyze bacterial growth in the presence of three of the most abundant proteins known to vary with the menstrual cycle[191]. Lactoferrin and mucin, both glycoproteins that could be used as carbon sources for bacteria, as well as albumin, which may facilitate bacterial adherence in the vaginal tract, were supplemented into growth media[192,193]. Growth profiles were analyzed as previously stated. While the addition of these proteins did not appear to have an effect on the growth of *L. crispatus* or *L. jensenii*, the presence of mucin appeared to notably enhance the growth of *L. iners* (Figure 20A) as compared to its growth on glucose (Figure 20D). Analyzing *G. vaginalis* and *P. bivia* in the same way, the addition of the three protein sources tested did not appear to markedly enhance growth as compared to glucose alone (Figure 21).

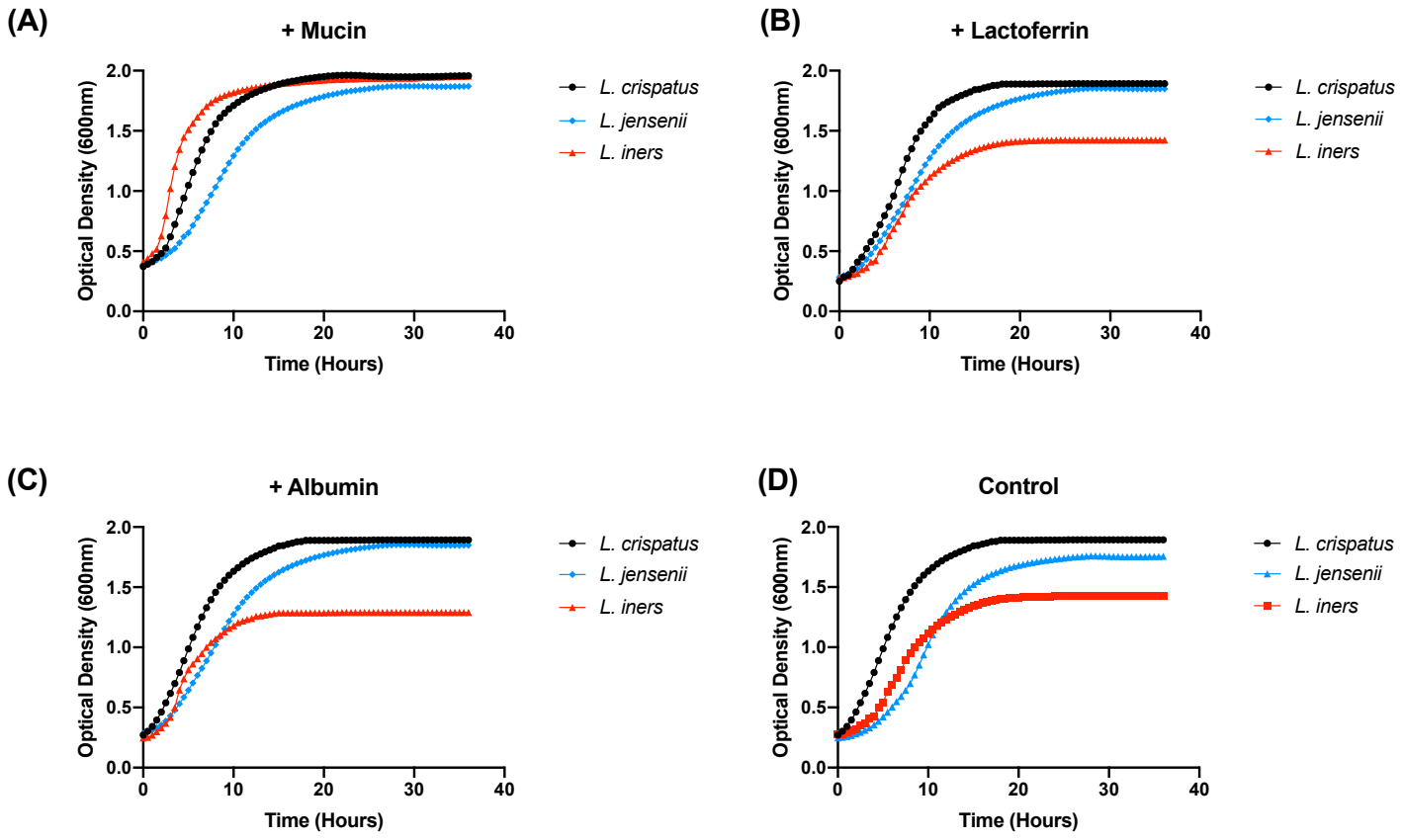

Figure 20: Growth of lactobacilli species in media with added proteins. *L. crispatus, L. jensenii* and *L. iners* were cultured in medium with added Oxyrase Enzyme and mineral oil in a 96 well plate that contained the addition of 10 mg/mL of **(A)** mucin, **(B)** lactoferrin, **(C)** albumin or **(D)** no added protein for 36 hours. The optical density at 600 nm was read every half hour. Each culture condition was run in triplicate and the triplicates were averaged over six separate experiments. Error bars represent SEM.

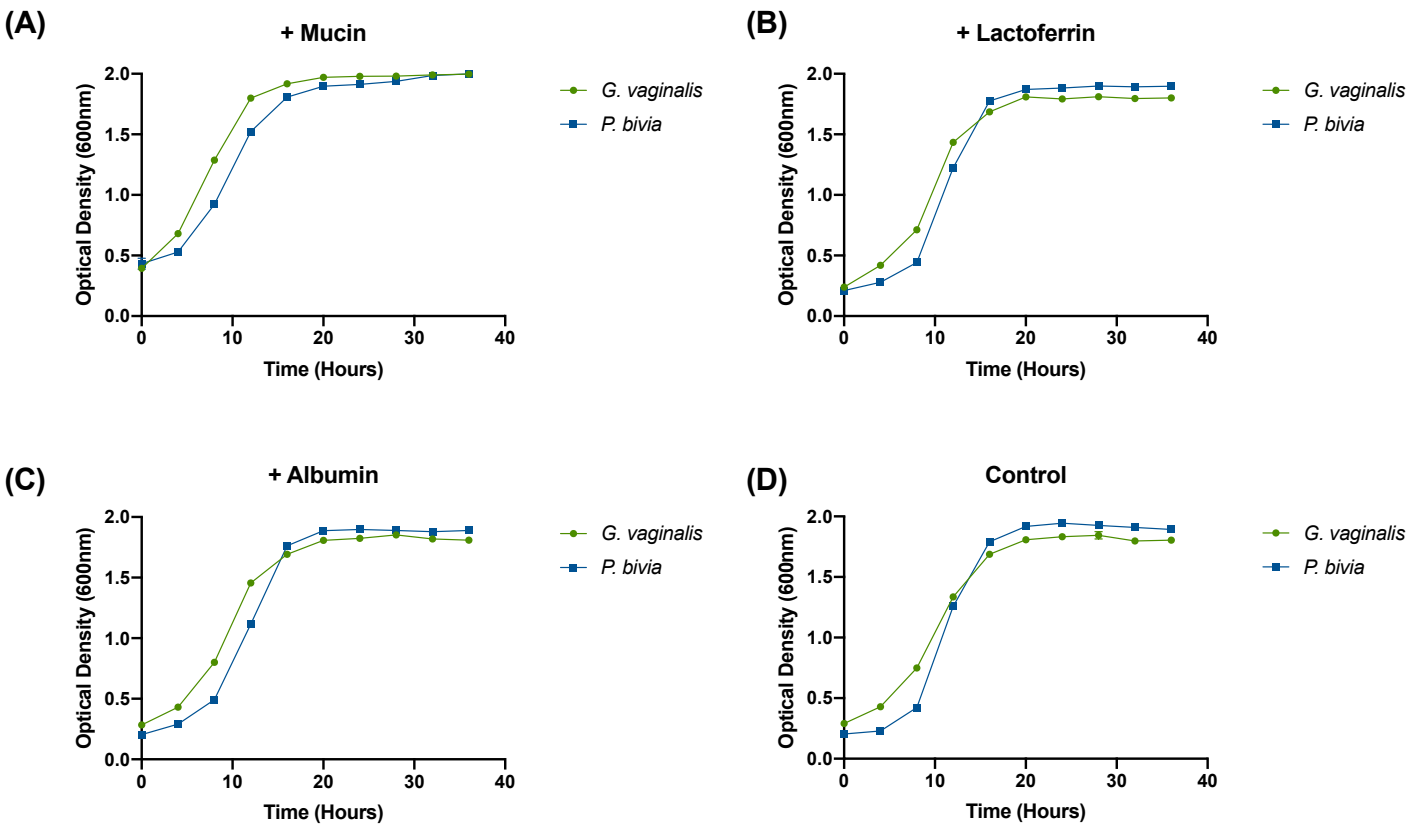

Figure 21: Growth of dysbiosis-associated bacteria in media with added proteins. *G. vaginalis* and *P. bivia* were cultured in medium in anaerobic conditions with the addition of 10 mg/mL of **(A)** mucin, **(B)** lactoferrin, **(C)** albumin or **(D)** no added protein for 36 hours. The optical density at 600 nm was read every four hours. Each culture condition was run in triplicate and the triplicates were averaged over four separate experiments. Error bars represent SEM.

As the media used before the addition of protein contained all the necessary components for growth of all vaginal species, we recognized that the bacteria may be saturated with nutrients under these conditions. Therefore, their ability to effectively utilize these proteins as resources or be enhanced by their presence could not be fully appreciated by this method. Therefore, we repeated this experiment in resource limited conditions, where there was no carbohydrate resource present. As all bacterial species tested were

unable to grow without a carbohydrate resource present, any growth observed could be attributable to the species ability to utilize the protein added. In comparing the lactobacilli species, all species were capable of utilizing mucin to grow (Figure 22A). However, in contrast to the growth curves observed for glucose and maltose (Figure 18A & 18C), *L. iners* displayed the most efficient growth on mucin, while *L. crispatus* and *L. jensenii* displayed markedly reduced growth. Conversely, all three lactobacilli were unable to grow when either lactoferrin or albumin was the only resource present (Figure 22D & 22C). Results from analysis of *P. bivia* and *G. vaginalis* gleaned similar results, whereby both could utilize mucin as a resource, but not lactoferrin or albumin (Figure 23). With this, *G. vaginalis* showed greater growth on mucin than *P. bivia* and was more reflective of optimal growth such as shown with glucose (Figure 23A).

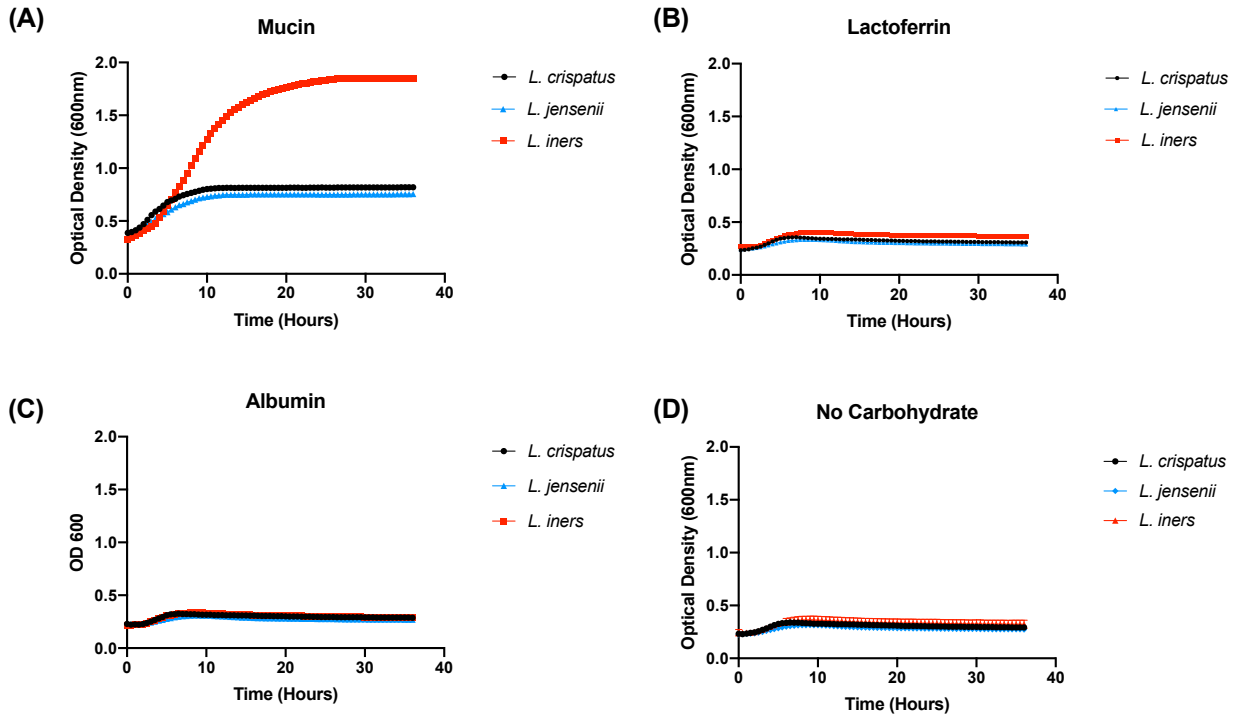

Figure 22: Growth of lactobacilli species in media with added proteins without any carbohydrate. *L. crispatus, L. jensenii* and *L. iners* were cultured in medium with added

Oxyrase Enzyme and mineral oil in a 96 well plate that contained no carbohydrate and the addition of 10 mg/mL of **(A)** mucin, **(B)** lactoferrin, **(C)** albumin or **(D)** no carbohydrate for 36 hours. The optical density at 600 nm was read every half hour. Each culture condition was run in triplicate and the triplicates were averaged over six separate experiments. Error bars represent SEM.

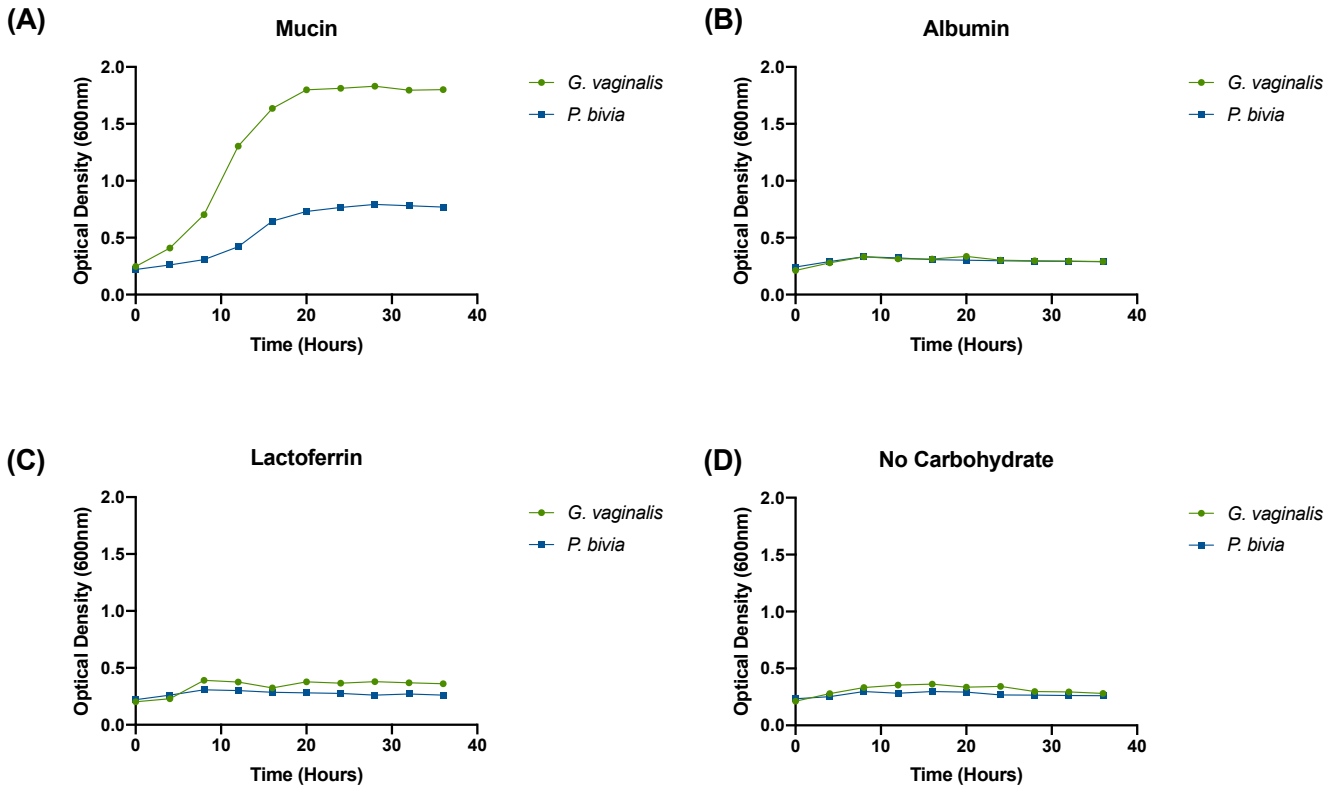

Figure 23: Growth of dysbiosis-associated bacteria in media with added proteins without any carbohydrate. *G. vaginalis* and *P. bivia* were cultured in medium in anaerobic conditions that contained no carbohydrates and the addition of 10 mg/mL of **(A)** mucin, **(B)** lactoferrin, **(C)** albumin or **(D)** no added protein for 36 hours. The optical density at 600 nm was read every four hours. Each culture condition was run in triplicate and the triplicates were averaged over four separate experiments. Error bars represent SEM.

Hormonal fluctuations across the menstrual cycle are known to influence alterations in cervicovaginal fluid viscosity, composition, immune cell populations, and VMB

composition[194]. As one of the best predictors of VMB fluctuations over time was in accordance with hormonal levels of the menstrual cycle, we wanted to assess if the presence of endogenous female sex hormones could directly influence the growth of vaginal bacteria[83]. Moreover, as our group has shown that the progestin-based hormonal contraceptive depotmedroxyprogesterone acetate (DMPA) increases diversity of the VMB in a cohort of sex workers, we wanted to assess if this exogenous hormone could likewise influence growth directly[195]. Cultures of vaginal bacteria were grown in the presence of estradiol (E2), progesterone (P4) and medroxyprogesterone acetate (MPA) to a final concentration of 10^{-9}M in NYC III media and growth was analyzed as previously described. The presence of these hormones did not appear to influence the growth of any *Lactobacillus* species as compared to control media without hormones supplemented (Figure 24). Likewise, these hormones did not have any discernable effect on the growth of *P. bivia* or *G. vaginalis* when supplemented to their respective media (Figure 25). This again may be an effect of the bacteria being saturated with their required nutrients and therefore already growing at maximal rates, and thus any enhancement in growth may not be recognizable. However, no growth from any of the vaginal bacteria was observed when bacteria were grown in hormone supplemented media in the presence of no carbohydrate (data not shown).

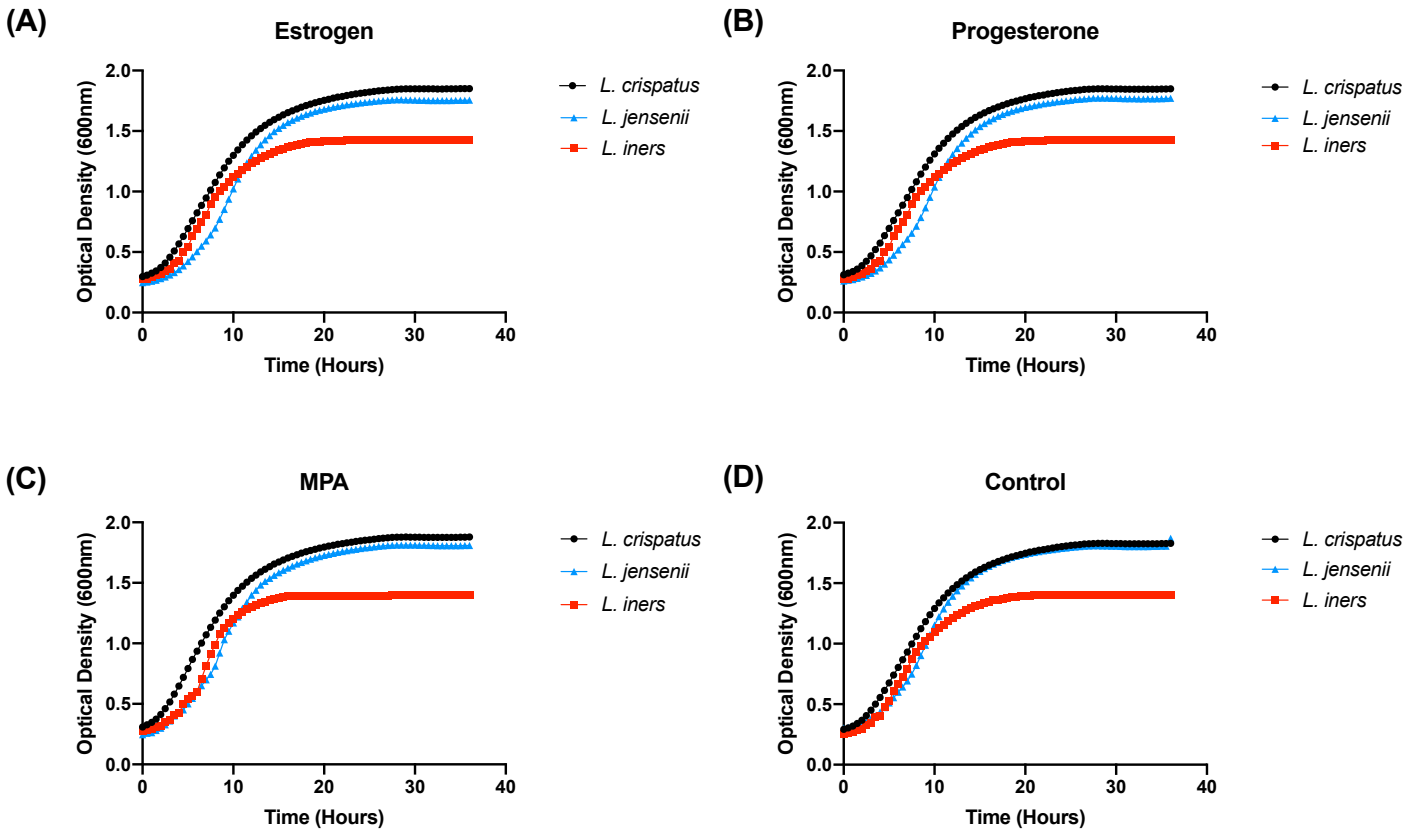

Figure 24: Growth of lactobacilli species with added hormones. *L. crispatus, L. jensenii* and *L. iners* were cultured in medium with added Oxyrase Enzyme and mineral oil in a 96 well plate that contained either the addition of **(A)** 10^{-9}M estradiol (E2), **(B)** progesterone (P4) or **(C)** medroxyprogesterone acetate (MPA) or **(D)** no added hormone for 36 hours. The optical density at 600 nm was read every half hour. Each culture condition was run in triplicate and the triplicates were averaged over three separate experiments. Error bars represent SEM.

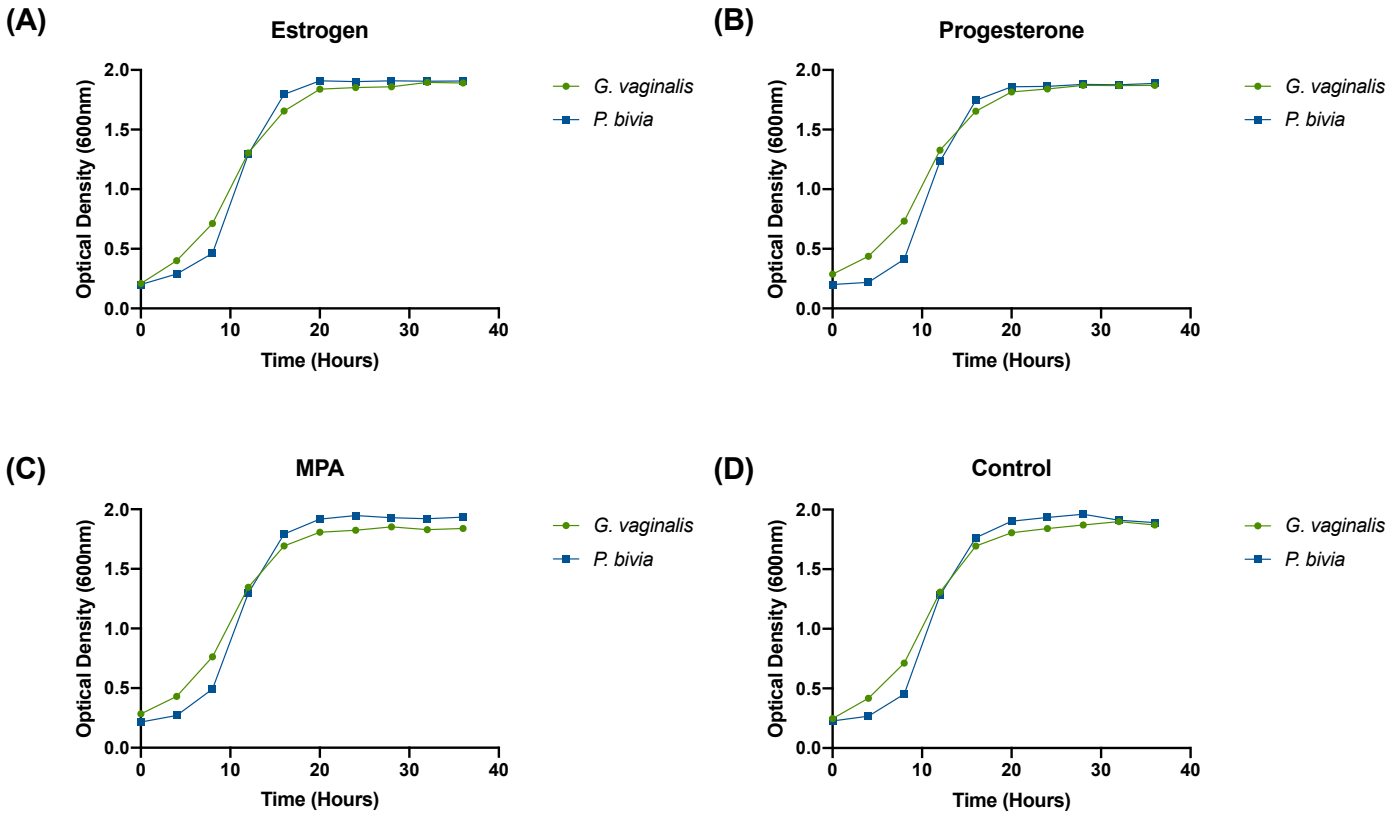

Figure 25: Growth of dysbiosis-associated bacteria with added hormones. *G. vaginalis* and *P. bivia* were cultured in medium in anaerobic conditions that contained either the addition of **(A)** 10^{-9}M estradiol (E2), **(B)** progesterone (P4) or **(C)** medroxyprogesterone acetate (MPA) or **(D)** no added hormone for 36 hours. The optical density at 600 nm was read every four hours. Each culture condition was run in triplicate and the triplicates were averaged over three separate experiments. Error bars represent SEM.

4.2.3: Analyze bacterial interactions in various abiotic conditions

To gain a greater understanding of the competitive and symbiotic interactions that we observed in our dual species cocultures, vaginal bacteria were analyzed in the presence of one another. As *L. crispatus* was deemed as the species conferring the most benefit to the host in our analyses and others, we wanted to focus on conditions that may alter competitive capabilities of this species with the other three non-optimal species. While non-*iners*

Lactobacillus species are rarely found to codominate a VMB, *L. iners* can often be found in appreciable amounts in the VMB in both non-*iners Lactobacillus* dominant VMBs and dysbiotic states[83]. As this was observed in our dual species cocultures with Vk2 cells, we wanted to analyze these interactions in liquid broth cultures, as this enabled us to alter growth resources present. In attempts to establish an *L. crispatus* and *L. iners* coculture, NYC III media containing either glucose, glycogen, maltose, sucrose or lactose as the carbohydrate source were evaluated and colonies of each species were later enumerated on agar plates. In all culture conditions, *L. iners* colonies were not observed in appreciable amounts at 4 and 8 hours and were not detectable at 12 and 24 hours. *L. crispatus* did not appear to be influenced by the presence of *L. iners* as it was able to grow at similar rates as observed in monocultures. As *L. iners* has been observed to grow in a slower fashion relative to *L. crispatus*, cocultures were attempted with bacteria harvested during their exponential phase of growth at inoculations of 6.0 x 10^6 CFU/mL each into NYC III media containing glucose in attempts to circumvent this potential disadvantage. While *L. iners* was detectable during all time points during these conditions, *L. crispatus* significantly dominated the culture by 8 hours, with increasing domination through to 24 hours. Once more, *L. crispatus* did not appear to be influenced by the presence of *L. iners*, with CFU/mL concentrations over 200-fold higher than the initial inoculum after 24 hours (Figure 26A). However, *L. iners* CFU/mL decreased in these co-culture conditions to over 100-fold lower than initial inoculum. This observation was replicated for NYC III media containing previously listed carbohydrates and combinations of these carbohydrates, however no significant enhancement of *L. iners* growth was observed after 24 hours. As mucin appeared to enhance the growth of *L. iners* in liquid broth cultures, 10 mg/mL of mucin was added to cultures. This addition did not appear to

enhance the growth of *L. iners* under these co-culture conditions, and a more than 100-fold decrease in growth was observed again. In attempts to give *L. iners* the upper hand at inoculum, *L. crispatus* was inoculated at 10^5 CFU/mL and *L. iners* was inoculated at 10^6 CFU/mL, however the results at 24 hours displayed similar findings as when inoculated with equal amounts. A summary of all conditions tested is listed in Table 3. When repeated for *P. bivia* and *G. vaginalis*, similar findings were observed, where *L. crispatus* always dominated after 24 hours (Figure 26B & 26C). In these cases, the resources tested included glucose, glycogen and the addition of mucin. While these findings support our previous observations that *L. crispatus* may effectively outcompete others as a result of better utilization of carbohydrate resources, this does not allow us to study the interactions between *L. crispatus* and other vaginal species under these conditions. While a model that better represents the dynamic interactions that occur with the VMB is required to study how perturbations and changing microenvironments may influence community structure and behaviour, the simplistic nature of liquid broth cultures as a means to accomplish this may not be the best suited model. With this, we were able to recover appreciable amounts of *L. iners*, and to a lesser extent *P. bivia* and *G. vaginalis* in the presence of Vk2 cells (Figure 6). With this, a Vk2 coculture model whereby we have the ability to alter the resources available for Vk2 media may allow for the analysis of differential competitive abilities between vaginal bacteria under various conditions.

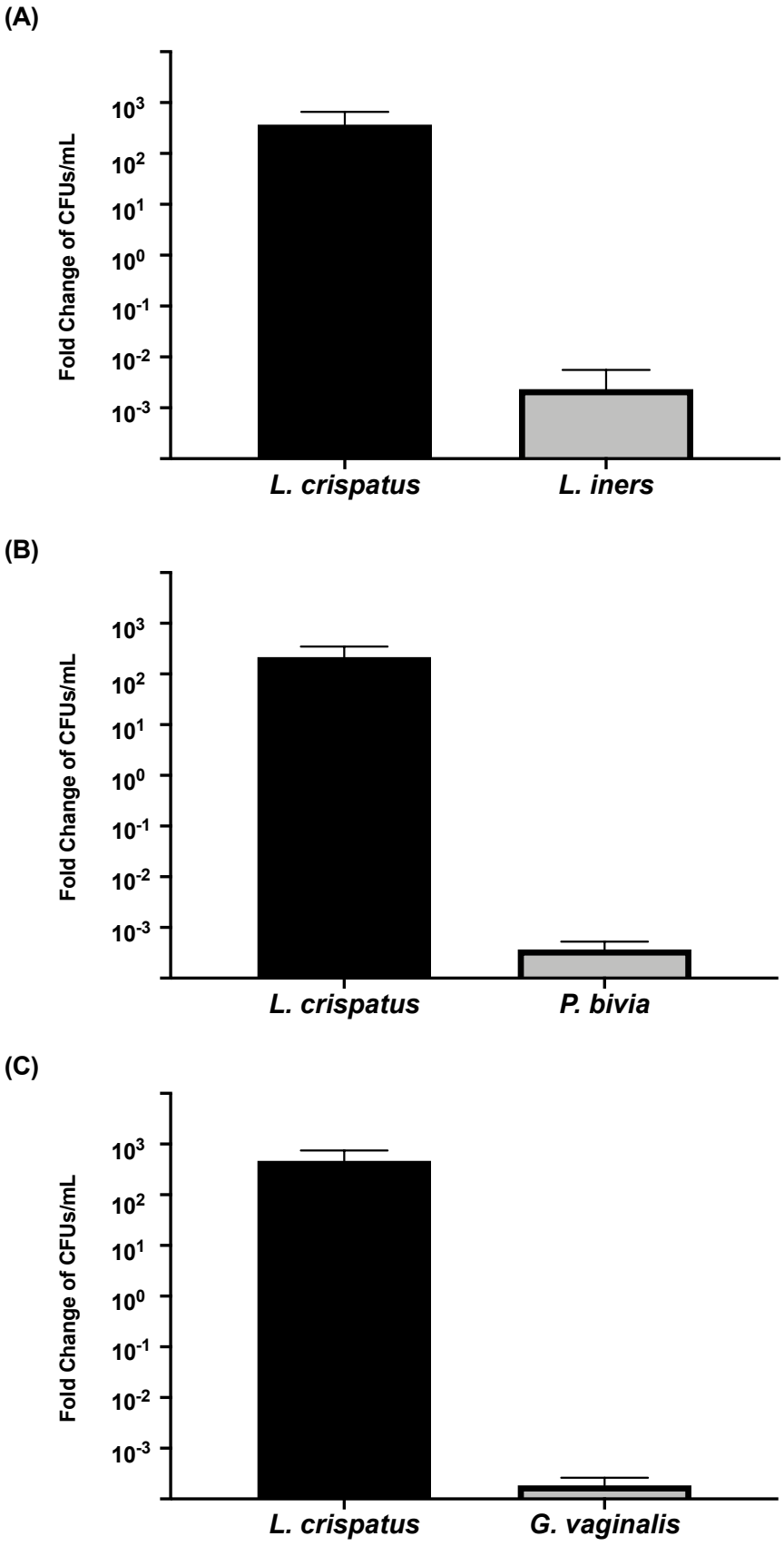
(A)
Fold Change of CFUs/mL
10^3
10^2
10^1
10^0
10^-1
10^-2
10^-3
L. crispatus
L. iners
(B)
Fold Change of CFUs/mL
10^3
10^2
10^1
10^0
10^-1
10^-2
10^-3
L. crispatus
P. bivia
(C)
Fold Change of CFUs/mL
10^3
10^2
10^1
10^0
10^-1
10^-2
10^-3
L. crispatus
G. vaginalis

Figure 26: Fold change of vaginal species CFU/mL in co-cultures after 24 hours of anaerobic cultivation in NYC III media containing 5.0% glucose. **(A)** Coculture of *L. crispatus* with *L. iners* **(B)** Coculture of *L. crispatus* with *P. bivia* **(C)** Coculture of *L. crispatus* with *G. vaginalis*. Initial inoculum (0 hours) was 6 x 10^6 CFU/mL for each species cultivated during exponential phase of growth. Data is representative of at least three experiments performed with three replicates. Error bars represent SEM.

Table 3: *L. crispatus* dominates co-cultures with *L. iners* in a variety of conditions.

NYC III Media content	Frozen Stock	Exponential phase: 10^6 CFU/mL each	Exponential phase: LC 10^5, LI 10^6 CFU/mL
Glucose	✓	✓	✓
Glycogen	✓	✓	✓
Sucrose	✓		
Maltose	✓	✓	✓
Glucose/Glycogen		✓	✓
Glucose/Sucrose		✓	✓
Glucose/Maltose		✓	✓
Glycogen/Sucrose		✓	✓
Glycogen/Maltose		✓	✓
Maltose/Sucrose		✓	✓
Mucin added		✓	✓

The table lists the conditions, substrates and inoculums used in the attempts at establishing a *L. crispatus* and *L. iners* co-culture in NYC III media over a 24 hour period. LC = *L. crispatus*, LI = *L. iners*.

4.3 STUDY SUMMARY:

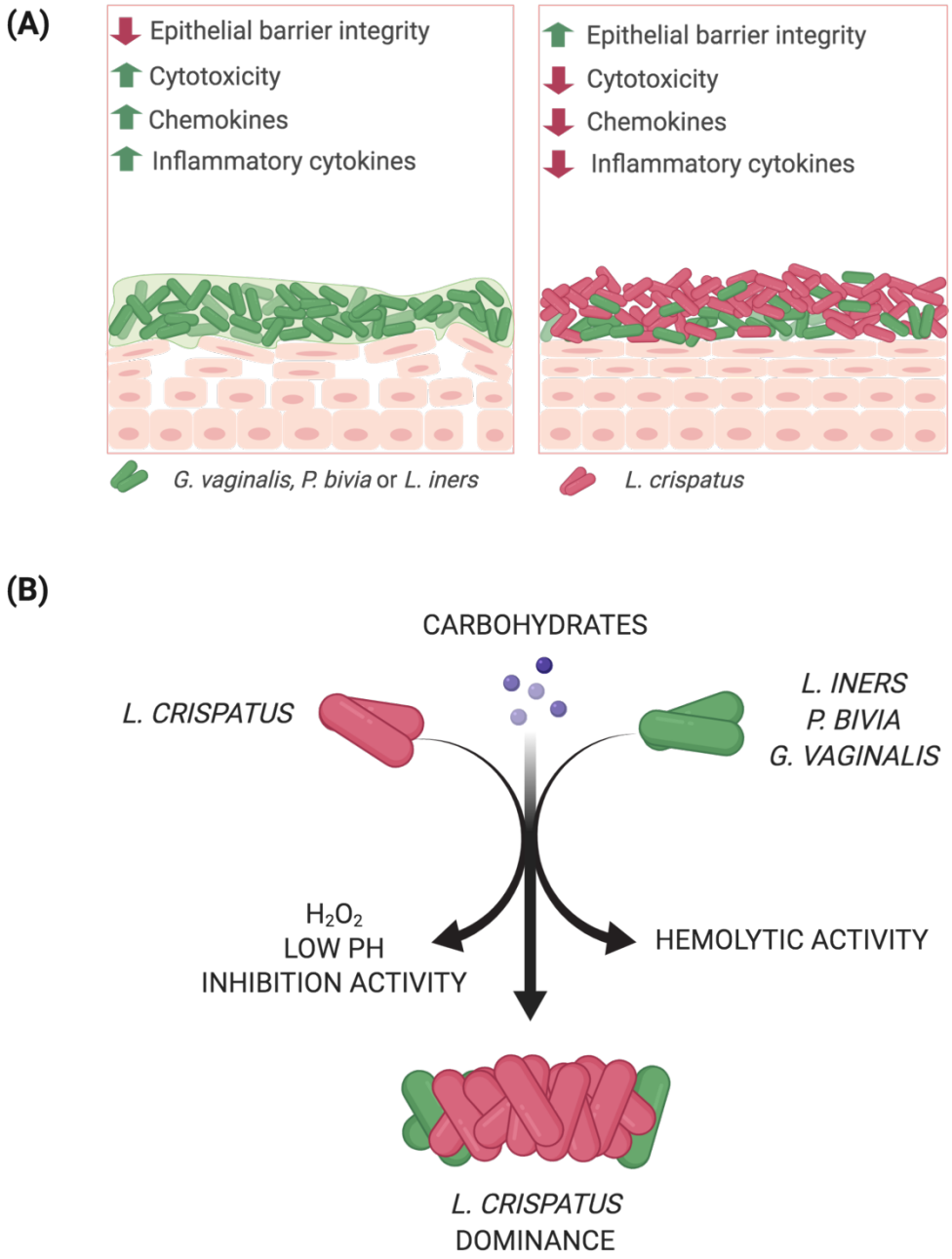

Figure 27: Study summary. **(A)** Our *in vitro* coculture system of VK2 vaginal epithelial cells cocultured with common vaginal bacteria resulted in cytotoxicity, decreased barrier function, and inflammation when either *P. bivia, G. vaginalis* or *L. iners* was present. When *L. crispatus* was cocultured with either of these other three bacteria in dual species cocultures,

these effects were largely negated. **(B)** Our phenotypic analyses found that *G. vaginalis* and *L. iners* had hemolytic capabilities, while *L. crispatus* produced H2O2, lowered the pH to the greatest extent, had inhibitory activity against *P. bivia* and *G. vaginalis* and was capable of growth on the widest range of carbohydrates. This translated to *L. crispatus* dominance over the other three species when cocultured in bacterial broth media.

CHAPTER 5: DISCUSSION

5.1: Discussion:

Optimization, management and restoration of a robust VMB that correlates with good reproductive outcomes and increased protection against STIs has the potential to vastly improve women's reproductive health and protect those most vulnerable to HIV-1 infection[31]. However, despite these important implications, surprisingly little is known about how the VMB regulates and alters susceptibility in the FRT and the factors that select for specific community compositions. These gaps in knowledge represent a major obstacle in the development of effective and practical strategies to treat bacterial dysbiosis or to optimize the VMB. In the work described in this thesis, I used a reductionist model system to deepen our understanding of the host-microbe and microbe-microbe interactions that may underlie the susceptibility associated with vaginal bacterial dysbiosis and the extrinsic factors that may drive community dynamics.

While our understanding of the VMB from a mechanistic and functional framework would provide critical information, much of the advancement in the field has been hampered by the lack of a quality experimental model. While no model system perfectly recapitulates all the complex biological, chemical and structural features of the human FRT, we could argue that appropriately designed simplified systems can inform, or even be a prerequisite to, understanding the more complex dynamics and interactions in the FRT. Herein, we modified an existing *in vitro* vaginal epithelium model system to more aptly and accurately reflect the *in vivo* conditions of the FRT. Using an immortalized cell line that closely mimics the functions of *in vivo* vaginal epithelial cells in an ALI culture system with that allows for vaginal epithelial cells to grow in a multilayered configuration reflective of the squamous

epithelium of the vaginal tract, and as well become terminally differentiated, we further mimicked the FRT by doing our coculturing experiments in microaerophilic conditions[140]. By maintaining viability and barrier function of the Vk2 cells analyzed (Figure 1), these conditions allowed us to better assess interactions within our cocultures with anerobic bacterial species that were conducive to these species growth. Our findings mirror those by Donnarumma et el, who examined Vk2 cells in microaerophilic conditions and likewise found viability was not reduced[196]. Moreover, global gene expression profiles of several bacteria, including *Lactobacillus* species, have shown that several hundred genes are found to be differentially expressed under anaerobic conditions. As such, the induction or repression of many of these genes under these conditions could have important implications in their interactions with the host and other bacterial species[197,198]. Thus, completing our assessments under these conditions that may better represent the interactions that occur *in vivo* was a priority for us. Recognizing the other limitations of our simplified system, establishing this model allowed us to subsequently analyze the interactions between common vaginal bacteria and host epithelial cells that may contribute to protection or susceptibility in the FRT.

Maintenance of a robust mucosal barrier in the female genital tract is critical for preventing invading microorganisms, including HIV-1, from penetrating into the tissues, establishing infection, and entering circulation[148,199]. In this context, interactions of vaginal bacteria with host epithelial cells is important in determining the course of resistance or assistance to the acquisition, establishment, and progression of HIV-1 infection. As such, while acknowledging that our model does not contain some important features of the mucosal barrier present in the FRT, such as a robust mucus layer, various cell types, and

many enzymes and proteins, our system does allow for a reductionist analysis of alterations in epithelial barrier integrity that may increase susceptibility. As the species most frequently associated with a dysbiotic VMB and driver of comorbidities, our findings of decreased cell viability and barrier integrity induced by *G. vaginalis* is consistent with a breadth of prior literature examining the cytotoxic nature of this organism and supported our hypothesis that dysbiosis-associated species would lead to decreased epithelial barrier function (Figure 2B & 5B)[146,200]. *G. vaginalis* is equipped with several virulence factors, chief among them being vaginolysin, which has been extensively characterized[201]. Vaginolysin is a cholesterol-dependent pore-forming cytolysin that can lead to rapid and complete cellular lysis, such as observed in primary vaginal epithelial cells and additional cell lines[202]. As such, the reduction in cell viability observed in our model is likely, at least in part, due to the cytotoxic nature of vaginolysin and expectedly contributes to the increase in LDH we observed (Figure 3B). While a 30% loss in Vk2 cells justifiably reduces the integrity of the epithelial barrier we observed, other mechanisms may also be at play. Namely, *G. vaginalis'* production of sialidase, a hydrolytic enzyme, has been shown to degrade several key protective mucosal factors, such as mucins and junction proteins, and has been suggested to contribute to exfoliation and detachment of vaginal epithelial cells[203,204]. This epithelial exfoliation in women with BV is clinically defined by the presence of "clue cells", which are epithelial cells coated in bacteria[205]. Until recently, this feature had not been reliably quantified or associated with specific species. However, a recent mouse model inoculated with *G. vaginalis* recapitulated several clinical features of BV, one of which was the presence of clue cells[206]. Herein, the authors determined that this epithelial exfoliation was due to sialidase activity. As such, the production of sialidase by *G. vaginalis* may also be a key factor contributing to

the reduction in barrier integrity we observed by this species. While our study did not address the presence and/or abundance of these proteins, an analysis of the relative contribution of these proteins to the decrease in barrier integrity may be an important avenue for future investigation.

The virulent nature of *P. bivia* in the FRT is much less clear. Though several *Prevotella* species are common vaginal commensals, the association between a dysbiotic VMB and *P. bivia* is well established[207]. Initial reports suggested that *P. bivia* may be a more inert bacterium and secondary colonizer, simply taking advantage of a hospitable environment induced by previously colonized *G. vaginalis,* without directly contributing to any pathogenicity[208]. However, several lines of recent evidence suggest that *P. bivia* may be more than a bystander in bacterial dysbiosis. Notably, *P. bivia* is an important source of lipopolysaccharide and ammonia in the vaginal mucosa, and has recently been shown to produce several proteases, including sialidase, collagenase, fibrinolysins and glycosidases[209,210,211,212]. These can all serve to degrade important proteins involved in mucosal barrier function and thus could potentially result in decreased integrity and promote vaginal sloughing. With this, while *P. bivia* did not induce the same extent of cytotoxicity in Vk2 cells as *G. vaginalis* in our model, it did significantly decrease barrier function (Figure 3B & 5B). As such, this is likely due to alternative mechanisms other than cytotoxicity and support its role as an important player in the overall pathogenicity and adverse outcomes associated with a dysbiotic VMB. The evidence that both *G. vaginalis* and *P. bivia* are capable of reducing mucosal barrier integrity are also in line with analyses of cervicovaginal fluid[213,214,215,216,217]. Several genomic, proteomic and metabolomic studies have identified several factors that may contribute to a weakened mucosal barrier in women

with a dysbiotic VMB that contain both *G. vaginalis* and *P. bivia*. Further supportive evidence comes from a recent study which found that with increasing bacterial diversity, there were decreases in cytoskeleton and cell wall proteins, decreases in adherence junction proteins, increases in lactate dehydrogenase as a marker of cell death, increases in proteases and decreases in antiproteases[213]. While the relative contribution of *G. vaginalis* and *P. bivia* to these alterations associated with barrier disruption are not known, our study suggests that they at least in part contribute to disruption of the mucosal barrier and thus may increase susceptibility to invading pathogens, such as HIV-1.

Having been described as somewhat of an enigma, *L. iners* propensity to behave as either a commensal, a neutral, or a virulent species has been increasingly put into question[36]. Its pathogenic potential was recently highlighted in a prospective VMB study, where 40% of the women who had an *L. iners*-dominant VMB became infected with HIV-1, opposed to the 0% who had an *L. crispatus*-dominant VMB[62]. However, questions still remained as to whether this was due to the absence of a more protective *Lactobacillus* species, or if *L. iners* played an active role in inducing susceptibility. In our model, *L. iners* induced a reduction in cell viability and barrier function that was comparable to *G. vaginalis*, suggesting that at least in isolation, this strain of *L. iners* has more pathogenic tendencies (Figure 2A & 5A). While *L. iners* is much less characterized than many other vaginal isolates, genomic studies have identified the presence of inerolysin, a cytolysin very similar to the vaginolysin produced by *G. vaginalis*[185]. This pore-forming cytolysin thus may contribute to the cytotoxic effects of *L. iners* observed in our model, and consequently the reduction in barrier function. With this, much remains to be characterized about other potential beneficial or detrimental capabilities of *L. iners*. In line with our findings, recent proteomic studies have highlighted

an increase in proteases, markers of cell death and a decrease in anti-proteases in VMBs dominated by *L. iners* in comparison to *L. crispatus*-dominated[213,216,]. While the mechanisms and conditions shaping the virulent nature or potential of *L. iners* warrants further investigation, our study provides *in vitro* evidence that *L. iners* can induce cytotoxicity in vaginal epithelial cells and decrease barrier integrity, and thus under certain conditions may be actively increasing susceptibility (Figure 3A). In contrast, *L. crispatus* undoubtedly has several protective mechanisms to aid in the defense against invading pathogens, including HIV-1, however its role in enhancing barrier function is less evident[29]. It is not certain whether the presence of *L. crispatus* is simply a proxy for the absence of dysbiosis-associated species and their damaging effects, or whether it can notably enhance the underlying barrier. While single species cocultures of *L. crispatus* did not decrease Vk2 viability or barrier function as observed by all other species tested, it also did not result in an enhancement of barrier function (Figure 2A & 4A). While this may suggest that *L. crispatus'* beneficial effects lie in alternative protective mechanisms, such as creating an inhospitable environment for invading pathogens or by mitigating the immunomodulatory effects of non-commensal species, our findings could also be due to several other factors. Firstly, one must consider the limited nature of our model system, which does not completely recapitulate the vast array of physiological features that work is concert to create a robust mucosal barrier in the FRT. As such, this species' potential beneficial impact on other cell types, proteins, enzymes and mucus components that may serve to enhance the barrier function cannot be elucidated by our model. Indeed, proteomic studies found that *L. crispatus*-dominated VMBs are associated with increases in antiproteases and adhesion junction proteins[213,216]. Additionally, a recent study found that *L. crispatus* supernatants had the greatest wound-healing properties as

compared to control media or other *Lactobacillus* species[217]. As our results are comparing the effect of bacteria on an already robust and intact epithelium, these beneficial effects may not be detectable by our measurements. Moreover, future assessments such as transcriptomic analysis may be able to elucidate more subtle alterations of epithelial barrier function that are not readily discernable by our measurements. Taking this into account, our findings do reflect those of other *in vitro* models, whereby the presence of *L. crispatus* does not induce cytotoxicity or obvious disruption of the epithelial barrier[138,147,218]. Despite many of these studies labeling them as beneficial in the absence of any noticeable negative impacts on barrier function, findings of any additional enhancement in barrier function were also not reported in these models. With this, these results do offer insights into the species-specific nature by which vaginal bacteria can influence host health and susceptibility to infection. Here, in the absence of any of the discernable detrimental effects induced by the other species, *L. crispatus* is positioned as the most optimal vaginal species. Additionally, our results also illuminated important characteristics about the other common vaginal species. While both *L. iners* and *P. bivia* are not routinely characterized by their pathogenic tendencies, our results suggest these species may play an active role in increasing susceptibility in the FRT. This is particularly important in the case of *L. iners*, whereby effective antibiotic treatment of a dysbiotic VMB often results in an *L. iners*-dominant VMB[219]. Comparable cytotoxicity and barrier disruption to the dysbiosis-associated species, and the findings of an HIV-1 acquisition rate of 40% in women with an *L. iners*-dominant VMB indicate that even effective antibiotic treatment of a dysbiotic-VMB may not result in notable enhanced protection[62]. Therefore, future efforts should focus on more deliberate selection of an optimal VMB composition in the prevention or treatment of VMB dysbiosis, rather than

rely on antibiotics to eliminate the dysbiotic bacteria. Our findings together with extensiveness prior literature suggest that this optimal VMB should be dominated by *L. crispatus*.

The adherence capabilities of vaginal bacteria have been described as protective in terms of *Lactobacillus* species, but virulent when referring to dysbiosis-associated species[151,146]. The contrast of these labels is differentiated by the nature of their implications. In the case of being a beneficial feature, adhesion to host tissue has long been considered an important factor and a prerequisite for long-term colonization of the FRT, stimulation of a tolerant immune system, and competitive exclusion against non-indigenous microorganisms[151]. Indeed, it has been shown that *L. crispatus* exhibits strong adherence capabilities to the surface proteins collagen and laminin in human epithelial cells[147,218,220]. Moreover, their ability to aggregate with themselves and other *Lactobacillus* species has been postulated to enhance the barrier function and protect the underlying mucosa[151]. While the adherent nature of *L. crispatus* in our model at approximately 50% did not appear to enhance barrier function by our measurements, our findings mirror those found in other *in vitro* epithelial cell models containing *L. crispatus* (Figure 7A)[151,220]. Once more, the lack of enhancement observed may be due to the reductionist *in vitro* nature of our model with an already healthy epithelium as the starting point. Due to the seemingly variable role *L. iners* can play in the VMB, the impacts of its adherence are less well known. In support of our findings of clear adherent capabilities at similar ratios to *L. crispatus*, *L. iners* has been found to strongly bind human fibronectin (Figure 7A)[221]. However, whether this adherence is a virulent factor or simply a mechanism utilized by *L. iners* to retain its presence in the vagina is unknown. Of note, fibronectin has been reported to increase 30-fold in the vaginal

epithelium during menses, which may explain the increased levels of *L. iners* observed during menses, especially relative to *L. crispatus*[83,222]. Nevertheless, the strong adhering capabilities of *L. iners* could potentially be deemed as a virulent trait, whereby it is used to facilitate its pore forming cytolysin and thus increase cytotoxicity. Its adhesion may be a mechanism used by the organism to sequester necessary nutrients such as iron for enhanced growth, which in turn results in damage to the epithelial barrier. As a requirement for most cholesterol-dependent cytolysins, the adherence of *L. iners* to Vk2 cells likely facilitated the cytotoxicity induced by this species in our model (Figure 3A)[223]. The adherence properties of *G. vaginalis* are much less debated, where it is described as the first step in the formation of a polymicrobial biofilm which is thought to play a key role in the pathogenesis and persistence of BV[114,146]. The presence of several adhesins and pili have been identified in *G. vaginalis*, and its adhesion to several human epithelial cell lines have been explored[224,225]. In these studies, it has been shown that *G. vaginalis* strains isolated from women with BV had greater adherent capabilities than non-BV isolates. As adherence is required for vaginolysin to exert its activity, in addition to the disparity between expression levels of vaginolysin, adherence could account for the differences in virulence observed between BV and non-BV isolates of *G. vaginalis*[201]. Taken together, the adherence of *G. vaginalis* we observed in our model may have been an important factor and prerequisite to inducing the cytotoxicity measured (Figure 7B).

The adhesive nature of *P. bivia* has been less explored, due to its common classification as a potential bystander in BV. While several studies have described the incorporation of *P. bivia* in biofilms previously established by *G. vaginalis*, the only independent adherence measurement of *P. bivia* has been described in HeLa cells[226]. Here,

while *P. bivia* was shown to adhere, it was not as great as *G. vaginalis*, which was likewise mirrored in our model (Figure 7B). Of note, the assessment of daily swabs in a study examining community composition prior to BV incidence found that *P. bivia* was the first BV-associated species to increase above baseline prior to symptomatic BV[227]. As such, its colonization, and thus prior adherence, may be an important factor in driving and establishing dysbiosis of the VMB. Taken together, with adherence to epithelial cells likely playing an important role in colonization and persistence of each species in the vaginal tract, it is unsurprising that these common vaginal inhabitants were all found capable of adherence in our model. However, as both the potentially beneficial and negative consequences of adherence have been outlined, one should not use this trait to distinguish a commensal versus a pathogenic species or likewise ascertain a causal link between adherence and pathogenicity.

While our single species coculture analyses provide the foundation and recognition of the species-specific manner in which vaginal epithelial cells respond to each species in isolation, very few bacteria grow axenically. As such, there is increasing awareness that bacterial-host interactions can be fundamentally altered in more complex multispecies communities[228]. There is thus a profound need to expand the biotic complexity of the current study systems to better understand the multifaceted and dynamic interactions that occur within the vaginal microbiota. Taking this into account, we sought to understand how host responses to these species may be altered in the presence of a simplified community, where bacterial-bacterial interactions could have important implications. Using the same species studied in isolation, we used a dual-species model whereby two species were simultaneously added in equal proportions to Vk2 cells. Our results indicated that when *L. crispatus* was

present in the dual-species model with any of the other three species, their ability to decrease cell viability and barrier integrity was mitigated (Figure 2-5). Conversely, *L. iners* was not capable of negating the effects of the dysbiosis-associated species *G. vaginalis* and *P. bivia*. Rather, cell viability and barrier integrity were further reduced when *L. iners* was present in cocultures with either one of these dysbiosis-associated bacteria. This was likewise observed when *P. bivia* and *G. vaginalis* were cocultured in the dual-species model. These findings point towards *in vivo* interactions whereby the presence of *L. crispatus* is capable of defending against potentially pathogenic species and restoring barrier function. With this, it is important to highlight a key deviation of our model from *in vivo* conditions. Namely, while lowering of the pH is thought it be a primary defense mechanism induced by *L. crispatus* by creating an inhospitable environment for many microorganisms, this was not a factor involved in our model (Figure 8)[153]. While this likely reflects the buffering capacity of the cell media we used, it is important to note that this divergence from *in vivo* conditions may have important implications on the observations made and may limit the translatability of our model. Within these limitations, our model does provide a unique vantage point to recognize the several other potential defense mechanisms at *L. crispatus*' disposal. By this framework, one can appreciate the breadth of conditions under which *L. crispatus* can out compete and induce potent antimicrobial effects to prevent the colonization of dysbiosis-associated species and their concomitant adverse effects. While our phenotypic characterizations of *L. crispatus* (Table 1 & Figure 17) can offer some insight into the mechanisms by which this may occur, it is important to highlight the broader suggestions of our findings. Hence, our model may help to explain why *L. crispatus*-dominated VMB display superior stability compared to other *Lactobacillus* species, experiencing fewer fluctuations

in community composition or increases in diversity[83]. This is especially true during times of increased pH in the vaginal tract, such as menses and sexual intercourse. Importantly, sexual intercourse is widely cited as a major means by which dysbiosis-associated species are introduced into the FRT and responsible for subsequent development of BV[229]. It is believed that the increase in pH provided by semen results in a transient environment that may be conducive to the colonization of dysbiosis-associated species[230]. Moreover, more frequent sexual intercourse or higher number of sexual partners is associated with an increased likelihood in the development of persistent diversity and symptomatic BV[95,231]. Our model supports these findings, wherein even in an environment with stable neutral pH, *L. crispatus* maintains its antimicrobial and killing activity, and therefore may be better equipped to deal with environmental disturbances to maintain stability and prevent the colonization of dysbiosis-associated species. It is also important to distinguish the differences in *L. crispatus'* ability to mitigate the adverse effects of the other three species. In the case of the dual species cocultures of *L. crispatus* with either *P. bivia* or *G. vaginalis*, the dysbiosis-associated species had greatly reduced viability after 24 hours (Figure 6C & 6D). In addition to many other properties, a key feature that has been attributed to *L. crispatus'* capacity to outcompete dysbiosis-associated species and a property measured in our model is their superior adherence ability[232]. It has been shown that *L. crispatus* is able to displace pre-adhered *G. vaginalis* and is continually cited as one of its key protective features, however, it is important to recognize another study that showed the ability for *G. vaginalis* to displace pre-adhered *L. crispatus*[233,234]. While there may indeed have been competition for adherence in our model, it appeared that adherence was a mechanism of survival by the dysbiosis-associated species, as of the surviving bacterium, the large majority were found to be

adhered (Figure 7C & 7D). These findings follow the notion that the ability for these species to form biofilms is advantageous and protective against a number of potential insults. Indeed, it has been shown that *in vitro* biofilms of *G. vaginalis* are far more tolerant of lactic acid and hydrogen peroxide than their planktonic forms[235]. It is difficult to assess whether viability would continue to decrease during a longer incubation time, or if the adhered bacteria would remain resistant and persist in our model. Moreover, due to the nature of our model whereby both species are added at the same time, it is difficult to say if the dysbiosis-associated species would be capable of adhering and colonizing if later introduced to an already *L. crispatus*-dominant environment. While these questions remain to be answered, it is still important to appreciate the viability of *P. bivia*, *G. vaginalis*, and especially *L. iners* that remained after 24 hours in our model. Here, nearly half of the starting *P. bivia* and *G. vaginalis* remained viable after 24 hours of coculture with *L. crispatus*, and *L. iners* was fully recovered (Figure 6). As such, the detection of these dysbiosis-associated bacteria in the absence of cytotoxicity and reduced barrier function suggests that mechanisms other than direct inhibition of growth and killing by *L. crispatus* may be at play (Figure 2-5). In support of this, a recent study found that the presence of *L. crispatus* repressed the expression for transcripts encoding both vaginolysin and sialidase in BV-associated stains of *G. vaginalis* after only 3 hours of exposure[236]. Potentially this may be a mechanism at work in our model, whereby even the surviving *G. vaginalis* were not virulent in nature due to the suppression of these factors by *L. crispatus*. This likewise may be a particularly important feature in the *L. iners* and *L. crispatus* dual species cocultures, as the viability of *L. iners* was not reduced after 24 hours (Figure 6A). The potential repression of *L. iners'* cytolysin by *L. crispatus* is supported by genomic comparisons of BV and non-BV isolates of *L. iners*[115]. Here, they found

the expression of inerolysin was significantly decreased in non-BV isolates, where *L. crispatus* was present in the VMB. A reduced expression of inerolysin may be why we no longer observed the cytotoxic effects of *L. iners* when *L. crispatus* was present, despite being recovered in equal proportions to *L. crispatus* (Figure 3A). While the mechanistic nature of our findings requires further investigation, these results highlight the potent antimicrobial and competitive capabilities of *L. crispatus*. Even under conditions where a low pH is not a contributing factor, which we have shown on its own inhibits the growth of *G. vaginalis* and *P. bivia* (Figure 17), several other factors appear to be involved in the mitigation of the negative effects induced by both dysbiosis-associated species, as well as *L. iners*. Together, these findings position *L. crispatus* as the species offering the greatest defense against the harmful effects induced by other common vaginal species. As such, its potent antimicrobial and competitive capabilities in combination with its natural colonization in the FRT make it an ideal species to consider for therapeutic or prophylactic interventions of the VMB.

While it is enticing and intriguing to focus attention on the great potential *L. crispatus* could have for the treatment and prevention of a dysbiotic VMB, our findings also suggest a cooperative relationship between *P. bivia*, *G. vaginalis* and *L. iners* that may result in increased damage to the vaginal epithelium (Figure 2-7). These findings of a synergistic or symbiotic relationship are supported by previous biofilm studies, where biofilm growth was significantly increased when both *P. bivia* and *G. vaginalis* were cultured together[237,238]. Likewise, the supernatants of *L. iners* was shown to increase the growth of *G. vaginalis*[234]. Our findings similarly suggest enhanced growth and increased pathogenic effects induced in the dual species cocultures of these three species. While the mechanism by which this synergy occurs is unknown, they may rely heavily on a reciprocal sharing of metabolic by-

products. It has been shown that *G. vaginalis* produces amino acids through its metabolism, which *P. bivia* can then use as a fuel source, creating ammonia as a by-product, which in turn can be used by *G. vaginalis* to enhance its own growth[239,240]. Additionally, in the case of both *G. vaginalis* and *L. iners*, their ability to lyse host cells can liberate nutrients for communal use by the other species, thus potentially enhancing the growth and virulence of other species[241,185]. While of course the species and interactions encompassing a dysbiotic VMB are much more abundant and multifaceted, our findings and others highlight how the culmination of these relationships are likely instrumental in determining community composition and downstream ramifications. Rather than targeting a single driver or initiator species, focusing on dismantling a network of species that cooperate to ultimately determine growth dynamics and virulence may be required to mitigate the condition. Our findings and others thus underscore the importance of further in-depth studies focused on bacterial communities. A greater understanding of the fundamental interactions and community dynamics underpinning dysbiosis of the VMB is likely to advance the field in the development of novel prophylactic targets and more efficacious therapeutics.

The potential for dysbiotic VMBs to cause mucosal inflammation in the FRT is not in dispute, however many questions remain concerning the species-specific nature of this inflammation and how it might be mitigated. Many studies have measured cervicovaginal cytokine levels in women with BV and have found incongruent results regarding a consistent cytokine profile[72,73,62,156]. The heterogeneity in the measurements is likely due to several factors, including variation in compositions of the microbial community, small sample sizes, differing dilutions and various assays used between studies, all of which make generalization challenging. It is difficult to say whether studies showing no difference in certain cytokine

levels are simply underpowered. Moreover, the cross-sectional nature of many of these studies introduces considerable uncertainty as to how representative correlations between single species and the immune milieu is at any given time. With this, understanding how the reproductive mucosa interacts with and responds to each member of the VMB can provide insights pertaining to the nature by which the host response could ultimately lead to increased susceptibility to HIV-1 infection. Herein, our study allowed for a comprehensive analysis of how common vaginal species may work in isolation or in concert with one another to modulate the mucosal immune response.

The majority of cross-sectional and longitudinal studies have reported an increase in proinflammatory cytokines in the cervicovaginal lavage samples of women with BV, or those harboring a dysbiotic VMB[62,72,156]. In our analysis, we found that both the dysbiosis-associated species, *P. bivia* and *G. vaginalis,* were associated with significantly elevated levels of TNF-α, IL-8, IL-6, IL-1β, IL-1α and RANTES (Figures 9-16). Additionally, compared to *L crispatus*, *L. iners* also had significantly elevated levels of TNF-α, IL-8, IL-1β, IL-1α, although with the exception of IL-8, these levels were all significantly lower than those found in cocultures of *P. bivia* or *G. vaginalis*. As such, these lower levels of proinflammatory cytokines may help explain why we didn't see the same extent of barrier reduction induced by *L. iners* as the dysbiosis-associated species (Figure 4&5). Unique to our study was the analysis of dual species cocultures of these species and their immunomodulatory implications. With this, we found that dual species cocultures of *P. bivia* and *G. vaginalis* resulted in certain cytokine levels that were significantly higher than their single-species cocultures, such as in the case of IL-6, IL-1β and IL-1α, once more highlighting a synergistic effect. Importantly, in our interrogation of the mitigatory effects of *Lactobacillus* species, we found that dual species

cocultures of *L. iners* with either *P. biva* or *G. vaginalis* resulted in similar or higher levels of cytokines, with the exception of TNF-α and IL-α where levels were lowered. While only IL-8 was significantly higher in these dual species cocultures, alternative mechanisms such as junction disruption may play a larger role in the additive decreases in barrier damage we observed in *L. iners* dual species cocultures (Figure 4&5). Conversely, cytokine levels were much lower when either *G. vaginalis* or *P. bivia* was combined with *L. crispatus*. Here, we found cytokine levels lowered to levels of single species *L. crispatus* cocultures or that of control. This trend was also observed in dual species cocultures of *L. crispatus* with *L. iners.* These findings recapitulate *in vivo* observations where *L. crispatus*-dominated VMBs are devoid of overt proinflammatory cytokines, while *L. iners*-dominated VMBs have heightened levels, and VMBs characterized by dysbiosis or BV are consistently associated with the highest proinflammatory profile, despite variations in individual cytokines[62,72,73,156]. As previously mentioned, this variation could be due to the differences in VMB community composition, and thus our study allowed for an analysis of the potential contributions made by specific species. With several comorbidities known to be associated with an inflammatory FRT, our findings once again highlight the beneficial role of *L. crispatus* and its ability to mitigate the negative effects of less-favorable vaginal species.

Importantly, the elevated cytokine concentrations observed in our model may facilitate HIV-1 infection in the FRT by reducing epithelial barrier integrity, recruiting and activating HIV-1 target cells, and promoting HIV-1 replication via NF-kB activation[177,242]. Indeed, elevated cytokine levels in the FRT have been associated with a threefold increase in HIV-1 susceptibility[243]. Our results indicate that each species of vaginal bacteria may be playing a different but important role in facilitating HIV-1 acquisition and decreasing the

mucosal barrier in the FRT. Supporting this, our group and others have shown that TNF-α can decrease integrity of an epithelial barrier, which can lead to increased susceptibility to HIV-1 infection by enabling the virus to penetrate the physical barrier through disruption of tight junction complexes[244,245]. While these analyses were done with epithelial cells which are joined by different junction proteins than vaginal cells, TNF-α may still have important implications for the vaginal epithelial barrier. While the TNF-α levels detected in our model were relatively low compared to those in women harbouring a dysbiotic VMB, this is likely a reflection of our model and the use of vaginal epithelial cells, in which other cell types or bacterial species may contribute to a greater induction of this cytokine (Figure 9)[163,246]. Likely a greater contributor to the decrease in barrier function in our model was due to the significant increase in IL-1β and IL-6 observed in both our single and dual species cocultures with all bacteria except for *L. crispatus* (Figure 11 & 12). Both cytokines have been implicated in decreased barrier integrity and disruption of adhesive junctions, and thus may be involved in the reduction of the vaginal epithelial barrier we observed in our model[247,248,249]. Moreover, upon cell death by necrosis, IL-1α precursor is released[250,251]. With this, it is unsurprising that we saw such a stark increase in IL-1α in single and dual species cocultures of *L. iners, G. vaginalis* and to a lesser extent *P. bivia* that were associated with cytotoxicity of Vk2 cells (Figure 3 & 13). This precursor is fully active and can function as an alarmin, which can in turn rapidly initiate a cascade of inflammatory cytokines and chemokines and thus may have contributed to the increase in other pro-inflammatory cytokines we observed[251]. In support of our findings, proteomic studies have shown that women with elevated cervicovaginal proinflammatory cytokine concentrations have unique protein signatures associated with reduced epithelial barrier function[252]. Several proteins that regulate actin

cytoskeleton organization and extracellular matrix components were found to be associated, suggesting that tissue remodeling may occur in women with inflammation at the expense of effective barrier function. Taken together, amongst many factors, the species-specific sensing of vaginal bacteria and induction of proinflammatory cytokines may contribute to the decreased barrier function we observed in our study and ultimately contribute to the increased HIV-1 susceptibility observed in women harbouring an *L. iners*-dominant or dysbiotic VMB.

In addition to contributing to barrier disruption, the elevated chemokines (represented in our study as IL-8 and RANTES) induced by *P. bivia*, *G. vaginalis* and to a lesser extent *L. iners* suggests a mechanism for inflammatory damage accompanied by recruitment of immune cells (Figure 10 & 16). This recruitment of immune cells is supported by examination of cervicovaginal samples which found women harboring a dysbiotic VMB had a 17-fold increase in activated CCR5+ HIV-1 target cells compared to women with a *L. crispatus*-dominant VMB[62]. Moreover, increased numbers of activated CCR5+ T cells were also found in the vaginal tract of germ-free mice following inoculation with *P. bivia*[62]. With this, studies evaluating increased immune cell recruitment in women with BV have been inconsistent[253,254]. This may be due to several factors, one of which may be due to the variability in bacterial community composition between women, where this recruitment may only be induced by certain species. While our findings require validation with more complex multispecies communities, on a background of decreased epithelial barrier integrity, this immune cell recruitment may have important implications for assisting HIV-1 virions in their productive infection of target cells. Moreover, the lack of recruitment of HIV-

1 target cells by *L. crispatus* may be another mechanism accounting for the lack of HIV-1 acquisition in women with an *L. crispatus*-dominant VMB.

In addition to recruiting more target cells for HIV-1 infection, some proinflammatory cytokines have been shown to directly upregulate HIV-1 replication through activation of the long terminal repeat promoter region in an NF-kB-dependent manner[160,255,256]. This feature has been shown for both TNF-α and IL-1β, which were both found to be significantly upregulated by *L. iners, P. bivia* and *G. vaginalis* in our model (Figure 9 & 12). Moreover, IL-8 was shown to increase HIV-1 replication in T cells and to an even greater extent in macrophages *in vitro*[257]. Additionally, IL-8 was shown to increase susceptibility to HIV-1 infection by more than fivefold in cervical explant tissues[258]. Importantly, both IL-1β and IL-8 were significantly increased in all non-*L. Crispatus* single species cocultures, and even more enhanced in their dual species cocultures (Figure 10 & 12). Collectively, these cytokines could serve to increase productive HIV-1 infection through direct upregulation of viral replication, and thus may be another factor resulting in increased susceptibility to HIV-1 infection in women harbouring these species. Together, the unique signatures in proinflammatory cytokine profiles induced by specific bacterial species and their combinations in our model suggests a primary role for vaginal bacteria in modulating the HIV-1 acquisition risk associated with inflammation. These findings once more underscore the importance of evaluating distinct multispecies bacterial communities to advance our understanding of the mechanisms regulating susceptibility in the FRT.

When trying to assess the baseline beneficial features of *Lactobacillus* species associated with protection, the data is less straight forward. At the gastrointestinal mucosal surface, the presence of lactobacilli is associated with downregulation of the immune

response to inflammatory stimuli[259,260,261]. A similar process has been defined in *in vitro* studies of vaginal or probiotic lactobacilli in the FRT using TLR-ligands and common FRT infectious agents, such as *Candida albicans* and *Neisseria gonorrhoeae*[262,263,264,265]. However, downregulation by endogenous vaginal *Lactobacillus* species has not been robustly assessed in response to dysbiosis-associated bacteria. Because many of the adverse health outcomes and symptoms associated with a dysbiotic VMB have been related to the concomitant inflammatory response, understanding whether the presence of *Lactobacillus* species could mitigate those responses is important to elucidate. While single species cocultures of *L. crispatus* did induce certain proinflammatory cytokine levels above baseline, this is likely due to the nature of using an *in vitro* model (Figure 9, 10 & 13). Here, we are exposing epithelial cells that have not been conditioned to become tolerogenic to bacteria in the absence of multiple immune cells involved in the mucosal regulatory response. As such, it is unsurprising that *L. crispatus* induced the production of proinflammatory cytokines above baseline and that this has likewise been observed in other *in vitro* models[147,266]. With this, the basis of labeling lactobacilli, and their lactic acid by-product, as anti-inflammatory in *in vitro* studies has largely come from their induction of high levels of IL-RA[57,267]. This anti-inflammatory cytokine occupies the same IL-1 receptor as IL-1-α and IL-1β, and thus can block their binding and reduce their inflammatory effects. In our analysis, the levels of IL-RA were much above the levels of detection of our assay (Figure 14)[172,173]. Taking this into account and understanding the limitations this poses on our interpretation of these results, it is interesting to note that *L. crispatus* induced the highest levels. This cytokine has been cited as a key mechanism by which *L. crispatus* induces an anti-inflammatory environment and thus protects against the HIV-1 risk associated with elevated inflammation in the

FRT[267,268]. Moreover, with IL-1RA levels even higher in *L. crispatus* dual species cocultures, this may be a mechanism by which *L. crispatus* was lowering inflammation and thus mitigating the adverse effects induced by other species. This may also be reflected in the lower levels of IL-1α and IL-1β observed in these dual species cocultures containing *L. crispatus* (Figure 12 & 13). While the mechanisms by which *L. crispatus* can lower inflammation is likely multifactorial, the presence of this species clearly has powerful immunomodulatory effects that could be harnessed to reduce the risk of HIV-1 acquisition.

Taken together, our single species and dual species cocultures with vaginal epithelial cells offer new insights into the species-specific responses elicited by host cells. Here, we demonstrate how *L. iners* and dysbiosis-associated species can work in isolation and together to induce cytotoxicity and a disrupted epithelial barrier, thereby weakening the physical and structural defences of the FRT. Moreover, with their induction of a number of proinflammatory cytokines, our findings position all three species as likely active contributors of increased susceptibility to a number of pathogens in the FRT. In the context of HIV-1, the presence of these species may thus weaken the epithelial barrier to increase viral translocation, increase immune cell recruitment to bring the virus in proximity with their target cells, and directly increase HIV-1 replication. Together, these findings may help elucidate the nature of why 50% of dysbiotic women and 40% of women harboring a *L. iners*-dominant VMB, acquired HIV-1[62]. Moreover, the lack of cytotoxicity, barrier disruption, and overt proinflammatory immune activation by *L. crispatus* may likewise help explain why 0% of women with an *L. crispatus*-dominant VMB become infected with HIV-1. Importantly, our dual species coculture findings that *L. crispatus* was able to largely mitigate the damaging effects of the other three species highlight its potential use as a therapeutic. Moreover, the

endogenous nature of *L. crispatus* and its propensity to naturally dominate the VMB places this as an ideal candidate over many lactobacilli not known to commonly colonize the FRT. The apparent synergistic effects of *L. iners, P. bivia* and *G. vaginalis* also highlight the profound need to study bacterial species in combinations and multispecies settings, as their interactions may have vast implications for the interactions and dynamics that govern susceptibility in the FRT. Lastly, with our results mirroring many of those found from cytokine profiling, metabolomics, proteomics and transcriptomics, our *in vitro* system provides a convenient model to build upon for evaluating further host-bacterial interactions in the FRT.

While our findings and others have supported the beneficial role of *L. crispatus* and its associated protection in the FRT, unraveling the complex dynamics and mechanisms involved in vaginal bacterial community formation and stability are essential to the advancement of the field and development of novel therapeutic targets. While these interactions in the VMB remain poorly characterized, the simplified experiments we employed in our study can help elucidate factors that may affect bacterial growth dynamics, interspecies interactions and resistance to environmental perturbations. In most cases, residential bacteria have developed efficient ways to process available nutrients as well as active mechanisms to protect their environment against competing bacterial species[269]. However, metabolic modulations within the host or times of nutrient-limited conditions can also be used by opportunistic pathogens or species to coordinately dominate and regulate virulence expression[270,271]. Based on the observations from our cocultures, we wanted to explore the factors that may be contributing to each species virulence, defense mechanisms, or ability to grow in the presence of Vk2 cells. In our phenotypic analysis, we found that both

L. iners and *G. vaginalis* were capable of lysing erythrocytes, while the other bacterial species were not (Table 1). This coincides with the presence of their cytolysins and the high levels of cytotoxicity detected in both our single and dual species cocultures of these bacteria[185,201]. This may also be a mechanism by which the bacteria can obtain and sequester certain nutrients from vaginal epithelial cells, which may give them a particular advantage during times of nutrient scarcity[272]. While only *G. vaginalis* was capable of lysing human erythrocytes, this mechanism may be principally important for its growth during menses but may not be a contributing factor of *L. iners* tendency to increase during this time[83]. However, with potential roles in growth, competition and pathogenicity, targeting these cytolysins responsible for lysis of cells could be an important target in a multiprong approach to dismantling a dysbiotic VMB. In our analysis of the defence and competitive mechanisms that these bacteria may possess, we found that both *L. crispatus* and *L. jensenii* were capable of producing hydrogen peroxide and lowering the pH to the greatest extent (Table 2). The ability for these species to produce lactic acid and lower the pH in the vaginal tract is likely a key mechanism for creating an inhospitable environment for non-commensal organisms and pathogens such as HIV-1[273]. Indeed, we found that media buffered to a pH of 4.0 significantly reduced the growth of both *P. bivia* and *G. vaginalis* (Figure 17). These findings reflect *in vivo* conditions, where *L. crispatus*-dominant VMBs have a pH below 4.0, *L. jensenii*-dominant of 4.2, *L. iners*-dominant above 4.5, and a dysbiotic VBM between 5-6[34,274]. While this lowered pH was not a factor contributing to *L. crispatus* dominance and protection in our coculture system (Figure 8), the production of lactic acid may have antimicrobial effects even in its deprotonated form at a higher pH. The antimicrobial activity of lactic acid, independent of pH, has been shown against yeast and HIV-1[275,276]. Indeed, a recent studying

examining the ability for *Lactobacillus* strains to suppress HIV-1 infection in cervicovaginal tissues *ex-vivo* found that lactic acid could inhibit HIV-1 replication independent of pH[275]. Here they found that when their media was diluted to a pH of 6.9 (similar to our coculture pH), media containing lactic acid could still inhibit HIV-1 replication, while media containing HCl could only inhibit HIV-1 at a pH of 4. Interestingly, they found that this sustained suppressive ability was dependent on the *D*-isomer of lactic acid, and that the *L*-isomer (the only isomer produced by *L. iners*) did not maintain the same level of HIV-1 suppression. As such, these findings offer support for sustained antimicrobial effects induced by *L. crispatus* by-products, even at a higher pH, such as observed in our system. Moreover, the notion that the *L*-isomer of lactic acid has less potent antimicrobial effects may be the reason why despite *L. iners* producing lactic acid, it did not induce the same antimicrobial or killing effects against *P. bivia* and *G. vaginalis* in our system. Instead, *P. bivia* and *G. vaginalis* were fully recoverable after 24 hours of cocultures, while this was not the case when *L. crispatus* was present (Figure 6). *In vivo*, lactic acid's sustained antimicrobial effects may be particularity important in the maintenance of VMB composition stability and protection during times of higher pH, such as during menses or sexual intercourse. Another mechanism that may be contributing to *L. crispatus'* antimicrobial effects in our system is the production of hydrogen peroxide. The relative contribution of hydrogen peroxide to defense in the FRT has been debated due to its microaerophilic environment, however this feature may still have important implications[277]. Namely, concentrations of hydrogen peroxide even in the low micromolar range have been shown to still be toxic to gram-negative species[278]. Perhaps more importantly, during times of increased oxygen in the vaginal tract, such as during menses, tampon use, sexual arousal and sexual intercourse, this feature could aid in

enhanced protection against the colonization and overgrowth of dysbiosis-associated bacteria[44,45,141]. As such, our findings that *L. iners* could not produce hydrogen peroxide further supports the notion that this species may provide a more conducive environment for diverse-associated species to survive and propagate than other lactobacilli. Together, the production of both hydrogen peroxide and lactic acid by *L. crispatus* and *L. jensenii* may provide these species with enhanced resistance to environmental perturbations and community fluctuations across a more diverse range of conditions common to the FRT. To determine the extent of contact-independent defense activity at the *Lactobacillus* species disposal, we examined the inhibition of growth employed by their supernatants (Table 2). Our findings that *L. crispatus* could inhibit the growth of both dysbiosis-associated species, while *L. iners* did not, once more positions *L. crispatus* as possessing a greater repertoire of competitive mechanisms against dysbiosis-associated vaginal species. To rule out the potential contributions of a low pH to this inhibition, we neutralized the media and likewise saw similar results. While this inhibition could be due to several factors, the production of bacteriocins have been proposed for vaginal lactobacilli[187,279]. Bacteriocins are antimicrobial proteinaceous substances secreted by some bacteria that may be active against both closely and distantly related microorganisms. While the production of bacteriocins has been extensively characterized for many lactobacilli species, this identification has been less well-defined for vaginal isolates[188]. However, recent gene sequencing analyses has identified the presence of genes related to bacteriocin production in *L. crispatus* vaginal isolates, but none were identified in *L. iners* genome[110]. While these identifications have not been examined for *L. jensenii*, our findings suggest its inhibitory effects are not as widespread as *L. crispatus',* as it could only inhibit the growth of *G. vaginalis*. While the functional importance of these

bacteriocins to vaginal community stability and competitive activity against the diverse range of anaerobes known to colonize a dysbiotic-VMB is unknown, these may be an important factor in the maintenance of *Lactobacillus* dominance. Together, the inability for *L. iners* to produce hydrogen peroxide, the *D*-isomer of lactic acid, and inhibit growth in a contact-independent manner may contribute to our observation of sustained growth of both dysbiosis-associated species in their cocultures with *L. iners* (Figure 6). These features may likewise help explain the detection of more fluctuations and overgrowth of dysbiosis-associated species in *L. iners*-dominated VMBs[83]. Together, these phenotypic assessments highlight relevant factors that may govern competitive interactions between vaginal species. These distinctions may provide the species with differential competitive abilities across a range of conditions, with *L. crispatus* appearing to possess the broadest array of defense mechanisms. As such, these differentiating characteristics suggesting *L. crispatus* may offer more protective benefits to the host may be important to consider in the development of therapeutics selecting for a specific *Lactobacillus* species.

In addition to these characteristics, little is known about the abiotic factors that might be relevant to competitive interactions between vaginal bacterial species. However, due to the variance in VMB composition within and between women, host physiology likely plays a critical role in shaping bacterial community composition[34,83]. As the vaginal tract varies in the nutrients available across the menstrual cycle, the high environmental adaptability of bacteria is contingent upon their ability to successfully and efficiently utilize resources available for growth[280,281]. Thus, understanding bacterial metabolic capabilities of resources common in the FRT can provide important clues about the conditions under which certain species will thrive and dominate. While local glycogen deposition in vaginal epithelial cells

has long been thought to be a key factor in determining *Lactobacillus* dominance, the prevailing assumption that lactobacilli were incapable of directly metabolizing this substrate was a perplexing caveat[100,101,102]. With a requirement of exogenous breakdown to simple sugars that could likewise be utilized by a majority of other bacterial species, this framework lacked a mechanistic explanation for glycogens role in selecting for lactobacilli[105,106]. With this, our novel findings of glycogen breakdown by both *L. crispatus* and *L. iners* may help explain why certain *Lactobacillus* species are more commonly found to dominate the VMB than others. In support of this, *L. crispatus* and *L. iners,* which were capable of directly metabolizing glycogen in our analysis (Figure 18E), are found to dominate the VMB of 26% and 34% women, respectively, whereas *L. jensenii*, unable to grow on glycogen, is only found as the dominant species in 5% of women[34]. The recent identification of the putative pullulanase type I enzyme capable of degrading glycogen detected in *L. crispatus* genomes supports our findings of direct utilization, and to our knowledge we are the first to report this observation for *L. iners*[108]. Together, our results highlight the necessary functional assessment of the metabolic capabilities of bacteria and the caution that should be taken when inferring absolutes from gene analyses. While both *L. crispatus* and *L. iners* can directly metabolize glycogen, *L. crispatus* was capable of growth on the widest range of carbohydrate resources. This likely allows it to function under a more diverse subset of environmental conditions common to the FRT and thus experience greater stability over *L. iners*. For example, should lactose, sucrose, galactose or fructose, which can be found in high abundance in male ejaculate, be introduced into the vagina, *L. crispatus* may be selected for given its unique ability to ferment these sugars[282]. Together, our findings can offer explanations concerning the propensity of certain *Lactobacillus* species to dominate over

others. However, while the current paradigm suggests that glycogen selects for *Lactobacillus* abundance, and is what we likewise hypothesized, our findings highlight that the mechanisms for this selection may be more complex than once thought. With this, we found that both *P. bivia* and *G. vaginalis* were capable of growth on glycogen, as well as some of its breakdown products. While their growth on glycogen was not as efficient as glucose or maltose, it does suggest that the mere presence of glycogen may not serve to exclusively select for *Lactobacillus* species, as has been previously suggested. Recognizing a more complex framework, our findings do not negate the suggestion that glycogen may be more effectively and efficiently utilized by *Lactobacillus* species. With this, *G. vaginalis, P. bivia* and even *L. iners* were found to grow more slowly on glycogen than *L. crispatus* or *L. jensenii*. Ideally, we would have established simple cocultures in liquid broth to more conclusively analyze how altered carbohydrate resources impacted community composition and dominance, however this could not be accomplished due to technical limitations. We found that cocultures with *L. crispatus* and any of the other bacterial species tested always resulted in stark *L. crispatus* dominance after 24 hours (Figure 26). While these results precluded us from analyzing how environmental perturbations may influence community composition, it does highlight the powerful competitive abilities of *L. crispatus* against these other bacterial species. With this, it seems that as long as any of these carbohydrates are present for *L. crispatus* to utilize, their various mechanisms of protection may be enough to sustain dominance. Thus, at least by our analysis, the ability for *G. vaginalis* and *P. bivia* to utilize glycogen or other carbohydrate resources may not be incredibly important in the presence of *L. crispatus*. However, this ability may be vital to their colonization and persistence in the FRT in the absence of *L. crispatus*. For example, if environmental conditions permit the

destabilization and depletion of *L. crispatus*, dysbiosis-associated species could utilize these resources to gain foothold in the FRT, form a resistant biofilm and thus preclude the recolonization of *Lactobacillus* species. Moreover, *G. vaginalis'* ability to use its vaginolysin to liberate resources from host cells may be particularly important during times of limited resources or glycogen deficiency, such as in a hypoestrogenic state[283]. These times could allow its establishment and formation of the multispecies biofilm, and thus even if glycogen and carbohydrate resources are replenished, they can still thrive and persist under these conditions. Of course, the presence of epithelial cells and other resources present in the vaginal tract would likely dismantle the simplicity of this framework. This is seen in the case of *L. iners*, whereby in liquid broth cultures it was not viable in the presence of *L. crispatus* but was completely recoverable in the dual species cocultures with Vk2 cells (Figure 6A). While *L. crispatus* reduced the viability of both *P. bivia* and *G. vaginalis* even in the presence of Vk2 cells, it is important to investigate if these findings are replicable in more complex systems. As such, alterations of carbohydrate resources in a model including Vk2 epithelial cells may provide a more comprehensive and relevant analysis of the conditions under which certain species may thrive and dominate. Altogether, our findings do highlight the seemingly natural propensity for *L. crispatus* to dominate the VMB under certain conditions. Our analysis of the direct influence of hormones and proteins abundant in the FRT did not detect differential influences on growth between the vaginal species, except for mucin. Here, it was found that all species could grow solely on mucin (Figure 22A & 23A). This may be owning to its glycosylation, whereby the species could be using this as a carbohydrate resource[284]. Overall, mucin appeared to enhance the growth of *L. iners* and *G. vaginalis* to the greatest extent, whereas growth of *L. crispatus*, *L. jensenii* and *P. bivia* were markedly less efficient.

This degradation of mucins is supported by the fact that BV is characterized by discharge with high concentrations of mucin-degrading enzymes and a watery consistency[285]. As mucins lubricate mucosal surfaces and can trap pathogens through mucoadhesions, degradation of the mucins may facilitate HIV-1 virion diffusion[286]. Indeed, while HIV-1 virions were trapped in cervicovaginal mucus from women with a *L. crispatus*-dominant VMB, virions diffused rapidly through mucus from women with a *L. iners*-dominant or dysbiotic VMB[287]. While the contribution of mucin degradation in determining community composition is unknown, one could envisage that this ability and proficiency may be important during times of limited resources, and may correspondingly reduce the mucosal barrier to increase susceptibility. While a deeper investigation into the metabolic capabilities of vaginal bacteria is still required, our analysis highlights the functional similarities and differences that may enable each vaginal species to thrive or compete under differing conditions.

Together our phenotypic characterizations of common vaginal species defense mechanisms, virulent traits and metabolic proficiencies provide novel insights into their functional capabilities that may contribute to their ability to compete and dominate in the FRT. Together, these characterizations can lay the groundwork for future investigations into novel therapeutic targets aimed at dismantling a dysbiotic VMB or selecting for an optimal VMB. These advancements are critically needed due to the ineffectiveness of current treatments and the rapidly developing resistance against existing antibiotics, including those used to treat BV[288]. Antimicrobial therapies interfering with specific bacterial metabolism or virulence factors may thus become an intriguing avenue. Moreover, the use of exogenous lactobacilli and the potential use of prebiotics to select for an optimal VMB are likewise

increasingly attractive. Overall, our findings of potent antimicrobial abilities, anti-inflammatory induction, diverse range of metabolic capabilities and propensity to naturally dominate the VMB situates *L. crispatus* as an ideal species to select for in the optimization of the VMB. Together, this body of work increases our understanding of the dynamic interactions that occur in the FRT, and thus advances the field in path towards harnessing the VMB to confer greater protection to the women most vulnerable.

5.2 Limitations:

While several limitations of our model have already been described thus far, it is important to reiterate the impacts these could have on our interpretations and the translatability of our findings. While using an *in vitro* model that recapitulated various important aspects of the vaginal epithelium, including a microaerophilic environment and an air-liquid interface allowing for the development of a multilayered squamous epithelium, several other fundamental features were not present. This includes multiple cell types, mucus components, enzymes, proteins and various carbohydrates, fatty acids and trace elements that bacteria may use to grow and survive. The nature and magnitude of how their presence could impact the interactions we observed in our model is not known. Additionally, if all immune components were present in the FRT, we may have observed an altered host immune response. This is likewise for structural components, including the presence of fibroblasts, in which their interactions with epithelial cells may alter their functions and thus how they interact with vaginal bacteria[148]. Also, with various resources absent that bacteria may use to grow off of and thrive, the interspecies interactions, competition, relative abundances and thus responses by host cells we observed may have also been modified from what is observed *in vivo*. Moreover, with recent links between metabolism and virulence

expression, it is uncertain whether the virulence we observed for certain species would have been found if alternative resources were present[270,271]. Another important limitation was that our analysis included a limited number of common vaginal species, and only assessed one strain of each species. With strain variability known to influence important aspects of virulence expression and metabolic capabilities, our findings may be restricted to only the strains tested[112,113]. As already discussed extensively, the buffering nature of the media used in our coculture system prevented the lowering of pH, and thus does not accurately reflect the environment *in vivo*. While this did allow us to observe factors at play outside of pH, it may have skewed our results. Together, there are various relevant abiotic and biotic factors not present in our model that could govern interactions between bacterial species and their host, and thus their absence may potentially distort how accurately our findings represent those *in vivo*.

5.3 Future Directions:

Our analysis provided a reductionist analysis of the interactions between common vaginal bacteria and host epithelial cells. While this provided insights as to how each species may increase susceptibility to infection, there are many possible factors that drive interactions between bacteria and with their host that were not present in our system. Thus, future efforts to more accurately represent these vital factors should be prioritized to gain a more comprehensive understating in future studies. Through the progressive inclusion of additional cell types, a mucus layer, and nutritional resources, this *in vitro* system can be built upon to better reflect *in vivo* conditions. Moreover, due to the limitations conferred by our buffered media, using an unbuffered media that also has the ability to be manipulated to vary the carbohydrate and nutritional resources present could provide additional

information regarding the factors that determine community composition and competition. With this, further characterization of the conditions and resources that select for specific species will define the components necessary to create stable representative communities. As our study highlighted the ways in which the combination of two species can significantly alter interactions with the host, it follows that increasing complexity to establish multispecies communities will allow for an even greater understanding the ways in which interspecies interactions can influence VMB functionality. In this same regard, a focus on establishing stable and natural bacterial communities isolated from women harbouring a dysbiotic VMB versus a *Lactobacillus*-dominant VMB will allow for a more representative and relevant analysis of how these communities interact. Analyzing these interactions in response to both abiotic and biotic perturbations that more closely mimic *in vivo* conditions could have important implications for future therapeutic design. Another avenue to pursue would be the further characterization of adherence properties of the different vaginal bacteria. As the majority of viable *P. bivia* and *G. vaginalis* after dual species coculture with *L. crispatus* were found to be adhered to vaginal epithelial cells (Figure 7), understanding the mechanistic nature and contribution by which adherence properties and biofilm formation contribute to the pathogenicity of dysbiosis-associated species may have important implications. With this, the potential that a robust biofilm is more resistant to *L. crispatus'* defenses may be an important factor to consider in dismantling the network of dysbiosis-associated species and allowing for a more optimal VMB to flourish without relapse. Together, our model system and findings provide a solid foundation to explore several avenues of future investigation that can ultimately advance our understanding of the VMB.

5.4 Conclusion:

Meeting the HIV-1 prevention needs of adolescent girls and young women who are at uniquely high risk of acquisition is imperative if we have to meet the goal of significantly decreasing and finally eliminating HIV-1, especially in Sub-Saharan Africa. As many women in these high-incidence populations have a dysbiotic VMB composition that is associated with a fourfold increased risk of HIV-1 acquisition, the ability to manipulate the VMB could have vast implications for women's health and altering the current trajectory of HIV-1 infection rates[34,35]. As such, the goal of our study was to gain a deeper understanding of microbe-microbe and host-microbe interactions that govern the VMB. Specifically, with the potential of harnessing and optimizing the VMB to prevent HIV-1 infection in the women most vulnerable, our analyses centered on mechanisms that may increase a woman's risk of HIV-1 acquisition. Together, our data provides novel mechanistic insight of the species-specific way in which certain common vaginal bacterial species may directly increase host susceptibility to infection through inducing cytotoxicity of host cells, decreasing epithelial barrier integrity, and inducing inflammation (Figure 27A). These adverse effects were expectedly induced by dysbiosis-associated species *G. vaginalis*, and of note, *P. bivia* and *L. iners*. To our knowledge this is the first report of these adverse effects directly associated with *P. bivia* and *L. iners* in vaginal epithelial cells. Further aiding in our increased understanding of host-microbe interactions was the establishment of dual-species cocultures with host epithelial cells, whereby combinations of these three species indicated additive consequences. Intriguingly, *L. crispatus* was able to largely mitigate their damaging effects, displaying the powerful benefits the presence of this species could have in the FRT. Together with our phenotypic analyses of potential defense mechanisms at each bacterium's disposal, our findings and others place *L. crispatus* as the endogenous species most capable

of providing protection in the FRT (Figure 27B). Moreover, we characterized the diverse metabolic capabilities and proficiencies of vaginal species to gain better insights into the exogenous factors that may help select for a specific community composition. Thus, with the prospect of using exogenous lactobacilli to optimize the VMB and prevent or treat bacterial dysbiosis, our findings can help elucidate the conditions under which certain species may thrive or when their growth may be discouraged. By delineating how functional configurations of microbes can impact vaginal health, we are able to lay the groundwork for more in-depth characterizations that will ultimately lead to a better understanding of the interactions that govern the dynamics of the VMB. This knowledge can thus be built upon to advance the field in the development of more rationale therapeutic or prophylactic interventions, and as such improve the reproductive health of many vulnerable women.

[1] Joint United Nations Programme on HIV/AIDS (UNAIDS) (2019)

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