THE EFFECT OF LACTOBACILLI AND FEMALE SEX HORMONES ON THE INNATE IMMUNE RESPONSES OF VAGINAL EPITHELIAL CELLS

THE EFFECT OF LACTOBACILLI AND FEMALE SEX HORMONES ON THE INNATE IMMUNE RESPONSES OF VAGINAL EPITHELIAL CELLS

By Jeffrey H.Y. Lam B.Sc. (Hon.)

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AUTHOR: Jeffrey H.Y. Lam, B.Sc. (University of Waterloo)

SUPERVISOR: Dr. Charu Kaushic

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Abstract

Background: The female genital tract represents the first line of defence against HIV. Biological factors such as female sex hormones, and the vaginal microbiota are known to affect HIV susceptibility at this site. The female sex hormone estradiol is known to play a protective role, whereas the progestin based contraceptive medroxyprogesterone acetate increases HIV susceptibility HIV. In addition, a Lactobacilli dominant vaginal microbiota is generally protective against HIV. Therefore, in this study, we aimed to elucidate the effects of female sex hormones, and Lactobacilli on the innate immune response of vaginal epithelial cells.

Method of Study: In this study, we utilized an *in vitro* vaginal epithelial cell culture system grown in an air-liquid interface. Subsequently, cells were either grown in the presence or absence of female sex hormones (estradiol, progesterone, medroxyprogesterone acetate), and/or co-cultured with or without Lactobacilli. We then assessed the barrier function, and cytokine production of the cell cultures, parameters which have been shown to be important in the context of HIV susceptibility. In addition, we also performed transcriptomic analysis on vaginal epithelial cells which have been co-cultured with Lactobacilli.

Results: We found that vaginal epithelial cells grown in the presence of medroxyprogesterone acetate had decreased barrier function, as seen in decreased transepithelial resistance measurements, and increased FITC-dextran

leakage. Cells grown in the presence of medroxyprogesterone acetate also produced higher levels of chemokine RANTES. In addition, we found that Lactobacilli co-cultured cells differentially expressed several gene clusters which may lead to decreased susceptibility to HIV.

Conclusions: In our *in vitro* system, we found that medroxyprogesterone acetate decreased barrier function, and increased chemokine production of vaginal epithelial cells. This study provides valuable insight to the potential mechanisms of how medroxyprogesterone acetate may affect the innate immune response of vaginal epithelial cells. Additionally, through transcriptomic analysis, we also gained insight into several possible mechanisms of how Lactobacilli may act on vaginal epithelial cells to decrease HIV susceptibility.

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DECLARATION OF ACADEMIC ACHIEVEMENT

All experiments were conceived and designed by Jeffrey Lam, and Dr. Charu Kaushic. Dr. Gregor Reid provided bacterial strain *L. rhamnosus* (GR-1) utilized in this study. Dr. Nuch Tanphaichitr provided bacterial strain *L. crispatus* (SJ-3C-US) utilized in this study. Jeffrey Lam performed all experiments. Jeffrey Lam wrote this dissertation with contributions from Dr. Charu Kaushic.

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ALI	Air-Liquid Interface
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BV	Bacterial Vaginosis
CFU	Colony Forming Units
CST	Community State Type
CVL	Cervicovaginal Lavage
DAVID	Database for Annotation, Visualization and Integrated Discovery
E2	Estradiol
ECM	Extracellular Matrix
FA	Focal Adhesion
FGT	Female Genital Tract
FITC	Fluorescein Isothiocyanate
FRT	Female Reproductive Tract
GR	Glucocorticoid receptor
HESN	Human Immunodeficiency Virus Exposed Seronegative
HIV	Human Immunodeficiency Virus
I-CAM1	Intercellular Adhesion Molecule 1
IgG	Immunoglobulin G
IL-10	Interleukin 10
IL-1RA	Interleukin-1 Receptor Antagonist
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8

IP-10	Interferon gamma-induced protein 10
ISG	Interferon-Stimulated Gene
kDa	Kilodalton
KLK	Kallikrein-Related Peptidase
KSFM	Keratinocyte Serum Free Media
LDH	Lactate Dehydrogenase
LGT	Lower Genital Tract
Mbp	Megabase Pair
MDS	Multidimensional Scaling Plot
MIP-1α	Macrophage Inflammatory Protein 1 alpha
MIP-1b	Macrophage Inflammatory Protein 1 beta
MPA	Medroxyprogesterone Acetate
MRS	De Man, Rogosa and Sharpe
N-9	Nonoxynol 9
P4	Progesterone
PRR	Pattern Recognition Receptor
RANTES	Regulated on activation, normal T cell expressed and secreted
SIV	Simian Immunodeficiency Virus
TER	Transepithelial Resistance
TLR	Toll Like Receptor
TNF-α	Tumor Necrosis Factor alpha
UGT	Upper Genital tract
ZO-1	Zonula Occludens 1

CHAPTER 1: INTRODUCTION

1.1 Epidemiology and Female Susceptibility to HIV

Despite over 30 years of extensive research, human immunodeficiency virus (HIV) remains one of the most prevalent sexually transmitted infections worldwide. According to a report published by UNAIDS, there were 36.9 million people living with HIV in 2017 (UNAIDS, 2018). Although great strides have been made in the form of antiretroviral therapy, a staggering 1.8 million new cases of HIV infection occurred in 2017 (UNAIDS, 2018). Women are disproportionately infected with HIV as opposed to their male counterparts (WHO, 2012). Women are more susceptible to HIV due to numerous biological, socio-economical, and political factors (Ramjee & Daniels, 2013). Sites of HIV transmission include the female and male genital tract, intestinal tract, placenta, and the blood stream (Hladik & McElrath, 2008). Although it is estimated that as many as 40% of HIV infections occur in the female reproductive tract (FRT) (Hladik & McElrath, 2008). Therefore, given that the FRT represents a predominant site of HIV infection through heterosexual intercourse, there is a need to further our understanding of biological factors that can alter HIV susceptibility within this site.

1.2 Human Immunodeficiency Virus

HIV is in the genus lentivirus inside the broader family of retroviruses. Retrovirus differ from DNA and RNA based viruses as they contain an RNA based genome, which is reverse transcribed into DNA and integrated into the host genome. The HIV virion is composed of two positive stranded RNA genomes at its core which are tightly associated with the nucleocapsid protein (NC/p7) (Georgiev., 2009). The capsid protein (CA/p24) encircles the NC associated with the viral RNA (Georgiev., 2009). The two viral enzymes, integrase and reverse transcriptase, are also enclosed within the capsid of the virion (Georgiev., 2009). The capsid is surrounded by the matrix protein p17, which acts to stabilize the virion (Georgiev., 2009). The viral protease and accessory proteins Vif, Vpr and Nef are also present in the virus (Georgiev., 2009). The envelope of the virion is composed of the plasma membrane of the host cell, surface protein glycoprotein 120 (gp120), and trans membrane glycoprotein 41 (gp41), both of which are responsible for the initial fusion of the HIV virion to the host cell's plasma membrane (Georgiev., 2009). In order for HIV to cause productive infection, the host cell must express the CD4 receptor, and a CXCR4, or CCR5 co-receptor (Wilen, Tilton, & Doms, 2012). HIV that utilizes the CXCR4 co-receptor for entry are termed X4 tropic viruses, while HIV that use the CCR5 co-receptor are R5 tropic viruses (Wilen et al., 2012). Although dendritic cells, and macrophages are susceptible target cells to HIV, $CD4^+$ T cells are generally considered to be the initial subset of cells which are infected by HIV (Hladik et al., 2007). Interestingly,

although both X4 and R5 tropic virus are capable of causing infection, the majority of productive HIV infections are caused by R5 tropic viruses, indicating the existence of a "gatekeeper" mechanism (Grivel, Shattock, & Margolis, 2011). However, it is not known what this "gatekeeper" mechanism is (Grivel, Shattock, & Margolis, 2011).

1.3 Female Reproductive Tract

In women, the FRT is often the first site of exposure to HIV. In order for productive HIV infection to occur in the FRT, the virus must traverse across the epithelial cell barrier that lines the FRT and reach target cells residing in the submucosa. Epithelial cells are not infected with HIV, but instead act as a barrier to target $CD4^+$ T cells (P. V. Nguyen, Kafka, Ferreira, Roth, & Kaushic, 2014). Therefore, the epithelium of the FRT represents an important first line of defence against HIV virions. The consists of the upper genital (UGT), and lower genital tract (LGT), both of which are sites which HIV infection could occur (P. V. Nguyen et al., 2014).

The UGT is composed of the fallopian tubes, uterus and endocervix, while the LGT is composed of the vagina and the ectocervix (Blaskewicz, Pudney, & Anderson, 2011; P. V. Nguyen et al., 2014). The UGT is lined by a single layer of columnar epithelial cells that separate the submucosa from the external environment (P. V. Nguyen et al., 2014). Tight junctions join neighbouring

columnar epithelial cells and strictly regulate the paracellular movement of molecules (Blaskewicz et al., 2011). In an experiment conducted by Blaskewicz et al, the authors demonstrate that fluorescently labeled IgGs were unable to penetrate the columnar epithelial cells to reach the underlying submucosa (Blaskewicz et al., 2011).

The LGT is characterized by the presence of multi-layered, non-keratinized squamous epithelial cells (D. J. Anderson, Marathe, & Pudney, 2014; Blaskewicz et al., 2011; P. V. Nguyen et al., 2014). The basal epithelial cell layer of the LGT is metabolically active and undergoes constant division (D. J. Anderson et al., 2014). As these metabolically active cells slowly differentiate into superficial cells, they lose their organelles and nuclei, and take on a superficial role on the apical side of the epithelial cell layer (D. J. Anderson et al., 2014). At the apical side, there is constant sloughing of superficial cells, which acts to remove any pathogens that may have adhered to the epithelium (D. J. Anderson et al., 2014; Kaushic, 2011). Although the LGT epithelium is composed of multiple layers, the apical epithelial cells are permissive to fluorescently labeled IgGs, indicating the absence of junctional proteins in this region(Blaskewicz et al., 2011). However, the suprabasal and basal layers of the LGT epithelium possess junctional proteins, which prevent full penetration of IgGs to the submucosa (Blaskewicz et al., 2011).

Although the LGT is generally not considered the main portal for HIV entry in the FRT due to the presence of a multi-layered epithelium, a study done by Stieh

et al suggests otherwise. In this study, female rhesus macaques were inoculated intravaginally with a reporter Simian Immunodeficiency Virus (SIV) with a R5 tropic envelope demonstrated that the virus was able to infect underlying target cells throughout the whole FRT (Stieh, 2014). In addition, the LGT is the site that comes in direct contact with infectious semen ejaculate (Dupont et al., 2018). Mechanical damage such as micro tears, caused by sexual intercourse can also compromise the barrier function of epithelial cells in the LGT, and create a route for HIV to reach underlying target cells (Dupont et al., 2018). As the LGT represents one of the key locations in which HIV transmission could occur during heterosexual intercourse, understanding factors that can either enhance or decrease susceptibility to HIV infection in this location would be beneficial for designing prophylactic strategies against HIV.

1.4 Mechanisms That Increase HIV Susceptibility in the Female Reproductive Tract

Although there are many mechanisms that can alter HIV susceptibility in the FRT, the role of inflammation and barrier integrity will be the primary focus of this thesis, and therefore will be reviewed in detail in this section. Biological factors, such as female sex hormones, and the vaginal microbiota which can alter these mechanisms will be discussed in a subsequent section.

1.4.1 Inflammation in the Female Reproductive Tract and HIV Susceptibility

The link between inflammation in the FRT, and HIV susceptibility has been studied extensively. Pro-inflammatory cytokines act to create a pro-inflammatory environment, whereas anti-inflammatory cytokines act to inhibit or resolve inflammation. For instance, the pro-inflammatory cytokines/chemokines IL-1α, IL-8, RANTES all act to promote influx of immune cells to the local area (Bickel, 1993; Gabay, Lamacchia, & Palmer, 2010; Appay & Rowland-Jones, 2001). A pro-inflammatory environment in FRT is a key contributing factor leading to increased HIV susceptibility at this site. A large scale study by Masson et al demonstrated that there was a direct correlation between the levels of pro-inflammatory cytokines/chemokines in cervicovaginal lavages (CVLs) (IL-1α, IL-1β, IL-6, IL-8, TNF-α, MIP-1α, MIP-1b, IP-10) and HIV, associating female genital inflammation and HIV susceptibility in women (Masson et al., 2015). In addition, several studies have demonstrated that individuals who are at high risk for HIV infection, but remain seronegative (HESN) have cytokine profiles characterized by lower levels of pro-inflammatory cytokines/chemokines in their reproductive tract (Chege et al., 2012; Lajoie et al., 2012; McLaren et al., 2010; Yao et al., 2014). An elevated level of pro-inflammatory cytokines in the FRT has been shown to increase HIV susceptibility through recruitment of additional HIV target cells to the surrounding area (Kaul et al., 2015). In a recent proteomic study published by Arnold et al, the authors demonstrate that women who had an elevated pro-inflammatory profile in the FRT also had increased numbers of $CD4^+$ cells (Arnold et al., 2016). Another

study by Kaul et al reported similar findings, where increased levels of RANTES in the FGT (Regulated on Activation, Normal T Cell Expressed and Secreted) correlated with the increased $CD4^+$ cells in the genital mucosa (Kaul et al., 2008). Findings from a macaque study also support this, as macaques treated with an anti-inflammatory agent (glycerol monolaurate) were protected from infection following subsequent SIV challenge (Li et al., 2009). Overall, these studies demonstrate that an elevated pro-inflammatory cytokine profile is associated with increased HIV susceptibility in the FRT, which might be due to inflammation-mediated recruitment of HIV target cells.

1.4.2 Barrier Function in the Female Reproductive Tract and HIV Susceptibility

As mentioned previously, the barrier function of the female reproductive tract is one of the key factors in the determination of HIV susceptibility. A robust epithelial barrier is key to preventing HIV from reaching underlying target cells in the submucosa. Our lab has previously shown the importance of barrier function in the context of HIV infection using an *in vitro* primary genital epithelial cell culture system (Nazli et al., 2010; Nazli et al., 2013). In this series of studies, we demonstrated that treatment of primary endometrial genital epithelial cells with HIV gp120 led to an inflammatory cascade, which resulted in impairment in epithelial barrier function 24 hours post exposure (Nazli et al., 2010; Nazli et al., 2013). When HIV was added on the apical side of the endometrial genital epithelial cell culture system, increased HIV translocation through the epithelial layer was correlated

with decreased barrier integrity (Nazli et al., 2010). In addition, we demonstrated that the impairment of epithelial barrier function was due to an inflammatory cascade that resulted in disruption of tight junction associated proteins (Nazli et al., 2010, Nazli et al 2013). Although these studies were performed in cells from the UGT, there are other studies that demonstrate the importance of barrier function in the LGT.

Nonoxynol-9 (N-9) is a spermicide which is applied to the vaginal tract prior to sexual intercourse. Interestingly, it appears that N-9 also diminished anti-viral activity against HIV (Malkovsky, Newell, & Dalgleish, 1988). Women who were regularly using the product had a two-fold increased risk of acquiring HIV (Van Damme et al., 2002). Further studies regarding N-9 revealed that regular application of the compound led to compromised vaginal epithelium (Hillier et al., 2005). Indeed, in an *in vivo* rabbit model, N-9 application led to increased vaginal permeability (Acaturk & Robinson, 1996).Thus, impairment of the vaginal epithelial barrier by N-9 provides a route for HIV to reach underlying target cells. Overall, these studies demonstrate that barrier integrity of the female epithelium (both upper and lower) is instrumental in preventing HIV from reaching target cells.

Other research groups have also focused on the importance of barrier function in the context of HIV infection in the FRT. Findings from a study published by Arnold et al found a correlation between the levels of pro-inflammatory cytokines and proteases which are responsible for breakdown of the epithelial barrier (Arnold

et al., 2016). An elevated level of proteases in the genital tract may lead to degradation of the tissue, causing injury; this may in turn lead to increased inflammation, due to the increased influx of immune cells to the site (Arnold et al., 2016). Furthermore, HESN individuals had proteome profiles characterized by increased levels of anti-proteases, which act to directly inhibit protease activity and decrease pro-inflammatory responses (Burgener, McGowan, & Klatt, 2015; Burgener et al., 2011).

1.5 Female Sex Hormones and HIV Susceptibility

Fluctuation of the levels of female sex hormones, such as estrogen and progesterone, play a key role in regulating the monthly menstrual cycle of women and in altering HIV susceptibility in women (Dupont et al., 2018; Wessels, Felker, Dupont, & Kaushic, 2018; Wira, Rodriguez-Garcia, & Patel, 2015). The menstrual cycle can be roughly divided into two phases, the proliferative/follicular phase, and the luteal/secretory phase. Estrogen levels are highest in the proliferative/follicular phase of the menstrual cycle. Estradiol (E2), the most potent form of estrogen, has the highest affinity for estrogen receptors (Files, Ko, & Pruthi, 2011), and serum levels that range from 10^{-10} M to 10^{-9} M during different phases of the menstrual cycle (Stricker et al., 2006). Progesterone levels are highest during the luteal phase of the menstrual cycle and can range from 10^{-9} M to 10^{-7} M during the menstrual cycle in the serum of reproductive age women (Stricker et al., 2006).

Collective findings from various studies suggests that estrogen plays a protective role against HIV, while progesterone may act to increase susceptibility to HIV (Dupont et al., 2018; Hel, Stringer, & Mestecky, 2010; Wessels et al., 2018). An early study done in macaques shows estrogen has a protective effect against SIV (Smith, Baskin, & Marx, 2000). In this study, the authors demonstrated that animals treated with estradiol were protected from SIV, whereas non-treated or progesterone treated animals were susceptible to SIV infection (Smith et al., 2000). Interestingly, when the virus was administered directly into the vaginal mucosa, estradiol treated animals were not protected from subsequent viral challenge (Smith et al., 2000). This suggests that the protective effect of estradiol treatment may be due to the vaginal epithelium. A follow-up study done by the same group subsequently showed that when estrogen was administered locally, in the form of a topical estriol, macaques were protected from SIV challenge (Smith et al., 2004). The authors from these studies also found that there was significant vaginal thickening and cornification when the animals were given estrogen (locally or systemically). Together these results suggest that estrogen is able to enhance the barrier function of the vaginal epithelium, thus diminishing the ability of the virus to transverse the barrier and reach target cells (Hel et al., 2010; Smith et al., 2000; Smith et al., 2004). Although these macaque studies provide solid evidence that estrogen plays a protective role against SIV, it is worth noting that these animals were all ovariectomized and therefore not naturally cycling. This was done so that the effect of sex hormones could be studied in isolation. However, a human *ex vivo*

study using cervical tissues obtained from normally cycling women confirmed these findings (Saba et al., 2013). This study shows that when challenged with R5 HIV, all productively infected cervical tissues were extracted from women in the secretory phase (progesterone high) (Saba et al., 2013). In contrast, no tissues extracted from the proliferative phase (estrogen high) were infected when challenged (Saba et al., 2013). Altogether, these studies provide solid evidence that estrogen plays a protective role against HIV infection, whereas progesterone may increase HIV susceptibility in women.

Although there is substantial evidence to demonstrate the protective effect of estrogen on HIV susceptibility, there are no well documented mechanisms for how this occurs. One mechanism that has been proposed, and which was mentioned previously, is that that is estrogen treatment leads to the thickening of the vaginal epithelium (Hel et al., 2010) as seen in macaques treated with estrogen (Smith et al., 2000; Smith et al., 2004). This suggests that estrogen may have a positive effect on the barrier function of vaginal epithelial cells. Although it was later observed that the thickness of vaginal epithelium was increased during the estrogen high phase, the increase was so small that it was most likely clinically irrelevant (Patton et al., 2000). Another proposed mechanism of estradiol-mediated protection is through enhancement of anti-inflammatory cytokines which prevents inflammation induced barrier leakage (Hel et al., 2010; Wessels et al., 2018). In one particular study, estrogen was shown to have a barrier enhancing effect on vaginal epithelial cells, as estradiol treated vaginal epithelial cells had

significantly higher transepithelial resistance (TER) compared to no hormone treated controls (Wagner & Johnson, 2012). In addition, estradiol also exerted an anti-inflammatory effect on vaginal epithelial cells, as estradiol treatment led to decreased transcription of pro-inflammatory related genes such as IL-1α, IL-1β, IL-6, IL-8, TNF-α when compared to control (Wagner & Johnson, 2012).

1.6 Medroxyprogesterone Acetate and HIV Susceptibility

Medroxyprogesterone Acetate (MPA) is an injectable progestin-based contraceptive that is widely used in the most HIV-impacted region, Sub-Saharan Africa (Hapgood, Kaushic, & Hel, 2018; Ross & Agwanda, 2012). Although MPA is a progestin-based contraceptive, several studies have suggested that it can also induce signalling through the Glucocorticoid receptor (Bamberger, Else, Bamberger, Beil, & Schulte, 1999; Govender et al., 2014). A large prospective study with over 1200 participants found that women using MPA had significantly increased HIV susceptibility compared to women who were not using any form of contraceptive (Lavreys et al., 2004). A recently published meta-analysis study examining the link between MPA and HIV susceptibility found a hazard ratio of 1.4, indicating that there is a 40% increase in HIV susceptibility when women are using MPA (Ralph, McCoy, Shiu, & Padian, 2015). Several mechanistic studies report conflicting findings regarding the effect of MPA on the production of pro-inflammatory cytokines and chemokines by epithelial cells (Hapgood et al., 2018). One study found that MPA exerted an overall anti-inflammatory effect (decreased

levels of IL-6, IL-8, and RANTES) on endocervical cells (Govender et al., 2014). A study from our lab showed similar findings, where MPA treatment of primary genital endometrial cells suppressed the production of pro-inflammatory cytokines such as IL-6, and TNF-α, but increased production of chemokines such as IP-10, and RANTES (Ferreira et al., 2015). Although cell culture studies show conflicting results, clinical studies examining human cervicovaginal lavage samples consistently detected increased levels of RANTES in women utilizing MPA (Deese et al., 2015; Fichorova et al., 2015; Morrison et al., 2014). However, more studies are required to examine how MPA treatment leads to increased RANTES levels in the CVLs of these women. In addition, women on MPA also have increased $CCR5^+$ target cells ($CD4^+$ T cells and macrophages) in vaginal tissues and cytobrush samples (Byrne et al., 2016; Chandra et al., 2013). However, it is important to note that there are conflicting reports regarding the association of MPA and the number of target cells (Mitchell et al., 2014).

In addition to increasing target cells, we have previously shown that MPA treatment of primary genital endometrial cells leads to increased transcytosis of HIV virions from the apical to the basolateral side of the cell culture system (Ferreira et al., 2015). Recently, we published a study that examined the transcriptomic changes of MPA treated primary endometrial genital epithelial cells and found that MPA treated primary endometrial genital epithelial cells upregulated pro-inflammatory and cholesterol/sterol synthesis related transcripts (Woods, Zahoor, Dizzell, Verschoor, & Kaushic, 2018). As cholesterol is required for

transcytosis, upregulation of cholesterol synthesis transcripts supports our previous finding that MPA treatment leads to increased transcytosis in these cells (Woods et al., 2018). It is worth noting that the studies discussed above were performed in cells of the UGT. In the LGT, one of the proposed mechanisms for how MPA increases susceptibility is through the thinning of the vaginal epithelium. Macaque studies indicate that MPA treatment leads to significant thinning of the vaginal epithelium (Butler et al., 2015; Butler et al., 2016). Further, a single human clinical study indicated that MPA usage leads to a slight thinning of the vaginal epithelium (Miller et al., 2000), however there are other studies which report no differences (L. Bahamondes et al., 2000; M. V. Bahamondes et al., 2014). Therefore, more studies are required to elucidate the impact of MPA on HIV susceptibility in the LGT.

1.7 Vaginal Microbiota and HIV Susceptibility

Another critical factor which can affect HIV susceptibility is the composition of the vaginal microbiota. Women with vaginal microbiota, which is not *Lactobacillus* dominated, are at higher risk of acquiring HIV through heterosexual intercourse (Petrova, van den Broek, Balzarini, Vanderleyden, & Lebeer, 2013). Women who have a vaginal microbiota which is not *Lactobacillus* dominant are also often at risk of developing bacterial vaginosis (BV) (Mirmonsef, Krass, Landay, & Spear, 2012; Petrova et al., 2013; Woodman, 2016), which is a clinical condition which results in an elevated pro-inflammatory vaginal cytokine profile

(Cauci, Driussi, Guaschino, Isola, & Quadrifoglio, 2002; St John, Mares, & Spear, 2007). Current literature indicates that BV is not caused by a singular bacterial genus/species, but by multiple genus/species of bacterial genus/species (Hickey & Forney, 2014; Onderdonk, Delaney, & Fichorova, 2016; Srinivasan, 2012). In contrast, numerous studies have demonstrated that women with a *Lactobacillus* dominant vaginal microbiota are less susceptible to HIV (Petrova et al., 2013). Therefore, there is a need to further our understanding of how Lactobacilli is exerting this protective effect.

Lactobacillus is a genus of gram positive, rod shaped bacteria. There are four primary species of *Lactobacillus* that inhabit the vaginal tract in women of reproductive age, Based on their relative dominance, these species can be separated into four different Community State Types (CST I-III,V) (Ma, Forney, & Ravel, 2012). *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus iners*, and *Lactobacillus jensenii* correspond to CSTs I,II,III and V respectively (Ma et al., 2012). A polymicrobial vaginal microbiota, or one which is not dominated by a *Lactobacillus* species and contains an abundance of strict and facultative anaerobes is classified as CST IV (Ma et al., 2012). CSTs are not static and transitions between CSTs occur within women (Petrova et al., 2013). *L. crispatus*, *L. gasseri*, and *L jensenii* dominant vaginal microbiota often transition into other CSTs which are also *Lactobacillus* dominated, whereas *L. iners* dominant vaginal microbiota often transition into a polymicrobial vaginal microbiota (CST IV) (Gajer et al., 2012).

1.7.1 Female Sex Hormones and Medroxyprogesterone Acetate Impact on *Lactobacillus*

In contrast to reproductive age women, pre-pubertal girls and post-menopausal women, often do not have a *Lactobacillus* dominant vaginal microbiota (Gupta, Kumar, Singhal, Kaur, & Manektala, 2006; Randelovic et al., 2012). This is largely attributed to a lack of estrogen, as pre-pubertal girls, and post-menopausal women have lower levels of estrogen than reproductive age women (Petrova et al., 2013). In agreement with this, estrogen treatment of post-menopausal women led to an increase in the abundance of Lactobacilli in the vaginal tract of these women (Shen et al., 2016). Although it is not fully understood how estrogen is associated with higher Lactobacilli abundance in the vaginal tract, it is hypothesized that estrogen leads to the thickening of the vaginal epithelium, which increases glycogen deposition. Glycogen can be broken down into glucose and other substrates utilized by Lactobacilli to facilitate their replication (Petrova et al., 2013). Indeed, studies have positively correlated glycogen levels in vaginal fluid with the abundance of Lactobacilli in the vaginal tract (Mirmonsef et al., 2014). Interestingly a study published by Spear et al, demonstrated that different *Lactobacillus spp* (including *L. gasseri* and *L. jensenii*) were not able to grow when glycogen was the only source of carbohydrates (Spear et al., 2014). However, these *Lactobacillus spp* were able to grow in the presence of glucose, a breakdown product of glycogen (Spear et al., 2014). This indicates that vaginal *L. gasseri* and *L. jensenii* do not have the capability to break down glycogen into useable

substrates. However, Lactobacilli were able to grow in broth containing glycogen as the sole food source when salivary α-amylase was added (Spear et al., 2014). Importantly, this study also showed that there is an amylase like enzyme present in the genital fluid of women which could degrade glycogen (Spear et al., 2014). However, the source of this a-amylase within the LGT is still unknown (Spear et al., 2014).

Numerous studies suggest that MPA usage in women may cause a decline in Lactobacilli populations in the vaginal tract (Mitchell et al., 2014). In agreement with this, our group has demonstrated that MPA usage is associated with a decrease in the abundance of Lactobacilli in the vaginal tract, as well as decreased glycogen and amylase levels (Wessels et al, 2018). A plausible explanation for why this occurs is that MPA usage in women leads to hypoestrogenism (Hapgood et al., 2018). As a result of low estrogen levels, the vaginal barrier thins, and glycogen levels may be reduced (Miller et al., 2000); however, more studies will need to be done to verify if this is true.

1.7.2 Protective Mechanisms of Endogenous *Lactobacillus* Against HIV

As mentioned above, a *Lactobacillus* dominant vaginal microbiota is more protective against HIV when compared to a polymicrobial microbiota. One of the well studied mechanisms for how Lactobacilli protects against HIV is through the production of lactic acid. Lactobacilli produce lactic acid which lowers the surrounding area to a pH ~4 (Ma et al., 2012; Petrova et al., 2013). Multiple studies

have shown that lactic acid is able to directly inactivate HIV virions (Aldunate et al., 2013; Tyssen et al., 2018). In addition, cervicovaginal mucus, which is acidified by lactic acid was shown to trap HIV virions more efficiently than mucus acidified by hydrochloric acid (Lai et al., 2009). In addition, hydrogen peroxide (H_2O_2) produced by some Lactobacilli was also suggested to play a role in inactivating HIV (Klebanoff & Coombs, 1991). It is however worth mentioning that there have been no follow-up studies regarding the potential potency of H_2O_2 against HIV (Petrova et al, 2013). Another speculated mechanism involve lectin molecules expressed on Lactobacilli, which can sequester the HIV virion and prevent it from reaching underlying target cells (Malik et al., 2016).

In addition to the direct mechanisms discussed above, multiple studies have also shown that Lactobacilli can modulate innate immune responses in epithelial cells. As discussed previously, the inflammatory response can affect HIV infection in the FRT. Recently, a published study showed that women with a *Lactobacillus* dominant vaginal microbiota had lower levels of inflammatory cytokines and decreased activated $CD4^+$ T cells when compared to women with a polymicrobial vaginal microbiota (Gosmann et al., 2017). In addition, other studies support the notion that the composition of the vaginal microbiota influences FGT inflammation, as women with *Lactobacillus* dominant vaginal microbiota have lower levels of pro-inflammatory cytokines compared to women with vaginal microbiota which is not Lactobacilli dominant (Anahtar et al., 2015). These studies show that endogenous Lactobacilli play a significant role in modulating the innate immune response of

epithelial cells and can exert an anti-inflammatory response on these cells, thus, protecting the site from HIV infection. However, the mechanism of how endogenous Lactobacilli performs this role is still unknown. Therefore, there is a need to further our understanding of how endogenous Lactobacilli are able to modulate the innate immune response of vaginal epithelial cells.

1.7.3 In Vitro Vaginal Lactobacillus Studies

There are several *in vitro* studies which examine the immunomodulatory effect of Lactobacilli on vaginal epithelial cells. In one study of interest, the authors show that the area where the Lactobacilli strain was isolated affects its immunomodulatory effects (Rose et al., 2012). In particular, a clinical vaginal isolate of *Lactobacillus jensenii* proved to exert the greatest anti-inflammatory on vaginal cells when stimulated with TLR agonists compared to other ATCC type strains (*Lactobacillus crispatus* (ATCC 33820), *Lactobacillus jensenii* (ATCC 25258)) (Rose et al., 2012). Specifically, the more recently isolated, low passage clinical isolate of Lactobacilli was most effective at reducing the production of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α in vaginal epithelial cell cultures stimulated with TLR agonists (FSL-1, PIC) compared to other ATCC strains. Although several studies show that *Lactobacillus casei* has a positive immunomodulatory effect on intestinal epithelial cells (Eun et al., 2011; Tien et al., 2006), Rose et al demonstrate *Lactobacillus casei* did not have any immunomodulatory effect on vaginal epithelial cells (Rose et al., 2012). This is

particularly informative, as there are numerous studies which outline the immunomodulatory effects of *Lactobacillus casei* on gut epithelial cells and its benefits against various gut pathogens (Aguero, Villena, Racedo, Haro, & Alvarez, 2006; Tiittanen, 2013; Vinderola, Matar, & Perdigon, 2005). Another study conducted by Libby et al also yielded similar findings, where they demonstrate that the addition of Lactobacilli to vaginal cells was able to reduce the pro-inflammatory response after stimulation with several TLR agonists (Libby, Pascal, Mordechai, Adelson, & Trama, 2008). Overall, findings from these studies indicate that vaginal Lactobacilli can reduce the pro-inflammatory response of vaginal epithelial cells when they are exposed to immunological insults, such as TLR agonists. Findings from the study conducted by Rose *et al* suggest that possibly a more recent, and low passage isolate may have enhanced immunomodulatory effects compared to ATCC type strains. In addition, although certain Lactobacilli strains such as *Lactobacillus casei* have immunomodulatory effects in the intestinal epithelial system, (Eun et al., 2011; Tien et al., 2006) these strains may not have any effect on vaginal epithelial cells (Rose et al ., 2013).

There are also studies that examine the effect of Lactobacilli on vaginal epithelial cells without any TLR stimulation. One study aimed to examine the effects of different Lactobacilli and bacteria associated with bacterial vaginosis on a 3D vaginal epithelial cell model (Doerflinger, Throop, & Herbst-Kralovetz, 2014). Results from this study indicate that the addition of Lactobacilli onto a 3D vaginal epithelial cell model did not increase production of pro-inflammatory cytokines

such as IL-6, IL-8, and TNF-α, while the addition of bacteria associated with bacterial vaginosis did, compared to control (non-treated) (Doerflinger et al., 2014). Another study conducted by Fichorova et al yielded similar results, where the addition of Lactobacilli onto vaginal epithelial cells did not elevate the production of pro-inflammatory cytokines/chemokines (IL-6, IL-8, RANTES) compared to untreated cells (Fichorova, Yamamoto, Delaney, Onderdonk, & Doncel, 2011). Further, Fichorova et al also found that the addition of bacterial vaginosis associated bacteria onto vaginal epithelial cells led to increased pro-inflammatory production when compared to Lactobacilli treated or non-treated cells (Doerflinger et al., 2014; Fichorova et al., 2011). Overall, these studies suggest that the addition of Lactobacilli does not alter the production of pro-inflammatory cytokines in vaginal epithelial cells when compared to control, whereas the addition of bacteria associated with bacterial vaginosis increases the production of pro-inflammatory cytokines compared to control.

While there are multiple studies examining the immunomodulatory effects of endogenous vaginal Lactobacilli on vaginal epithelial cells, most of them have several major limitations. For instance, a study conducted by Rizzo et al aimed to examine the effects of *Lactobacillus crispatus* (ATCC 33820) on the epithelial cell responses to *Candida albicans*. However, the authors of this study utilized HeLa cells, a cancer cell line of cervical origin, rather than cells of vaginal origin (Rizzo, Losacco, & Carratelli, 2013). In addition, many of these studies utilized *Lactobacillus crispatus* (ATCC 33820) as it is one of the four major Lactobacilli

species found in the vaginal tract (Niu, Li, Zhang, Wang, & Liu, 2017; Rizzo et al., 2013). However, this particular strain of Lactobacilli (*Lactobacillus crispatus* (ATCC 33820)) was isolated from the eye, and not the vaginal tract (DSMZ, 2018). As previously discussed, results from a study conducted by Rose and colleagues suggest that the origin of Lactobacilli is important when examining its effects on vaginal epithelial cells (Rose et al., 2012). In conclusion, although there are multiple studies which aim to examine the effect of endogenous Lactobacilli on vaginal epithelial cells, there are few which utilize Lactobacilli isolated from the vaginal tract, and vaginal epithelial cells.

1.7.4 Probiotic Vaginal *Lactobacillus* Studies

There are also several studies that examine the immunomodulatory effect of probiotic vaginal Lactobacilli on vaginal epithelial cells. Probiotics are live microorganisms that are capable of conferring health benefits when administered in appropriate doses (Sanders, 2008). Probiotic vaginal Lactobacilli differ from endogenous vaginal Lactobacilli as they do not represent major constituents of the vaginal microbiota, however, they have been found to confer certain beneficial effects discussed below. There are two vaginal probiotics (*Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14), which have been studied extensively by Dr. Gregor Reid's group (Western University) for their ability to interfere with the colonization of sexually transmitted pathogens such as *Candida*

spp. (Malik et al., 2016; R. C. Martinez et al., 2009; Reid et al., 2003). Several studies also demonstrate that these two vaginal probiotic strains have the capacity to modulate vaginal epithelial cell immune responses (R. C. R. Martinez et al., 2009; Wagner & Johnson, 2012). Findings from the study conducted by Wagner and Johnson demonstrated that a combination of probiotic Lactobacilli (GR-1 + RC-14) was able to decrease transcription of pro-inflammatory associated genes, such as TLR2, TLR6, and TNF-α in vaginal epithelial cells (Vk2) (Wagner & Johnson, 2012). Treatment with the same probiotics also led to an increase in IL-1α production in Vk2 cells (Wagner & Johnson, 2012). This may indicate that these probiotic bacteria may induce a pro-inflammatory response in the form of IL-1α. In addition, Martinez et al show that treatment with GR-1, and RC-14 led to a significant increase in IL-8 production in Vk2 cells (R. C. R. Martinez et al., 2009). In addition, these studies also show that these two probiotics (GR-1, RC-14) are able to reduce the colonization of *Candida spp*. by inhibiting their growth in vaginal epithelial cells. Although these studies show that vaginal probiotics are able to exert an immunomodulatory effect on vaginal epithelial cells, it is not clear whether this effect is pro-inflammatory or anti-inflammatory due to conflicting results. More studies are needed to elucidate the immunomodulatory effects of these probiotics on vaginal epithelial cells.

1.7.5 *Lactobacillus* Impact on Barrier Function

In addition to their immunomodulatory effects, Lactobacilli are also hypothesized to enhance barrier functions of the vaginal epithelium (Macklaim et al., 2012; Petrova et al., 2013). Studies which examine the barrier function of vaginal epithelial cells often correlate pro-inflammatory cytokine profiles, particularly expression of TNF-α to diminished barrier function of vaginal epithelial cells (Doerflinger et al., 2014). One study from our lab, for instance, showed a direct correlation between increased TNF-α levels and impaired barrier function as assessed by tight junction associated proteins, paracellular leakage, and HIV translocation in primary genital endometrial cells (Nazli et al., 2010).

The majority of literature suggesting that *Lactobacillus spp.* can positively impact the barrier function of epithelial cells are from studies performed in the intestinal tract. For instance, *in vivo* mouse studies of inflammatory bowel disease show that administration of probiotic Lactobacilli is able to attenuate the effect of dextran sulfate sodium induced colitis (Castagliuolo et al., 2005; Osman, Adawi, Ahrne, Jeppsson, & Molin, 2004). In one study, oral administration of probiotic *Lactobacillus plantarum* to human volunteers led to an increase in the co-localization of tight junction associated proteins ZO-1 and occludin in duodenal samples, indicating increased barrier function. In addition, *in vitro* functional studies show that *Lactobacillus plantarum* treated Caco-2 cells (an intestinal cell line) had increased transepithelial resistance (TER) and decreased paracellular

leakage compared to control, indicating enhanced barrier function of intestinal epithelial cells (Karczewski et al., 2010). A similar study found that *Lactobacillus plantarum* treatment of Caco-2 cells led to increased TER, and increased transcription and translation of tight junction associated proteins (R. C. Anderson et al., 2010). In addition, previous experiments conducted in our lab indicate that probiotic *Lactobacillus rhamnosus* (GR-1), and *Lactobacillus reuteri* (RC-14) enhanced barrier functions in primary endometrial genital epithelial cell cultures (Dizzell et al, unpublished). Overall, there are numerous studies which demonstrate the positive impact of probiotic Lactobacilli on the barrier function of intestinal epithelial cells. However, similar studies in vaginal cells are lacking. More studies are required to determine the role of endogenous and probiotic vaginal Lactobacilli in enhancing the vaginal barrier function.

1.8 Vaginal Epithelial Cell Experimental Models

Many *in vitro* studies of vaginal physiology utilize a vaginal epithelial cell line (Vk2/E6E7) (Hearps et al., 2017; Niu et al., 2017; Wagner & Johnson, 2012). Vk2/E6E7 cells were isolated from fresh, discarded vaginal tissues (Fichorova, Rheinwald, & Anderson, 1997; Hearps et al., 2017) and then immortalized using a retroviral vector containing the E6E7 antigen. (Fichorova et al., 1997). The selective expression of different cytokeratins in the Vk2 cell line is similar to normal vaginal tissue (Fichorova et al., 1997). Thus, creation of this cell line provided

researchers with a cell line that is more representative of the vaginal tract than HeLa cells, which were previously utilized for vaginal studies. As previously mentioned, the vagina is defined by multi-layered squamous epithelium. However, many studies utilizing Vk2 cells grow these cells in a monolayer, which does not accurately reflect physiological conditions (Hearps et al., 2017; Niu et al., 2017; Wagner & Johnson, 2012). Those studies which do utilize multi-layered vaginal cells, either use high-cost (3D) systems (Fichorova et al., 2011), or cell lines that are not commercially available (Rose et al., 2012). Therefore, our lab recently developed a method to culture the commercially available Vk2 cell line in a multi-layered setting (Lee et al., 2016). We demonstrated that by growing these cells in an Air-Liquid interface (ALI) culture, the Vk2 cells are able to differentiate into multiple layers (Lee et al., 2016). In addition, we showed that there is a differential effect of estradiol and progesterone on the cytokeratin expression of ALI grown Vk2 cells, mimicking the cytoskeletal changes during the different stages of the menstrual cycle (Lee et al., 2016). This further supports that this system represents physiological conditions more accurately than Vk2 cells grown in a monolayer system.

CHAPTER 2: RATIONALE AND HYPOTHESIS

In a healthy female reproductive tract, it has been shown that *Lactobacillus* is an important constituent of the human vaginal microbiome (Petrova et al., 2013). There is clear evidence which outlines the importance of the protective effects that endogenous vaginal *Lactobacillus* exert against HIV in the female reproductive tract (Gosmann et al., 2017; Petrova et al., 2013; Wessels et al., 2018). *Lactobacillus* has been thought to play a barrier enhancing role in the vaginal tract, although this has not been definitively proven. Most of this evidence stems from studies conducted in the intestinal tract (Karczewski et al., 2010). However, we recently found observed that probiotic vaginal Lactobacilli were able to enhance the barrier function of primary endometrial genital epithelial cells (Dizzell et al, unpublished). Whether *Lactobacillus* is able to enhance the barrier function of vaginal epithelial cells has not been examined. In addition, *Lactobacillus* has also been shown in multiple studies to exert an anti-inflammatory effect in vaginal epithelial cells (R. C. Anderson et al., 2010; Gosmann et al., 2017; Rose et al., 2012). Similarly, estrogen has been shown to exert an anti-inflammatory effect, and enhance barrier function of vaginal epithelial cells (Wagner & Johnson, 2012). In contrast, MPA has been associated with increased HIV susceptibility in women, as indicated in a meta-analysis study (Ralph et al., 2015), and many other studies (Hapgood et al., 2018). There are however, limited studies which examine the

direct effect of physiological levels of MPA on vaginal epithelial cells (Africander, Verhoog, & Hapgood, 2011; Irvin & Herold, 2015) .

I therefore hypothesize that endogenous vaginal Lactobacillus, and estrogen are able to improve the barrier function, and lower the pro-inflammatory response of vaginal epithelial cells. In contrast, MPA will decrease the barrier function, and increase the pro-inflammatory response of vaginal epithelial cells.

To test this hypothesis the following aims will be addressed

Aim 1: Establishing the co-culture model of Vk2/E6E7 cells and different *Lactobacillus spp.*

Aim 2: Determine the effect of *Lactobacillus spp*, and hormones on Vk2 barrier function and cytokine production

Aim 3: Identifying of the effect of different Lactobacilli species on the transcriptome of vaginal epithelial cells

CHAPTER 3: RESULTS

3.1 Aim 1: Establishing a co-culture model of Vk2/E6E7 cells grown in ALI culture and *Lactobacillus spp.*

To fully explore our hypothesis, we first needed to establish an *in vitro* system to examine the effects of female sex hormones (E2, P4), contraceptives (MPA), and Lactobacilli on the growth and viability of vaginal epithelial cells (Vk2/E6E7). As mentioned, we have previously adapted an *in vitro* air-liquid interface (ALI) cell culture system for vaginal epithelial cells that resembles physiological conditions of the vaginal tract better than a classical liquid-liquid interface system (Lee et al., 2016). Moving forward, we chose to expand this ALI culture system to examine the effects of Lactobacilli, with and without hormones, on vaginal epithelial cells. We first established an experimental timeline for the culture system to determine the optimal time (culture days) for further experiments.

Vk2 cells were grown in ALI culture conditions and the barrier integrity of each Vk2 cell culture was monitored over time (days of culture) by measuring transepithelial resistance (TER). Viability was assessed by trypan blue staining. The TER of Vk2 cells in each cell culture insert (n=8) reached a maximum value on culture day 9 (Figure 1A). Vk2 cell viability showed a similar trend with significant decreases between culture days 10 and 11 in ALI culture compared to culture day 8 and 9 (Figure 1B). Data shown represents one experiment done in 8

replicates. Thus, the data suggested that any future experiments should be performed prior to day 9, as cell viability and cell integrity decline after this time.

A previous study showed that immunomodulatory effects of Lactobacilli can differ based on the time, and the location of isolation (Rose et al., 2012). *Lactobacillus casei* isolated from yogurt was shown to have no immunomodulatory effect on vaginal epithelial cells, whereas other vaginal Lactobacilli exerted an anti-inflammatory effect (Rose et al., 2012). In addition, the time of isolation is also of importance, as it was shown that a low passage, vaginally isolated *Lactobacillus jensenii* strain had the largest immunomodulatory effect on vaginal epithelial cells compared to other ATCC Lactobacilli strains (Rose et al., 2012). Therefore, for our study, we utilized four different strains of Lactobacilli to compare their effects. These Lactobacilli strains are *Lactobacillus rhamnosus* (GR-1), *Lactobacillus crispatus* (ATCC 33820) *Lactobacillus jensenii* (ATCC 25258), and *Lactobacillus crispatus* SJ-3C-US. *Lactobacillus rhamnosus* (GR-1) is a vaginal probiotic, which has been studied extensively for its ability to modulate cytokine expression of vaginal epithelial cells and its ability to reduce *Candida albicans* colonization. The two strains of *Lactobacillus crispatus* we selected for our experiments were isolated from different locations; *Lactobacillus crispatus* (ATCC 338200) was isolated from the eye whereas *Lactobacillus crispatus* (SJ-3C-US) was isolated from the vaginal tract. *Lactobacillus jensenii* was also of vaginal origin. For other studies examining the effects of Lactobacilli on vaginal epithelial cells, researchers utilized vaginal epithelial cell to Lactobacilli ratios of 1:1 to 1:1000 and co-cultured

Lactobacilli with vaginal cells anywhere from 6 to 48 hours (R. C. R. Martinez et al., 2009; Rose et al., 2012). In one particular study, the authors found that a ratio of 1:10 led to saturation of bacterial adhesion (Donnarumma et al., 2014). Therefore, moving forward, we utilized ratios of 1:1 (6×10^5 CFU/mL of Lactobacilli), and 1:10 (6×10^6 CFU/mL of Lactobacilli) in our studies. Subsequently, we chose a culture time of 24 hours, as this was often the midpoint of what was observed in literature. Since bacteria produce different bacterial products during their various growth phases, most experimental studies are performed using log phase bacteria (Gefen, Fridman, Ronin, & Balaban, 2014). To utilize log phase bacteria for our studies, we required a method to accurately enumerate the bacterial concentration of Lactobacilli when they are replicating in De Man, Rogosa and Sharpe (MRS) broth. Therefore, optical density and respective bacterial count (CFU/mL) were correlated using a linear equation (Figure 2). In this manner, the bacterial concentration of Lactobacilli in MRS broth could be accurately quantified, and the appropriate amount could be extracted for subsequent studies.

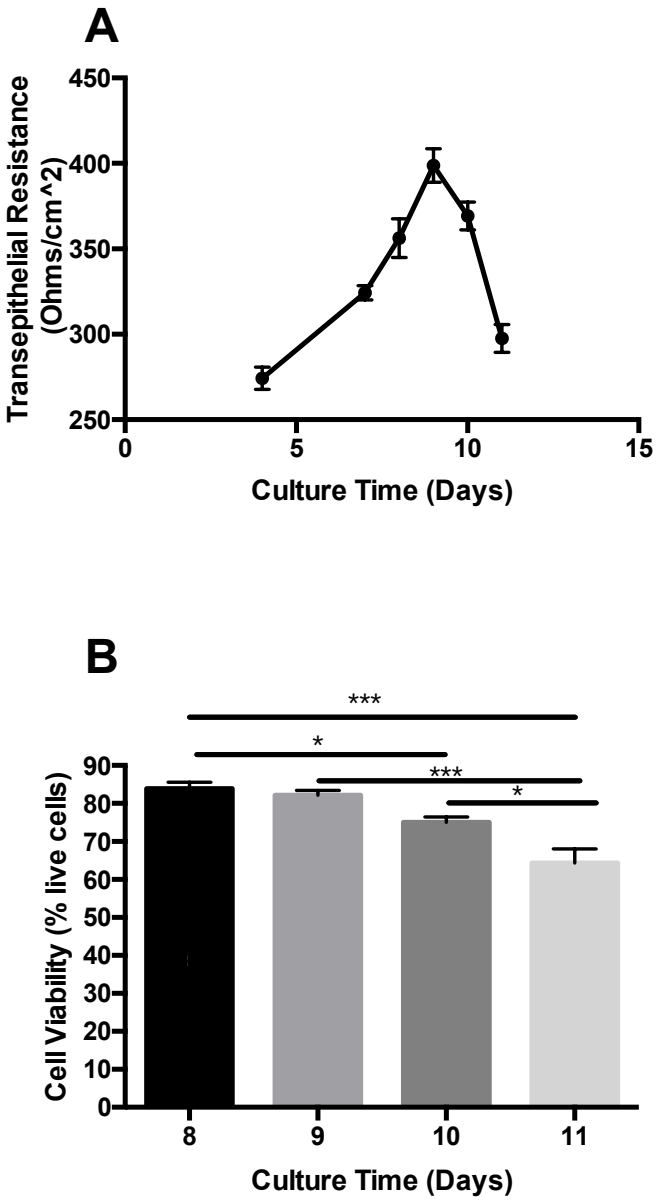

Figure 1: Maximal barrier function of Vk2 cells grown in ALI Culture is reached at culture day 9. Vk2 cells were grown in ALI culture as described in Materials and Methods. **(A)** TER measurements were taken for eight separate well inserts from one experiment on different culture days. (B) Cell viability was assessed using trypan blue viability assay on different days of culture. Viability data is representative of one experiment performed with four 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, ***p<0.001. Error bars represent SEM.

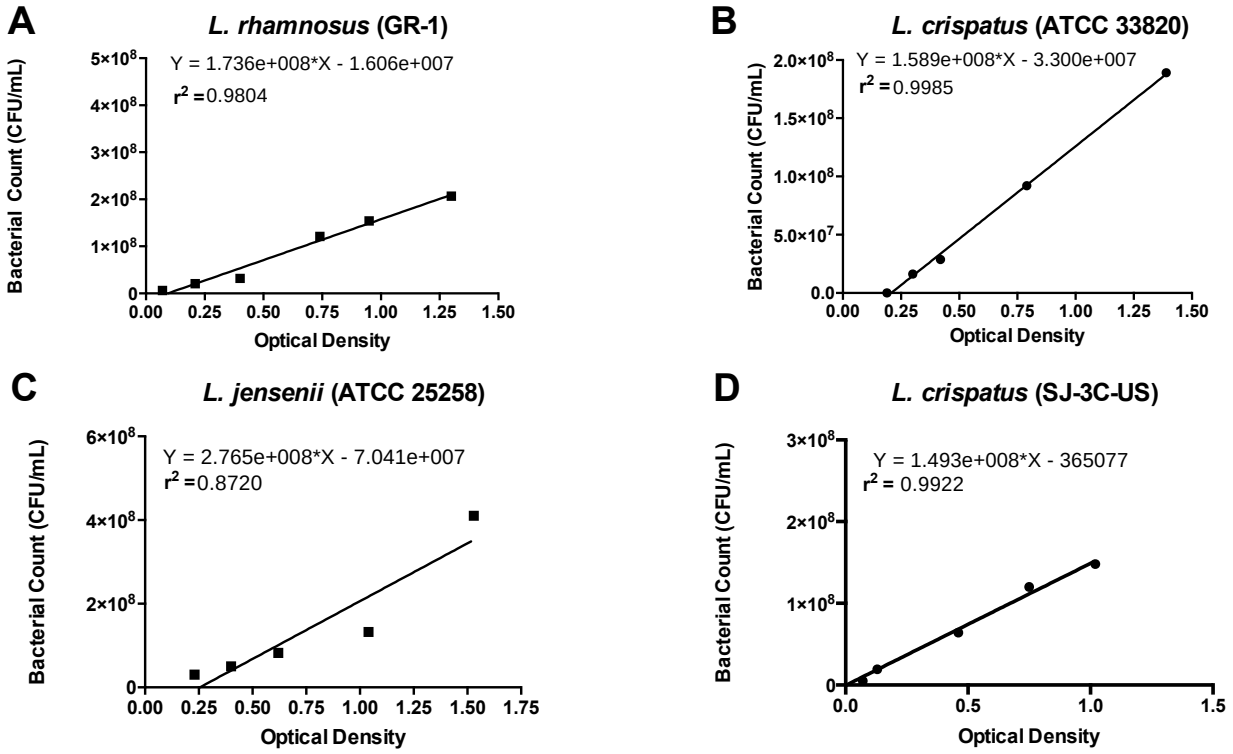

Figure 2: Linear equations of bacterial count (CFU/mL) versus optical density. Different Lactobacilli spp. were inoculated in MRS broth. Optical density measurements and bacterial count were quantified and plotted to fit a line. Linear equation of bacterial count versus absorbance corresponding to GR-1 **(A)** LC (ATCC 33820) **(B)** LJ (ATCC 25258) **(C)** and LC SJ-3C-US (**D)**. The coefficient of determination, r^2 is shown below the linear equation. Data shown represents one experiment.

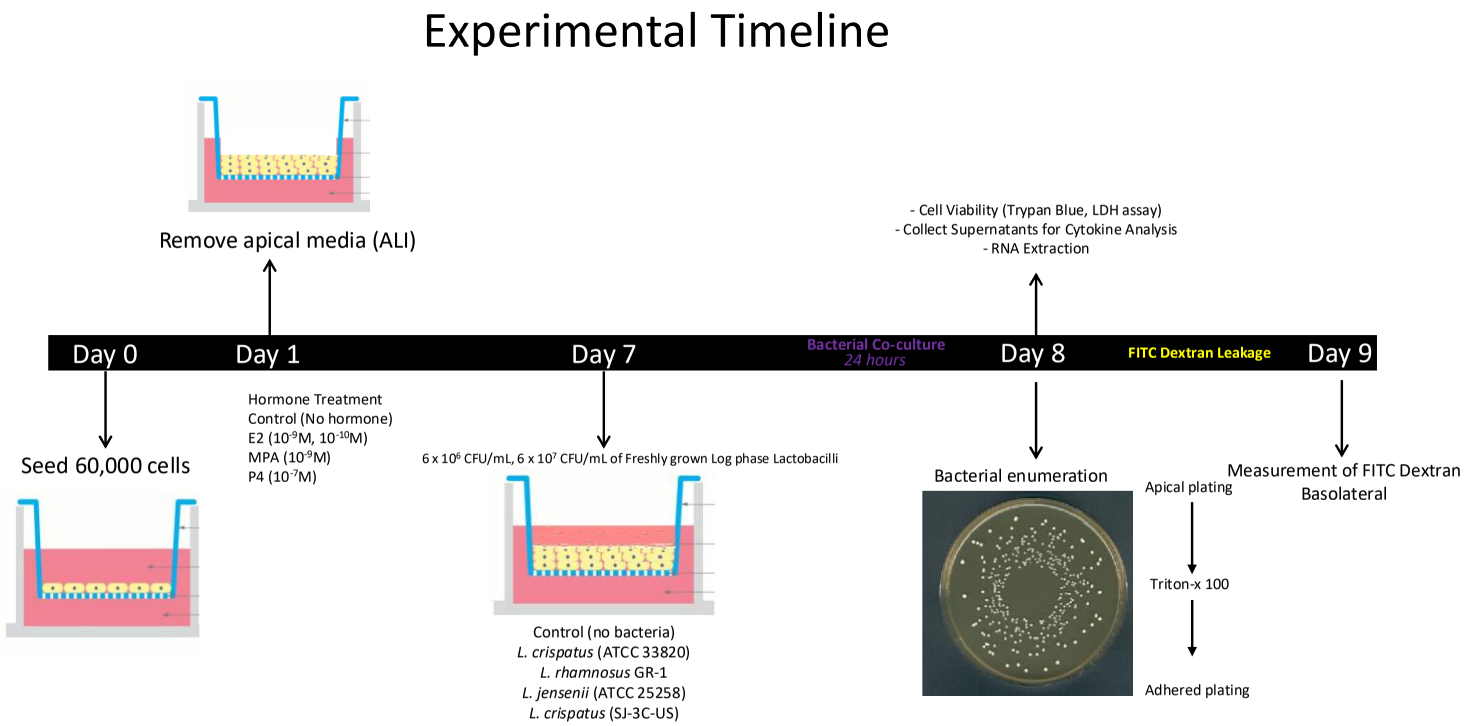

Figure 3: Experimental Timeline of Vk2/Lactobacilli co-culture experiments. 60,000 Vk2 cells were seeded into transwells on day 0. On day 1, apical media was removed and basolateral media was replaced with media containing hormones (E2, P4, MPA, no hormone). On day 7 of culture, 6 x 10^5 CFU of log phase Lactobacilli was added to the apical side of Vk2 cultures. After 24 hours, bacteria were enumerated through serial dilution, and plating. Vk2 culture supernatants were collected for cytokine analysis. Cell viability was assessed. FITC-Dextran dye was added on day 8 for 24 hours. After 24 hours, FITC-Dextran was measured in the basolateral media to determine cell permeability. RNA extraction was done for transcriptomics analysis.

To examine the effects of hormones and hormonal contraceptives on vaginal epithelial cells we used the following treatments: E2 (10^{-9} M, 10^{-10} M), P4 (10^{-7} M), MPA (10^{-9} M). As previously mentioned, estrogen is associated with protection against HIV. Estradiol concentrations in serum range from 10^{-10} M to 10^{-9} M during different phases of the menstrual cycle in reproductive age women (Stricker et al., 2006). Therefore, two estradiol concentrations, 10^{-10} M and 10^{-9} M, were utilized to compare the effects of high and low physiological concentrations on vaginal epithelial cells. In contrast to estradiol, the progesterone high phase (luteal or secretory phase) of the menstrual cycle is associated with increased susceptibility to HIV in *in vivo* macaque and *ex vivo* human studies. Therefore, to evaluate the effects of progesterone on Vk2 cells, a higher physiological concentration of progesterone was utilized (10^{-7}M) (Stricker et al., 2006). The mean MPA level in the serum 60 days after sub-cutaneous injection of Depo-SubQ Provera 104™ was found to be approximately 10^{-9} M (Mishell, 1996). Therefore, we used an MPA concentration of 10^{-9} M moving forward.

3.1.1 Assessment of Cell Viability of Vk2 cells in co-culture with different Lactobacilli with and without hormones

Cell viability of ALI Vk2 cultures was examined by trypan blue exclusion assay when they were grown in the presence or absence of various hormones (E2,P4,MPA). In the trypan blue exclusion assay, cells which are viable will have

an intact cell membrane, and thus will not be stained by the trypan blue dye. On the contrary, cells which are not viable do not have an intact cell membrane, and will be stained (Strober, 2001). As seen in Figure 4, there were no significant differences observed in cell viability between Vk2 cells grown in hormones compared to control (no hormones). Next, we assessed cell viability of Vk2 cells co-cultured with 6 x 10^6 CFU/mL Lactobacilli spp. for 24 hours. As seen in Figure 4B, there was no impact on cell viability with Lactobacilli when compared to no bacteria controls. Finally, the combined effects of hormones and Lactobacilli on cell viability was examined. No significant differences were observed between any treatment groups when compared to no bacteria, no hormone control (Figure 4C). Figure 4 represents data collected from one single experiment, with two/three experimental replicates.

To confirm that Vk2 cell viability is not negatively affected by hormones and/or Lactobacilli, Lactate Dehydrogenase (LDH) cell viability assays were performed on all experimental conditions to verify the results of the trypan blue exclusion assay (Figure 4). This assay is based on the principle that non-viable cells will release their intracellular contents due semi permeable cell membrane which is not intact (Kumar, Nagarajan, & Uchil, 2018). By quantifying of LDH enzymatic activity in the cell supernatant, cell viability of the culture can be determined (Kumar, Nagarajan, & Uchil, 2018). In this assay, a positive control was utilized to determine the LDH activity following 100% lysis in one Vk2 cell culture transwell. LDH activity of different treatments were not significantly different

from and were extremely minimal compared to the 100% lysis control (Figure 5A). The LDH activity of each treatment was normalized by first converting values to cell viability percentage and then dividing by the LDH activity of the 100% lysis control (Figure 5B). Overall, Vk2 cell viability was not negatively affected by the different treatments. Data from Figure 5 represents data collected from one single experiment, with three experimental replicates. Collectively, data from cell viability experiments indicated that Vk2 cell viability was not negatively affected when co-cultured with different Lactobacilli species (GR-1, LC (ATCC 33820), LJ, LC (SJ-3C-US)) in the presence or absence of different hormones.

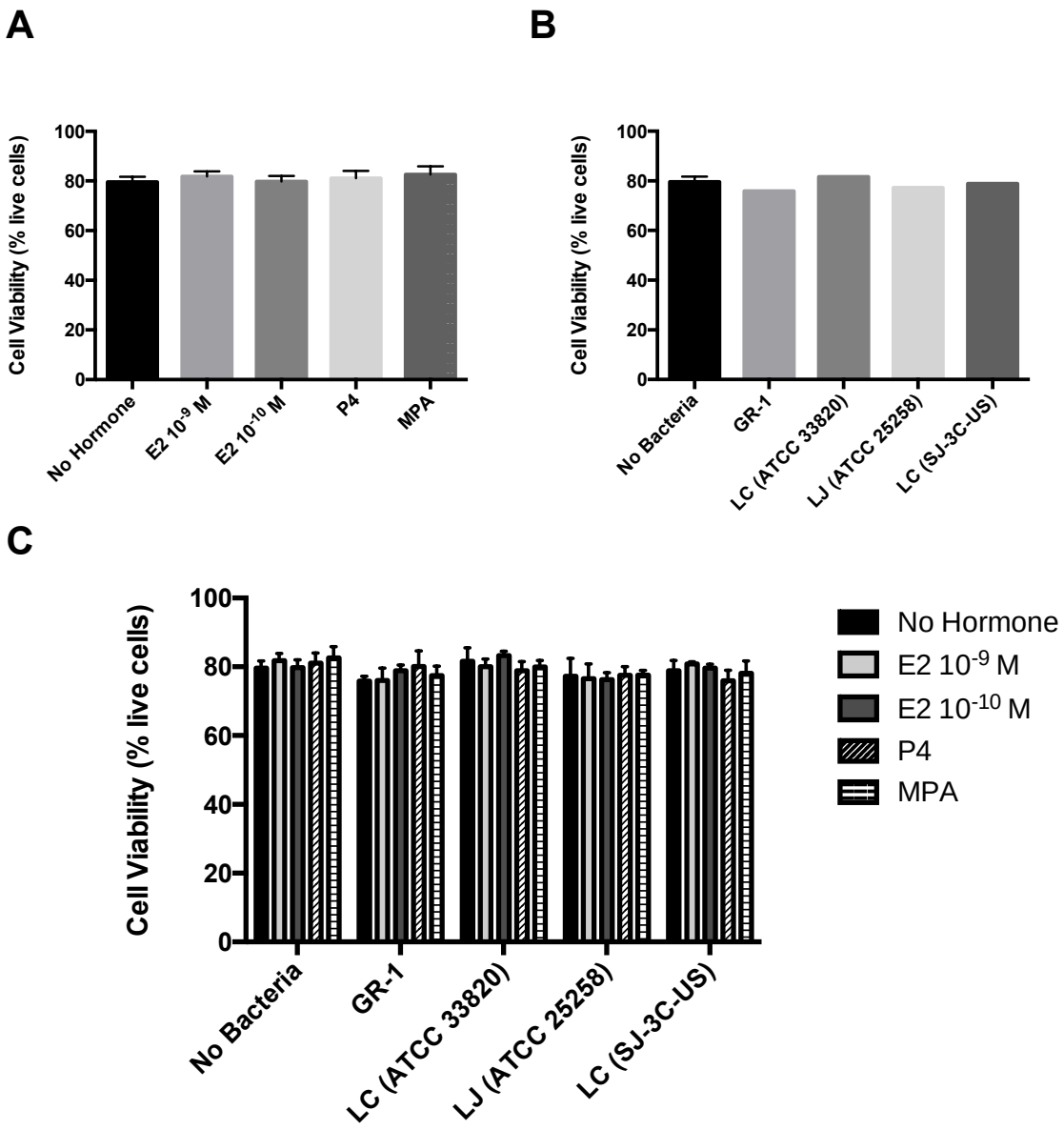

Figure 4: Cell viability of Vk2 cells are not affected by hormones, Lactobacilli or their combination (Trypan Blue). **(A)** Cell viability was assessed by trypan blue exclusion assay in the presence of different hormones. Data shown represents one experiment done in duplicates **(B)** Cell viability of Vk2 cells co-cultured with different Lactobacilli assessed by trypan blue exclusion assay. Data shown represents one experiment done in triplicates. **(C)** Cell viability of Vk2 cells co-cultured with different Lactobacilli, and/or grown in the different hormones was assessed by trypan blue viability assay. Data shown represents one experiment, hormone only conditions were done in duplicates, all others were done in triplicates. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. Error bars shown represent SEM.

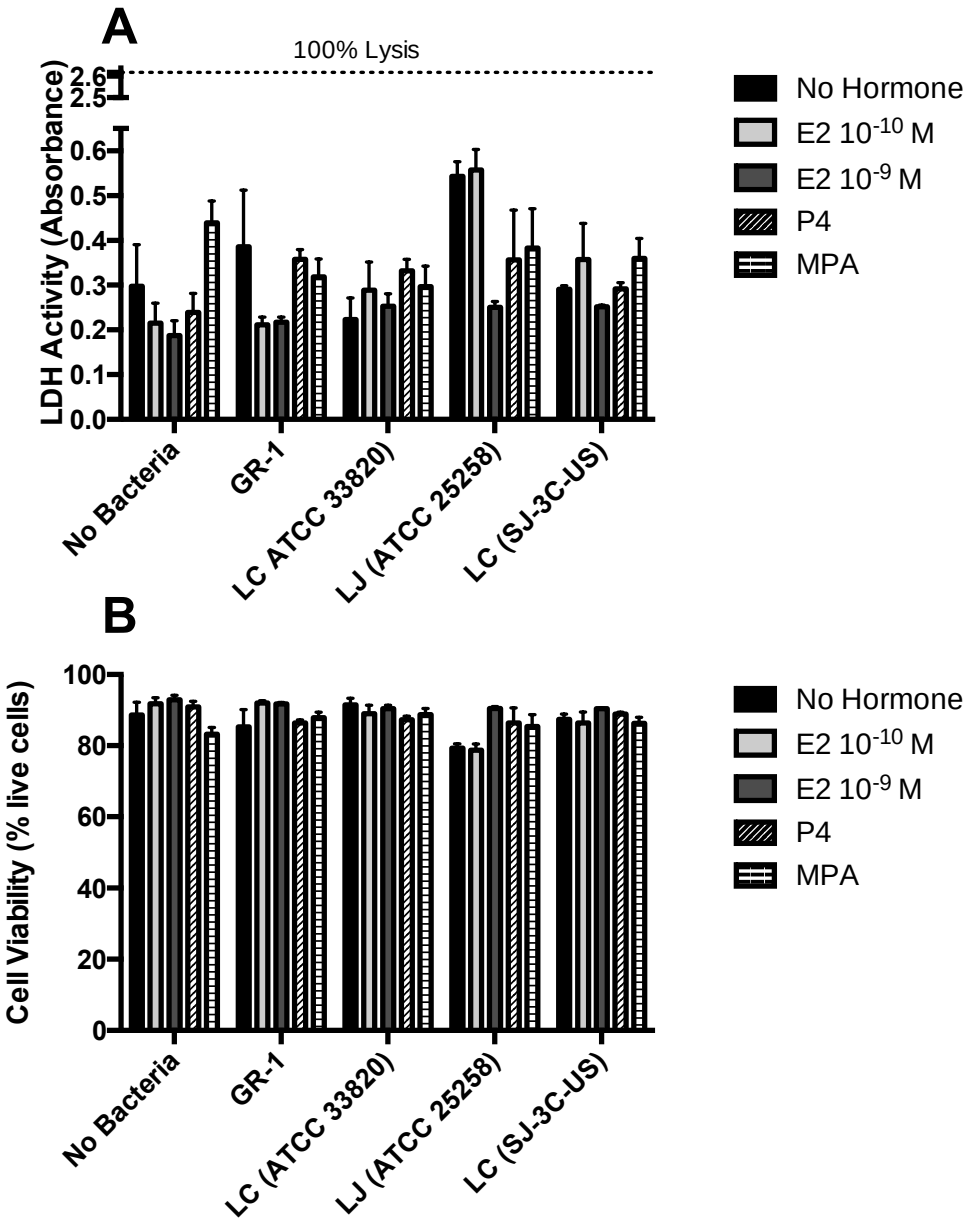

Figure 5: Cell viability of Vk2 cells is not affected by hormones, Lactobacilli or their combination (Lactate dehydrogenase assay) **(A)** Cell viability of Vk2 cells co-cultured with different Lactobacilli and/or grown in hormones as assessed by LDH assay, dotted line represents LDH activity of 100% lysis control of one single Vk2 cell transwell. **(B)** Cell Viability of LDH assay expressed as cell viability. LDH activity of different conditions were divided by the LDH activity of the positive lysis control to yield a percentage. Data shown represents one experiment done in triplicates. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. Error bars shown represent SEM.

3.1.2 Assessment of the Growth and Adherence of Lactobacilli in Co-culture with Vk2 cells under Different Hormonal Conditions

The growth of different Lactobacilli species was examined when bacteria were resuspended in keratinocyte serum free media (KSFM), the media in which Vk2 cells are grown. Starting with bacterial concentrations of 6 x 10^6 CFU/mL and 6 x 10^5 CFU/mL, the bacterial enumeration was done at 0 (prior to addition), 4, 8, 24, and 48 hours in KSFM (Figure 6). Probiotic GR-1 bacterial count was significantly higher at 4, 8, 24 and 48 hours compared to bacterial count prior to addition (0 hours), for both 6 x 10^5 CFU/mL and 6 x 10^6 CFU/mL (Figure 6A). When examining *L. crispatus* (ATCC 33820) isolated from the eye, bacterial counts had slight discrepancies in the results depending on the initial bacterial concentration (Figure 6B). With an initial concentration of 6 x 10^6 CFU/mL, bacterial count was significantly increased at 4, and 8 hours while, in contrast, bacterial count was significantly decreased at 8 hours if 6 x 10^5 CFU/mL of bacteria was added (Figure 6B). At 24 hours, both starting bacterial concentrations follow the same pattern, where the bacterial counts drop significantly when compared to the bacterial count prior to addition (Figure 5B). Although bacterial count did continue to decrease at 48 hours for the starting bacterial concentration of (6 x 10^6 CFU/mL), it did not decrease much compared to the bacterial count at 24 hours (Figure 6B). However, the starting bacterial concentration of 6 x 10^5 CFU/mL at 48 hours was non-detectable (below 10^2 CFU/mL).

As seen in Figure 5C, *L. jensenii* (ATCC 25258) bacterial count was significantly increased at 8 hours compared to 0 (6 x 10^6 CFU/mL). At 24 hours, there was no significant differences in bacterial count compared to time 0 for both starting bacterial concentrations, but at 48 hours, bacterial counts were significantly decreased (Figure 6C). Finally, *L. crispatus* (SJ-3C-US), isolated from the vaginal tract had a significant decrease in bacterial count at 24 hours for the higher starting concentration (6 x 10^6 CFU/mL) and a similar trend was seen for the lower starting concentration (6 x 10^5 CFU/mL) (Figure 6D). After 48 hours in KSFM, bacterial count for both starting bacterial concentrations was significantly decreased (Figure 6D). Data shown in Figure 5 represents three separate experiments, each with three replicates.

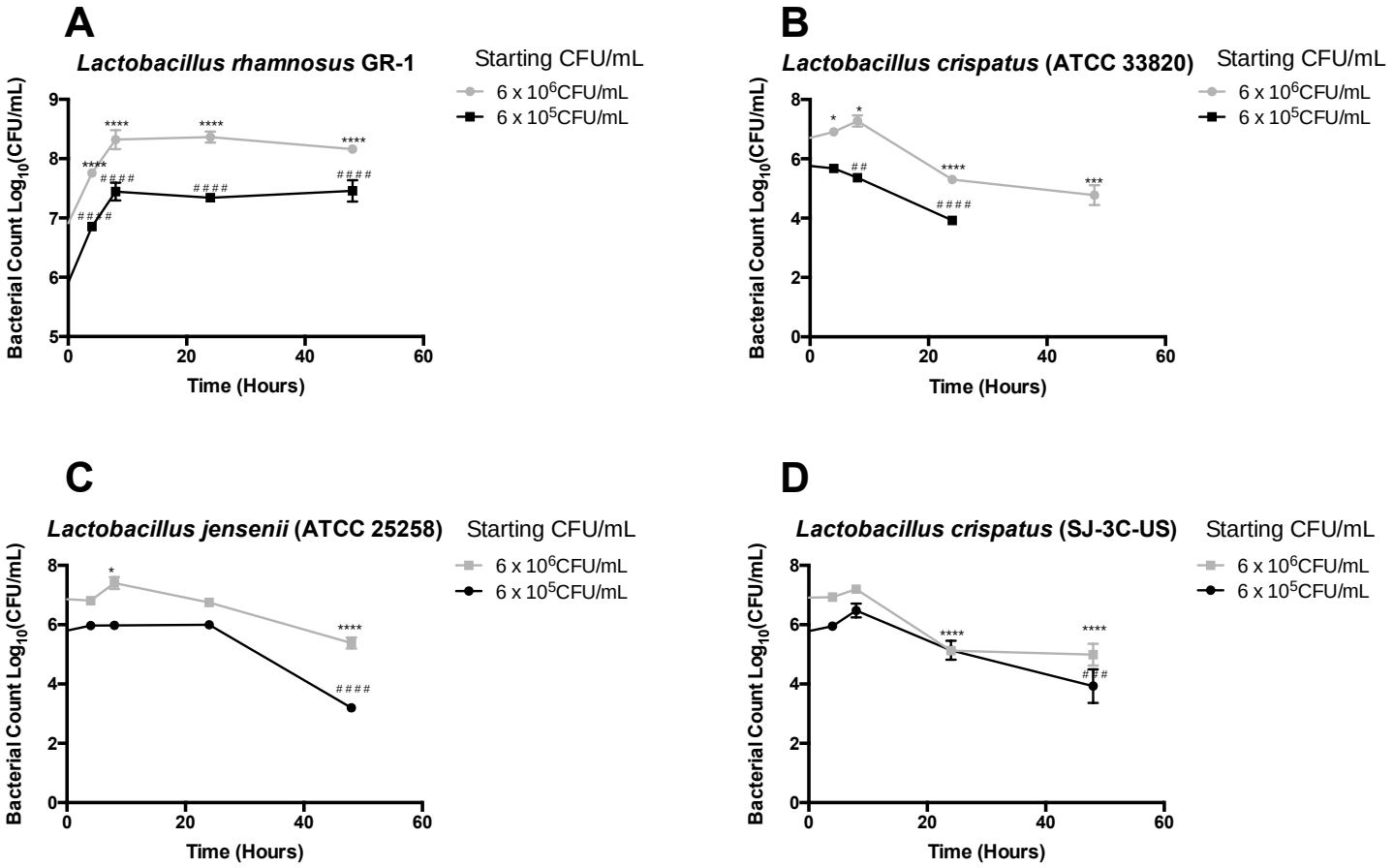

Figure 6: Bacterial count of different *Lactobacillus spp.* in KSFM at 0 (prior to addition) ,4 ,8 ,24 and 48 hours. **(A)** bacterial count of *Lactobacillus rhamnosus* GR-1 **(B)** bacterial count of *Lactobacillus crispatus* (ATCC 33820) **(C)** bacterial count of *Lactobacillus jensenii* (ATCC 25258) **(D)** bacterial count of *Lactobacillus crispatus* (SJ-3C-US). Data shown represents three different experiments, each done in triplicates. Data was analyzed using one-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. * represents significance compared to bacterial count prior to addition (0h) at starting bacterial concentration of 6 x 10^6 CFU/mL (0h) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. # represents significance compared to bacterial count prior to addition (0h) at starting bacterial concentration of 6 x 10^5 CFU/mL (0h) #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001. Error bars shown represent SEM.

Next, experiments were done to evaluate the bacterial count of different Lactobacilli after co-culture with Vk2 cells for 24 hours (resuspended in KSFM). The 1:10 cell to Lactobacilli ratio (6 x 10^6 CFU/mL) (Figure 7) was first examined. There was a significant increase in the bacterial count of probiotic GR-1, and *L. jensenii* after 24 hours in Vk2 co-culture (Figure 7A, B). *L. crispatus* (ATCC 33820) bacterial count was not altered after 24 hours in Vk2 co-culture (Figure 7C) but *L. crispatus* (SJ-3C-US) bacterial count was significantly decreased over this time (Figure 7D). Data from this figure represents data combined from two separate experiments, each done with three replicates. The same experiments were then then performed with, a lower starting bacterial concentration (6 x 10^5 CFU/mL), or 1:1 Vk2 cell to Lactobacilli ratio (Figure 8). Similar to the higher bacterial concentration, GR-1, and *L. jensenii* bacterial count was significantly increased after 24 hours in Vk2 co-culture (Figure 8A, C), and *L. crispatus* (ATCC 33820) bacterial count was not significantly altered (Figure 8B). Interestingly, unlike what we observed with the higher starting concentration (Figure 6D), the bacterial count of *L. crispatus* (SJ-3C-US) was not significantly different after 24 hours in Vk2 co-culture, (Figure 7D). Bacterial count in KSFM alone and in Vk2 co-culture is summarized in Table 1. Of note, certain Lactobacilli co-cultured with Vk2 cells have higher bacterial counts than when they are resuspended in KSFM alone after 24 hours. As seen in Table 1, *L. crispatus* (ATCC 33820) was significantly decreased after 24 hours in KSFM alone, but this decrease was ameliorated when bacteria was co-cultured with Vk2 cells. Similarly, there was no change in *L.*

jensenii bacterial count after 24 hours in KSFM alone, but bacterial count was significantly increased after 24 hours in Vk2 co-culture. However, the addition of Vk2 cells did not appear to alter *L. crispatus* (SJ-3C-US) bacterial count (Table 1). Overall, this data suggests that certain Lactobacilli, such as *L. crispatus* (ATCC 33820), and *L. jensenii* (ATCC 25258) are able to better maintain their bacterial count when co-cultured with Vk2 cells.

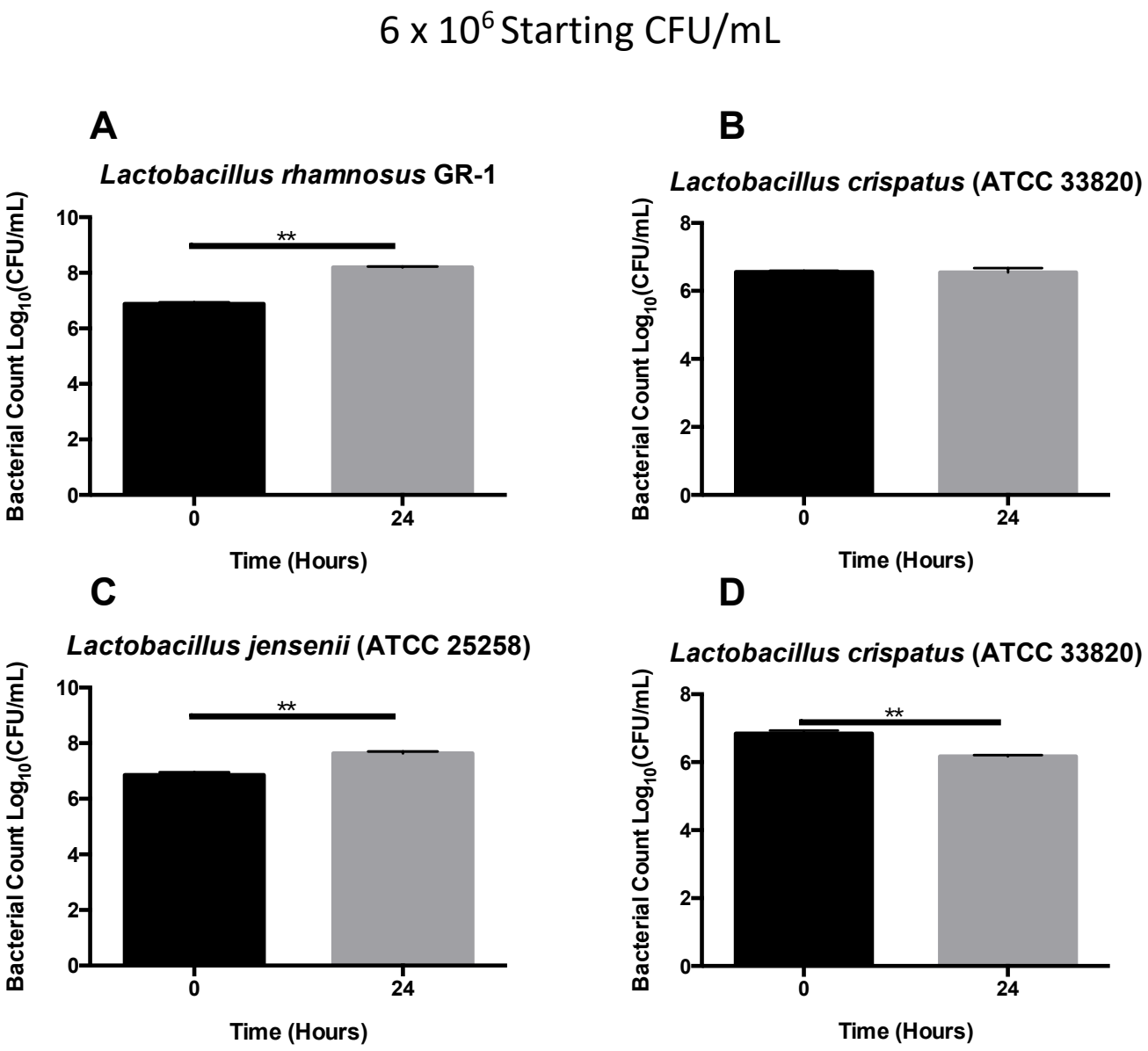

Figure 7: Bacterial count of different *Lactobacillus spp*. after 24 hours of co-culture with Vk2 cells 6 x 10^6 CFU/mL. Bacterial count at 0 hour represents bacterial count prior to addition. **(A)** Bacterial count of *Lactobacillus rhamnosus* GR-1 **(B)** Bacterial count of *Lactobacillus crispatus* (ATCC 33820) **(C)** Bacterial count of *Lactobacillus jensenii* (ATCC 25258) **(D)** Bacterial count of *Lactobacillus crispatus* (SJ-3C-US). Data shown represents two separate experiments, each done in triplicates. Data was analyzed by Mann-Whitney test. *p<0.05, **p<0.01. Error bars shown represent SEM.

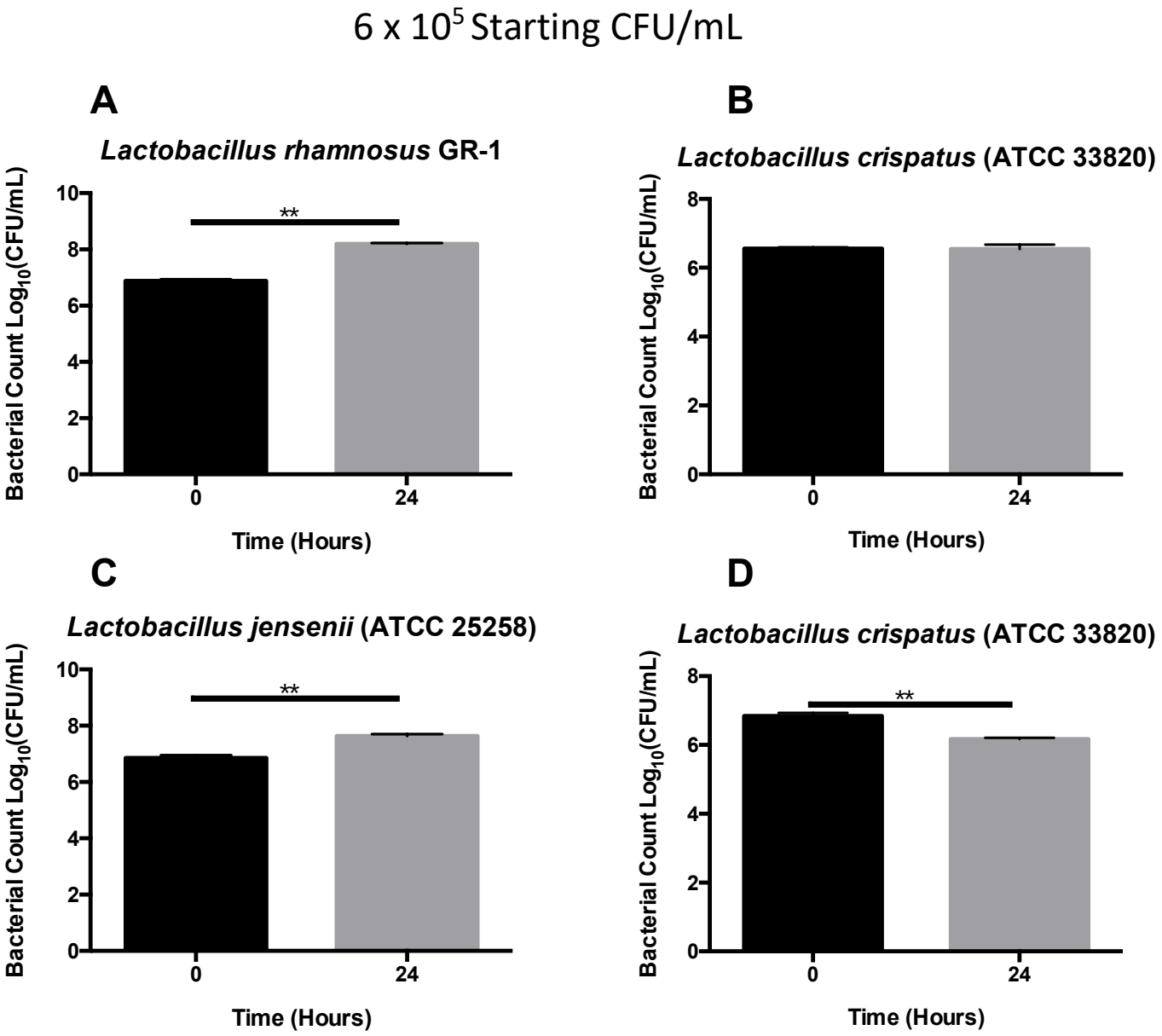

Figure 8: Bacterial count of different *Lactobacillus spp*. after 24 hours of co-culture with Vk2 cells with the starting bacterial concentration of 6 x 10^5 CFU/mL. Bacterial count at 0 hour represents bacterial count prior to addition. **(A)** Bacterial count of *Lactobacillus rhamnosus* GR-1 **(B)** Bacterial count of *Lactobacillus crispatus* (ATCC 33820) **(C)** Bacterial count of *Lactobacillus jensenii* (ATCC 25258) **(D)** Bacterial count of *Lactobacillus crispatus* (SJ-3C-US). Data shown represents three separate experiments, each done in triplicates. Data was analyzed by Mann-Whitney test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

Table 1: Summary of bacterial count of different *Lactobacillus spp*. in KSFM alone or with Vk2 cells after 24 hours

Condition	Starting Bacterial Concentration	Lactobacilli Species			
		***L. rhamnosus* (GR-1)**	***L. crispatus* (ATCC 33820)**	***L. jensenii* (ATCC 25258)**	***L. crispatus* (SJ-3C-US)**
Media Alone (KSFM) 24 hours	**6 x 10^6 CFU/mL**	****** Increase**	***Decrease**	**No Significance**	****** Decrease**
	6 x 10^5 CFU/mL	****** Increase**	***Decrease**	**No Significance**	**No Significance**
Co-Cultured with Vk2 cells 24 hours	**6 x 10^6 CFU/mL**	**** Increase**	**No Significance**	**** Increase**	**** Decrease**
	6 x 10^5 CFU/mL	****** Increase**	**No Significance**	****** Increase**	**No Significance**

Significance represents significant difference in the bacterial count prior to addition, and at 24 hours. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data shown represents 2-3 experiments each done in triplicates.

In vivo, the adherence of Lactobacilli onto vaginal epithelial cells is integral to its ability to colonize the vaginal tract. Therefore, we examined the adherence of the four *Lactobacillus spp*. After 24 hours in Vk2 co-culture, the non-adhered Lactobacilli count was first quantified by serial dilution and plating of the apical cell media. The Vk2 cells were subsequently disrupted using triton-X, a detergent, in order to quantify the rest of the Lactobacilli which was adhered to the Vk2 cell layer. The adhered Lactobacilli bacterial count was divided by the total bacterial count (non-adhered count + adhered count) to obtain an adherence ratio. As seen in Figure 9A, the ratio of adhered *L. crispatus* (SJ-3C-US) was significantly higher than of the other three Lactobacilli, with an initial bacterial concentration of 6×10^6 CFU/mL. Data shown represents data combined from two separate experiments, done in triplicates. Interestingly, at a lower starting bacterial concentration, *L. jensenii* (ATCC 25258), *L. crispatus* (ATCC 33820), and *L. crispatus* (SJ-3C-US) seem to be able to adhere significantly better than *L. rhamnosus* GR-1 (Figure 9B). Next, we evaluated whether or not different the ratio of adhered Lactobacilli at different starting bacterial concentrations would differ, and we observed that although the ratio of *L. rhamnosus* GR-1 did not significantly differ between the two starting concentrations, however, the other three Lactobacilli species had significantly higher adhered ratios at the lower starting concentration (6×10^5 CFU/mL) when compared to the higher starting bacterial concentration (6×10^6 CFU/mL) (Figure 9C). Data shown in this figure represents a minimum of three separate experiments, each done in triplicates.

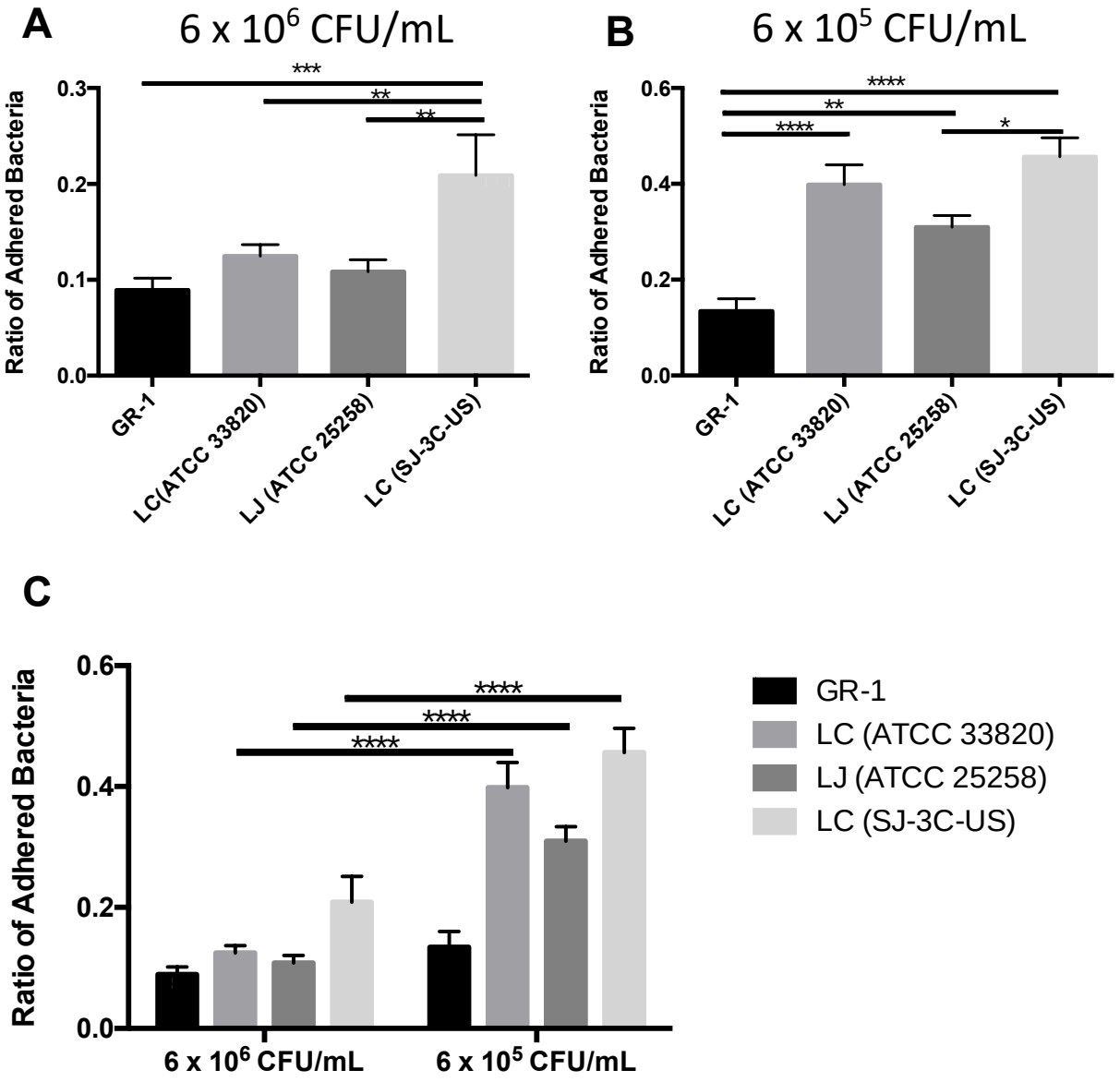

Figure 9: Adherence ratios of different *Lactobacillus spp.* after 24 hours in Vk2 co-culture at different starting bacterial concentrations. **(A)** Comparison of adherence ratios of different *Lactobacillus spp* at the starting bacterial concentration of 6 x 10^6 CFU/mL. **(B)** Comparison of adherence ratios of different *Lactobacillus spp* at the starting bacterial concentration 6 x 10^5 CFU/mL. **(C)** Comparison of adherence ratios between 6 x 10^6 CFU/mL, and 6 x 10^5 CFU/mL of each *Lactobacillus spp.* Data was analyzed with one-way ANOVA **(A)(B)**, or two-way ANOVA **(C)**, with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

As mentioned previously, estrogen is associated with increased abundance (Shen et al., 2016) and adherence of Lactobacilli (Silva, Rey, & Elena Nader-Macias, 2004). In contrast, MPA usage in women is associated with decreased abundance of Lactobacilli (Mitchell et al., 2014). Data from our lab also corroborate these findings, as MPA usage is associated with decreased abundance of Lactobacilli in the vaginal tract (Wessels et al, 2018). In order to assess the impact of hormones on Lactobacilli adherence, Vk2 cells were grown in E2, P4, and MPA and co-cultured with different *Lactobacillus spp*. for 24 hours at a starting bacterial concentration of 6 x 10^6 CFU/mL. The total, non-adhered, and adhered bacterial fractions of Lactobacilli were examined, and no differences were observed for any of the four Lactobacilli species examined under different hormone concentrations (Figure 10). Data shown is representative of 1-3 experiments, with three experimental replicates.

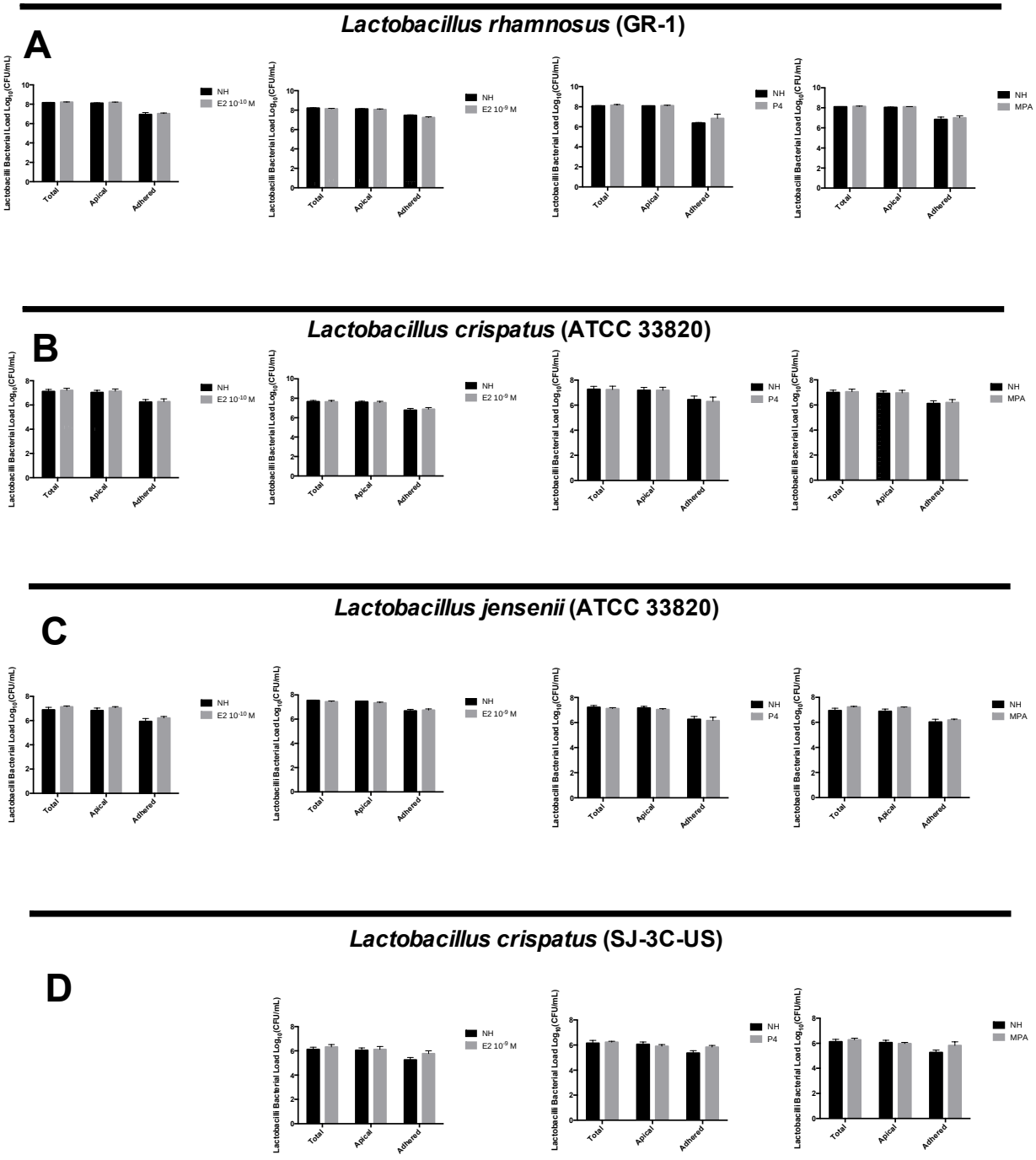

Figure 10: Lactobacilli growth and adherence in Vk2 co-culture is not affected by hormones. Bacterial growth and adherence were quantified by serial dilution and plating (See Materials and Methods). **(A)** GR-1 growth and adherence in the presence of different hormones **(B)** LC (ATCC 33820) growth and adherence in the presence of different hormones. **(C)** LJ (ATCC 25258) growth and adherence in the presence of different hormones. **(D)** LC SJ-3C-US growth and adherence in the presence of different hormones. Data shown represents a minumum of one experiment, done in triplicates. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisions. Error bars shown represent SEM.

3.2 Aim 2: Determining the Effect of Lactobacillus, and Hormones on Vk2 Barrier Function and Pro-Inflammatory Cytokine Production

3.2.1 Evaluating the effect of Lactobacilli and Hormones on the Barrier Function of Vk2 Cells

As shown above, the addition of different *Lactobacillus spp*. at the concentration of 6 x 10^6 CFU/mL did not negatively affect Vk2 cell viability (Figure 4,5), thus, this concentration was used for subsequent functional experiments.

A robust vaginal epithelial cell barrier is integral to prevent HIV translocation to target cells, thus the barrier function of Vk2 cells was examined in the presence of different hormones and Lactobacilli. TERs of Vk2 cell cultures grown in different hormonal conditions were measured from day 0 (prior to the addition of hormone treatment) to day 8 (Figure 11). Interestingly, the TERs of Vk2 cells grown in MPA were significantly lower on day 6 and 7 of culture when compared to control Vk2 cells (no hormone) (Figure 11D), indicating that MPA may negatively impact the barrier function of vaginal epithelial cells. There were no significant differences observed with any other hormonal treatment (E2 10^{-10}M, E2 10^{-9}M, P4 10^{-7}M) (Figure 11A-C). Data shown in Figure 11 represents one representative experiment with a minimum of three replicates for each day. Next, the effect of Lactobacilli co-culture on Vk2 cell barrier function was examined. Vk2 cells were co-cultured with different *Lactobacillus spp.* for 24 hours and TER measurements were taken prior to co-culture (Day 7) and 24 hours after co-culture (Day 8).

Results show that TERs of Vk2 cells were not changed after 24 hours of co-culture with any Lactobacilli spp. (Figure 12A). Next, the TERs of Vk2 cells in co-culture with Lactobacilli in the presence of different hormonal conditions was examined on Day 7 of culture and after 24 hours of Lactobacilli co-culturing. The only significant difference occurred when Vk2 cells were grown in the presence of MPA and co-cultured with *L. rhamnosus* (GR-1). Under these conditions there was a significant decrease in TER compared to control (no bacteria, no hormone) (Figure 12B). Data shown represents three separate experiments, each done in triplicates.

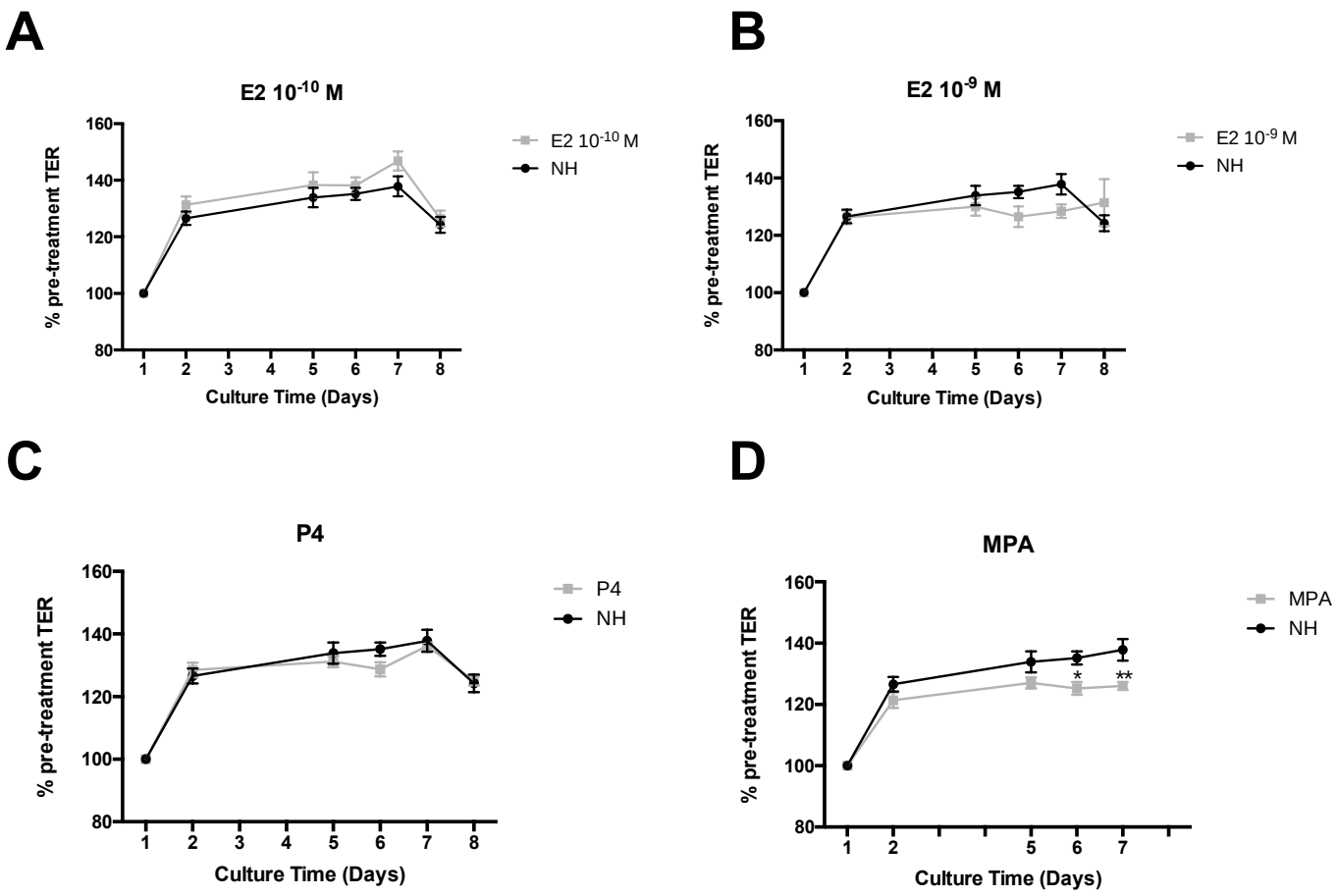

Figure 11: Transepithelial Resistance (TER) of MPA treated Vk2 cells are significantly lower than control. **(A)** TER of E2 (10^{-10} M) treated Vk2 cells compared to control **(B)** TER of E2 (10^{-9} M) treated Vk2 cells **(C)** TER of P4 treated Vk2 cells compared to control **(D)** TER of MPA treated Vk2 cells compared to control. Data shown represents one representative experiment, with a minimum of three experimental replicates done on each specified day. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. * represents significance compared to control at the corresponding day. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

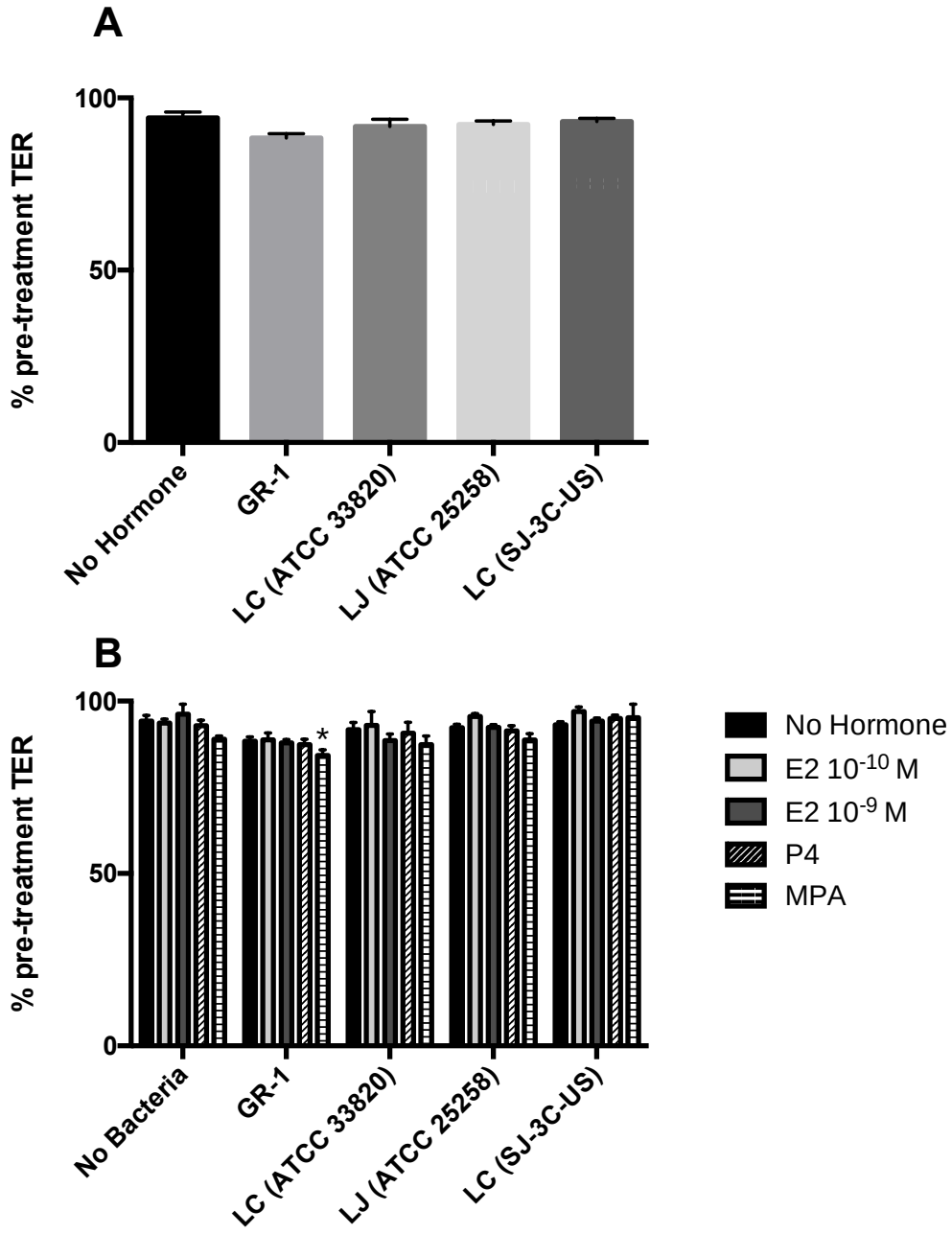

Figure 12: TER of Vk2 cells treated with different hormones, and/or co-cultured with different *Lactobacillus spp.* **(A)** TER of Vk2 cells co-cultured with different *Lactobacillus* spp. for 24 hours. **(B)** TER of Vk2 cells co-cultured with different *Lactobacillus spp.* and/or treated different hormones. Data shown represents three separate experiments, each done in triplicates. Data was analyzed by one-way ANOVA **(A)**, or two-way ANOVA **(B)** with Holm-Sidak Test to correct for multiple comparisons. * represents significant difference in TER of Vk2 cells after 24 hours of Lactobacilli co-culture (Day 8) compared to TER prior to bacterial addition (Day 7). *$p<0.05$. Error bars shown represent SEM.

Another assay that is commonly used to measure barrier integrity in cell cultures is the measurement of paracellular permeability. Since HIV must traverse the vaginal epithelium to infect underlying target cells, a paracellular leakage assay is an appropriate quantitative assay to assess potential HIV leakage across the epithelium. To do this, 10kDa FITC-dextran was added to the apical side of the 8 day old cell culture transwells. After 24 hours, the FITC-dextran concentration in the basolateral compartment was measured and the percentage of FITC-dextran leakage was determined (See Materials and Methods). In this manner, the paracellular leakage of FITC-dextran could be quantified in each cell culture. First, we needed to standardize a leakage assay for Vk2 cells grown in ALI cultures. We added 10 kDa of Fluorescein isothiocyanate–dextran (FITC-dextran) to the apical side of 8 day old Vk2 cell cultures as well as transwells without cells to measure paracellular leakage, and 8 day old Vk2 cells treated with 1% nonoxynol-9 to induce leakage. After 8 days of culture, ALI grown Vk2 cells formed epithelial layers which were capable of significantly reducing the paracellular leakage of 10kDa FITC-dextran compared to transwell only control (No cells present, transwell only) (Figure 13A). In addition, this experiment shows that the Vk2 cell layer can be disrupted by an agent such as N-9 (1%), as N-9 treatment for 24 hours led to significant increases in FITC-dextran leakage compared to control (Figure 13B). Thus, this leakage assay standardized in ALI grown Vk2 cells provides a good measure of barrier function *in vitro*.

To test whether hormones impact the Vk2 barrier function in the context of paracellular permeability, Vk2 cells were grown for 8 days under different hormonal conditions. FITC-dextran leakage assays were performed at 4, 8, and 24 hours after addition onto the apical compartment of Vk2 transwells. Results from this experiment demonstrate that estradiol (10^{-9} M, 10^{-10}M), and progesterone did not have any significant effect on FITC-dextran leakage of Vk2 cells at 4, 8 or 24 hours when compared to no hormone control (Figure 14A-C). On the contrary, FITC-dextran leakage of MPA treated Vk2 cells was significantly higher at 4, and 24 hours when compared to control (Figure 14A, 13C). However, data gathered at 24 hours was of higher significance compared to 4 hours (Figure 14A, 13C). In addition, data gathered from this experiment demonstrate that there is a linear rate of FITC-Dextran leakage over 24 hours of both control (no hormone), and MPA conditions, however, it is clear that FITC-dextran leakage occur at a higher rate in MPA treated Vk2 cells as seen in the steeper slope (Figure 14D). Altogether, this indicates that the 24 hour timepoint is the optimal timepoint for FITC-dextran leakage assays, as this timepoint yielded the most significant difference compared to control (Figure 14A, 13C). Data shown in this figure represents one representative experiment performed in at least two experimental replicates. To confirm that Vk2 cells grown in MPA have increased FITC-dextran leakage when compared to non-treated cells, a series of repeat experiments were performed using multiple hormonal conditions. Data suggests that MPA significantly increases paracellular permeability across Vk2 cells, while other hormonal

treatments, such as estradiol and progesterone do not (Figure 15A). Data from multiple independent experiments show that MPA consistently, significantly increases FITC-dextran leakage compared to control (n=13, two-four replicates/experiment) (Figure 15B). This indicates that MPA is able to compromise barrier integrity in increasing paracellular permeability. Finally, the effect of 24-hour Lactobacilli co-culture, with no added hormones, on FITC-dextran leakage was examined. Results demonstrate that Lactobacilli co-culturing of Vk2 cells does not significantly impact FITC-dextran leakage (Figure 15C), which indicates that Lactobacilli does not alter paracellular permeability of Vk2 cells in our system.

Lastly, we examined the combined effect of Lactobacilli and different hormonal conditions on leakage. Our results indicate that MPA is the primary cause of increased paracellular leakage, as any treatment condition that included MPA had significantly higher FITC-dextran leakage when compared to control (Figure 16A). Data shown in this figure represents one representative experiment, performed in two/three experimental replicates. To confirm our findings, data from 4 separate experiments with different hormonal conditions, and Lactobacilli spp. were normalized to the each of their respective experimental controls (n=4, two-three replicates/experiment) (Figure 16B). MPA treatment increased the paracellular permeability of Vk2 cells compared to control (no hormone, no bacteria), regardless of the presence of absence of Lactobacilli. Altogether, results

gathered from these barrier function experiments provide strong evidence that MPA is negatively affecting the barrier integrity of vaginal epithelial cells in culture.

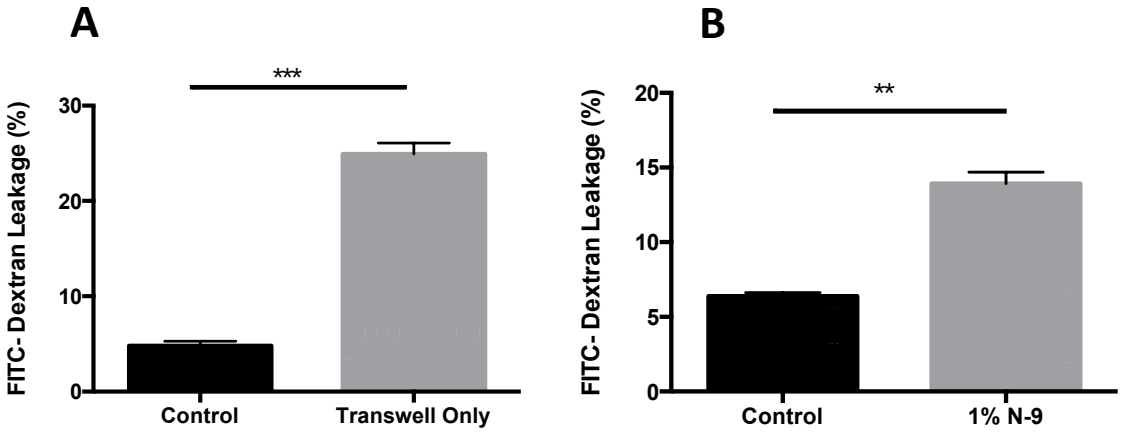

Figure 13: FITC-dextran leakage of Transwells without Vk2 cells, and N-9 treated Vk2 cells compared to Vk2 cells alone. **(A)** FITC-dextran leakage of transwells without Vk2 cells compared to transwells seeded with Vk2 cells **(B)** FITC-dextran leakage of 1% N-9 treated (24 hours) Vk2 cells compared to untreated Vk2 cells (control). Data shown represents one experiment done in triplicates. Data was analyzed by an unpaired T-Test. *p<0.05, **p<0.01, ***p<0.001. Error bars shown represents SEM.

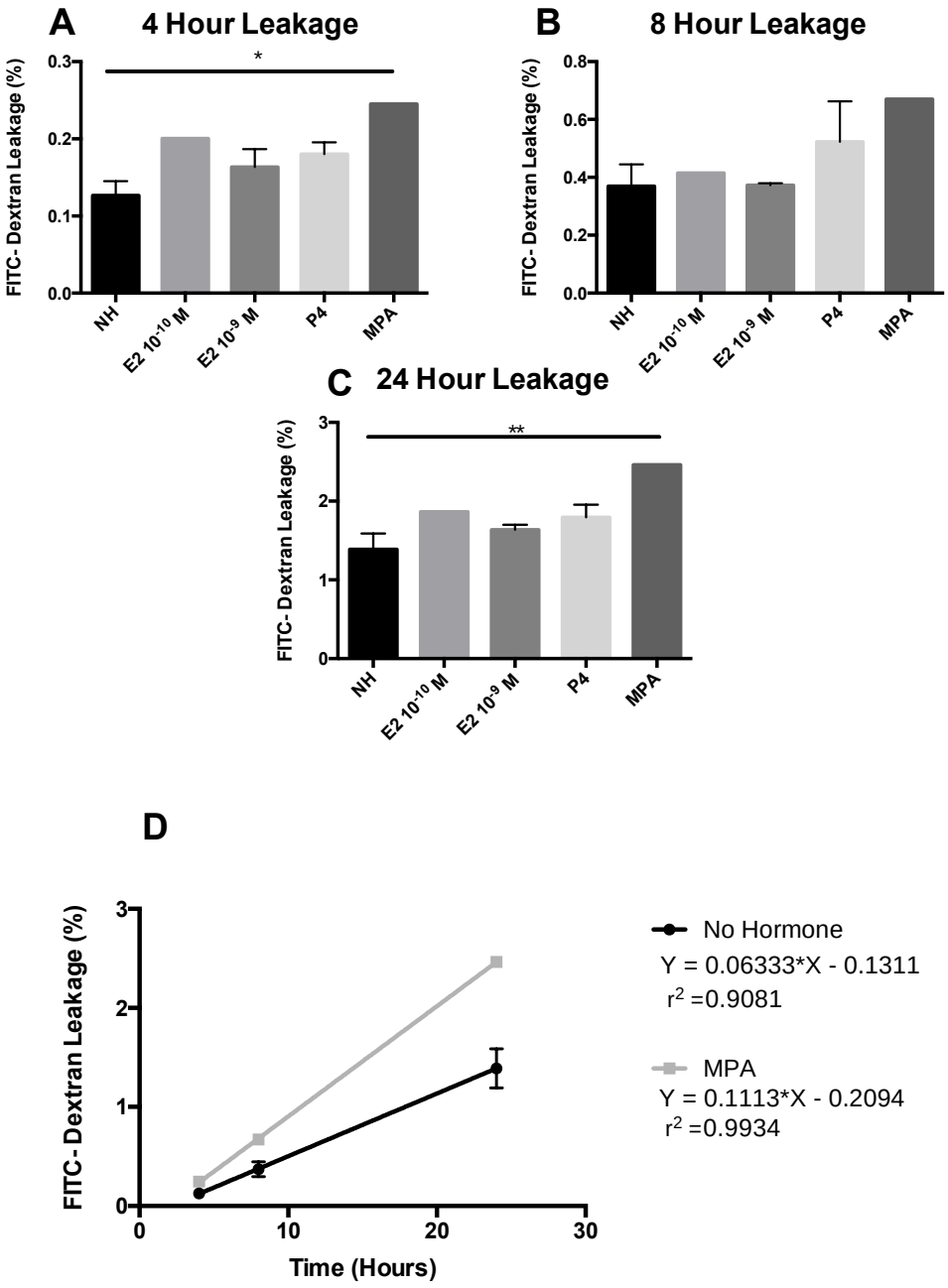

Figure 14: FITC-dextran leakage of Vk2 cells treated with different hormones at different timepoints. **(A)** FITC-dextran leakage of hormonal treated, and control Vk2 cells at 4 hours. **(B)** FITC-dextran leakage of hormonal treated, and control Vk2 cells at 8 hours. **(C)** FITC-dextran leakage of hormonal treated, and control Vk2 cells at 24 hours. **(D)** FITC-dextran leakage of untreated Vk2 cells, and MPA treated Vk2 cells at different timepoints, plotted as linear equations, r^2 represents coefficient of correlation. Data shown represents one representative experiment done in two/three replicates. Data was analyzed by one-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 (triplicate).

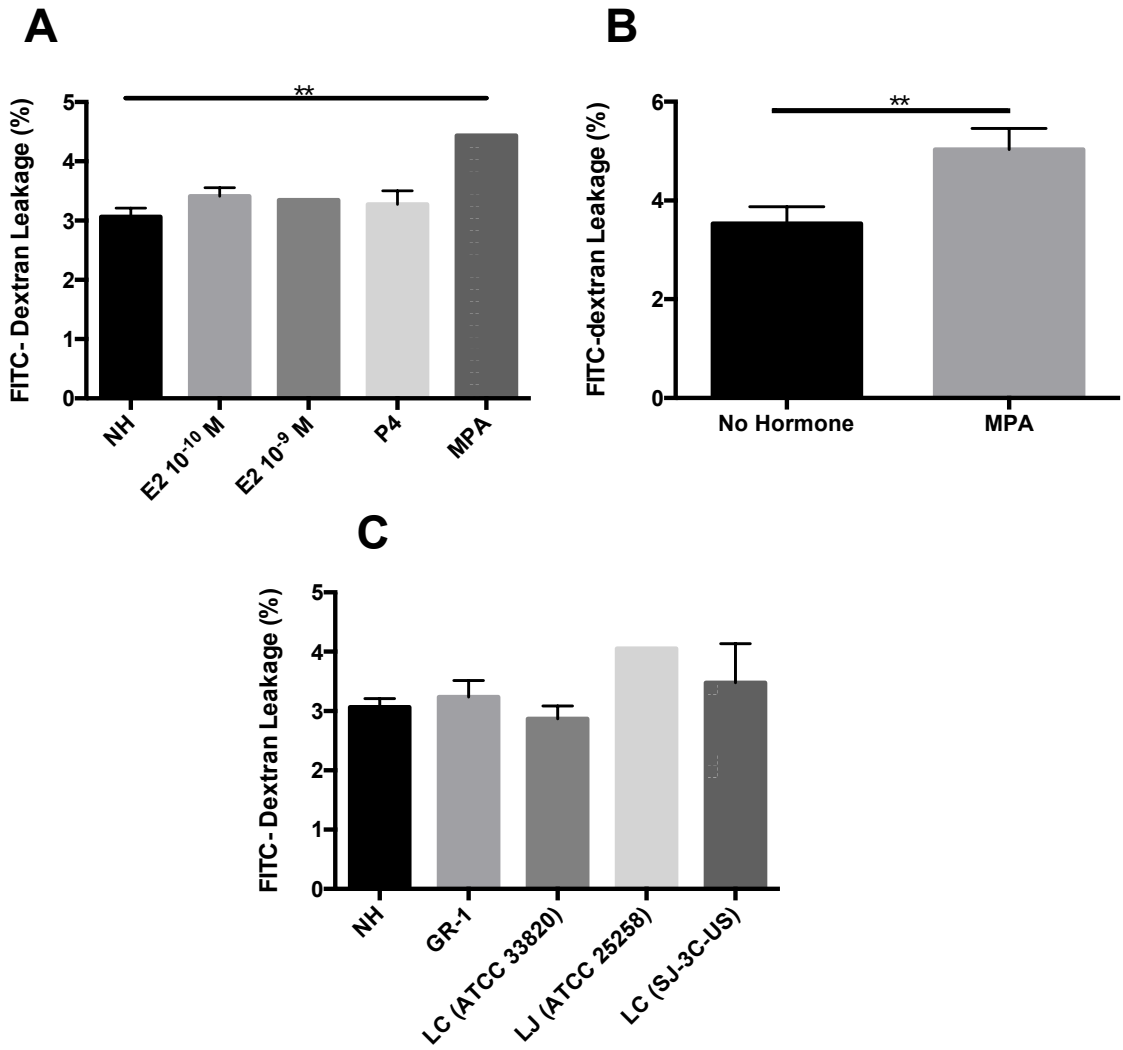

Figure 15: Vk2 cells treated with MPA has significantly higher FITC-dextran leakage (24 hours) compared to control. **(A)** FITC-dextran leakage of Vk2 cells treated with different hormones. Data shown represents one representative experiment done in two/three replicates. **(B)** FITC-dextran leakage of Vk2 cells treated with MPA compared to control. Data shown represents data combined from thirteen different experiments, each done in two/three replicates. **(C)** FITC-dextran leakage of Lactobacilli co-cultured Vk2 cells, and control. Data shown represents one representative experiment done in two/three replicates. Data was analyzed using one-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons **(A)**,**(C),** or Mann-Whitney unpaired T Test **(B)**. *p<0.05, **p<0.01. Error bars shown represent SEM.

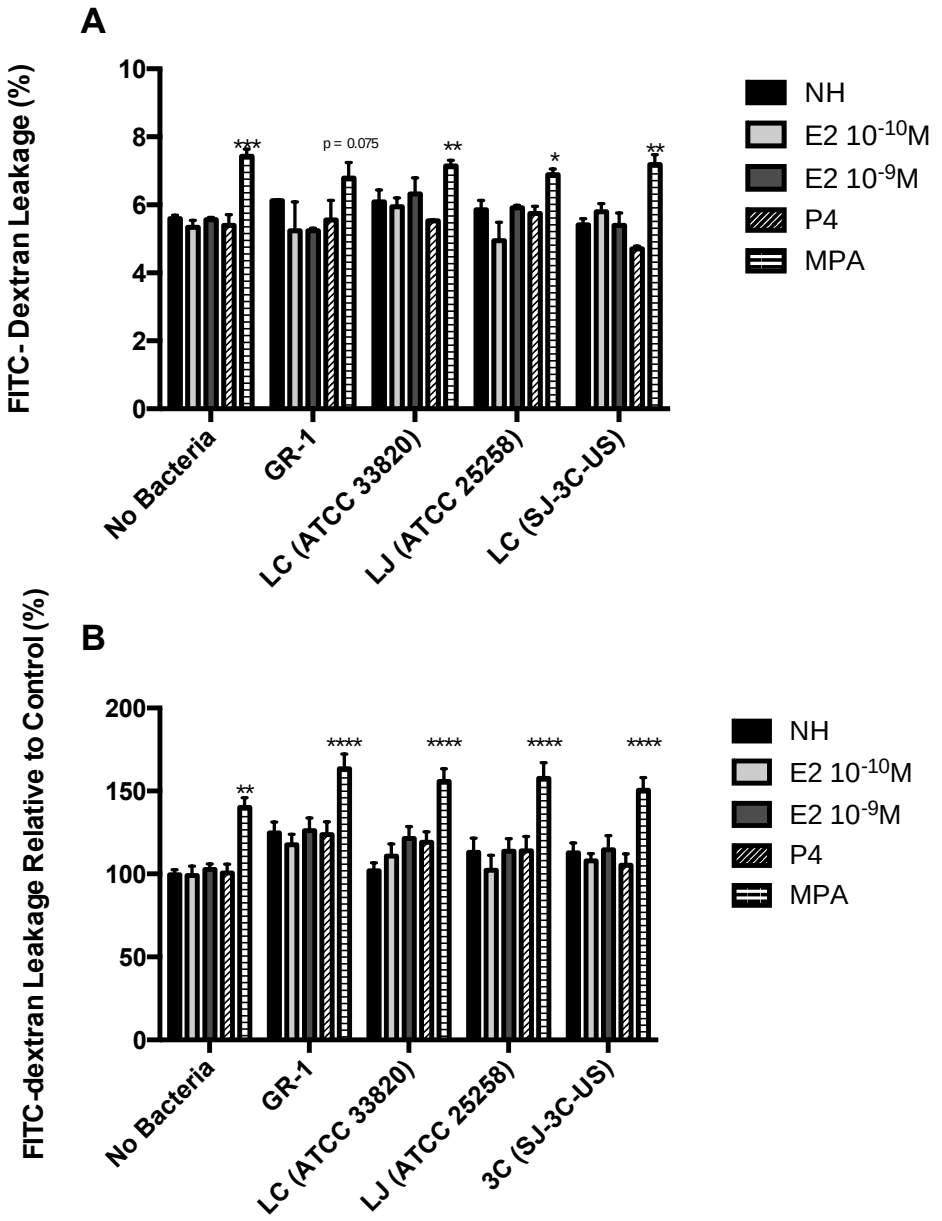

Figure 16: MPA is the main driver in increasing FITC-dextran leakage of Vk2 cells. **(A)** FITC-dextran leakage of Vk2 cells under different hormonal condition and/or different Lactobacilli treatment. Data shown represents one representative experiment done in two/three replicates. **(B)** Combined and normalized FITC-dextran leakage of Vk2 cells under different hormonal condition and/or different Lactobacilli treatment. Data shown represents data normalized to control from each respective experiment (no hormone, no bacteria) and data combined from four separate, each with two/three experimental replicates. Data was analyzed by two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. * represents significance compared to control (no hormone, no Lactobacilli). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

3.2.2 Evaluating the Effect of Hormones and Lactobacilli on Pro-Inflammatory Cytokine Production by Vk2 cells in ALI culture

As previously discussed, a key mechanism that could affect HIV susceptibility in the FRT is the inflammatory state of the vaginal microenvironment as a pro-inflammatory environment is associated with enhanced susceptibility to HIV. Indeed, increased levels of pro-inflammatory cytokines such as IL-1α, lL-8, TNF-α, and chemokine RANTES are associated with increased HIV susceptibility (Kaul et al., 2008; Masson et al., 2015) while cytokines with anti-inflammatory function, such as IL-10, and IL-1RA are associated with decreased HIV susceptibility (Corley, 2000; Kwon & Kaufmann, 2010). Therefore, the effect of hormonal treatments (E2, P4, MPA) and different Lactobacilli species on the cytokine production of Vk2 cells was examined. Based on literature we selected and quantified IL-1α, lL-8, TNF-α, RANTES, lL-10, and IL-1RA in the apical and basolateral supernatants of Vk2 cells grown in ALI conditions.

Of the six cytokines analyzed, IL-10 levels in both basolateral and apical supernatants were below the detectable limit of the assay (data not shown) so no follow-up experiments were performed for this analyte. TNF-α levels in the basolateral media were also below the detectable limit, whereas the levels of TNF-α in apical media were relatively low and ranged from approximately 2-5 pg/mL in different treatment conditions (Figure 17). There were no significant differences in

the levels of TNF-α between any of the treatment conditions or the control in the apical media (Figure 17).

The levels of IL-1α in the basolateral media of the Vk2 cell cultures were also below the minimal detectable limit (not shown), in one experiment run in triplicate. However, the apical media contained IL-1α levels that were well above the detectable range in different experimental conditions (Figure 18A), however, there was no significant difference between any of the treatment groups. A follow up repeat experiment verified that there was no significance across different hormonal and Lactobacilli treatment groups. Data was normalized within each experiment to the no hormone, no bacteria control and combined results verify that there was no significant difference in the levels of IL-1α across any treatment groups (Figure 18B).

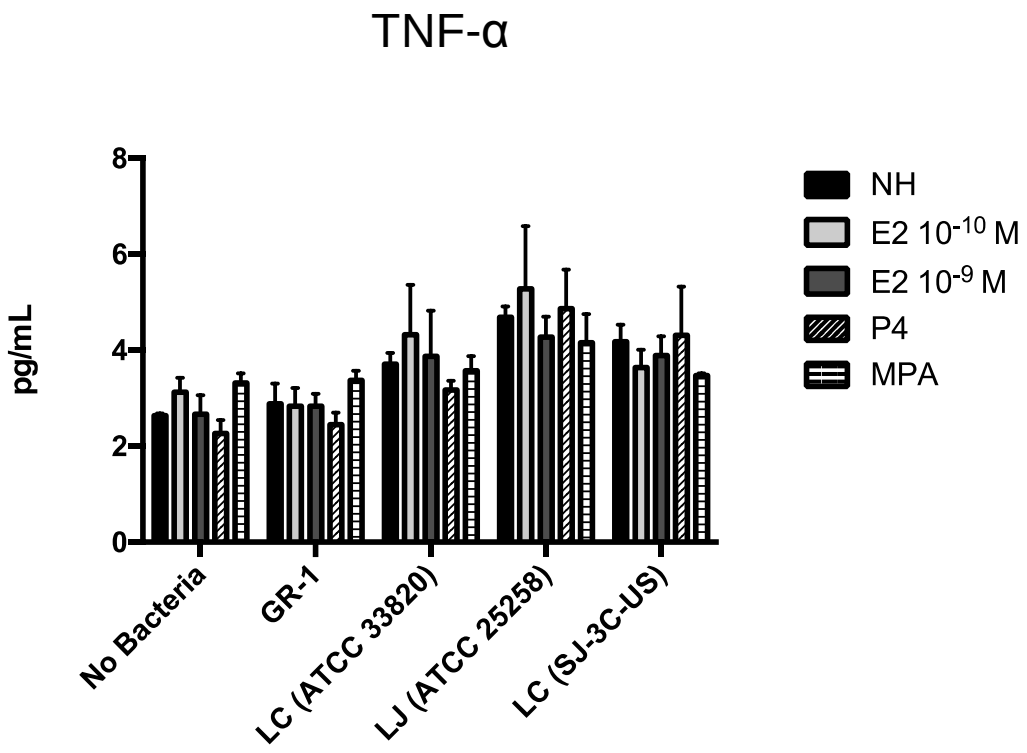

Figure 17: TNF-a levels in the apical media of Vk2 cells are not affected by hormones and/or Lactobacilli. Data shown represents one experiment, done in two/three replicates. Data was analyzed using a two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. Error bars shown represent SEM.

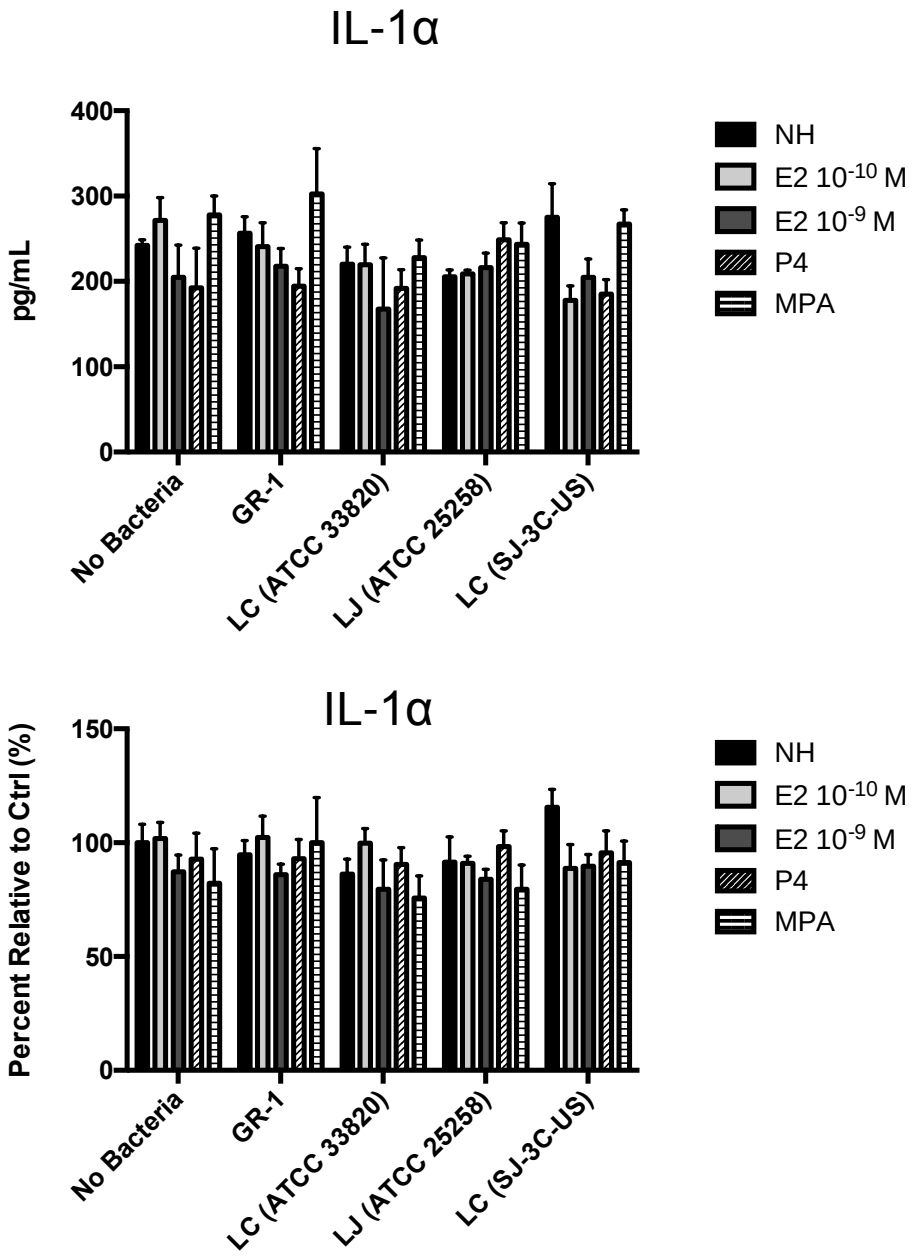

Figure 18: IL-1α levels in Vk2 cells are not affected by hormonal or Lactobacilli treatments. **(A)** Levels of IL-1α in the apical media of Vk2 cells treated with different hormones, and/or different Lactobacilli. Data shown represents one representative experiment, each done in two/three replicates. **(B)** Levels of IL-1α in apical media of Vk2 cells treated with different hormones and/or different Lactobacilli. Data shown represents data normalized to control from each respective experiment (no hormone, no bacteria) and combined from two separate experiments, each done in two/three replicates. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparison. Error bars shown

When examining the levels of RANTES in the apical media of Vk2 cells treated with different hormones, we observed that MPA led to a dramatically significant increase in level of RANTES in the apical supernatant (Figure 19A). Lactobacilli treatment did not alter RANTES production in the same manner (Figure 19B). Finally, the combined effect of Lactobacilli and hormones was explored and it was confirmed that the significant increase in RANTES production was due to MPA alone, as any treatment group containing MPA had significantly increased RANTES production compared to control (regardless of whether or not Lactobacilli was present) (Figure 19C). Experimental data from the basolateral media also showed a strong effect of MPA on the production of RANTES in Vk2 cells, although the concentration of the analyte was much lower than that in the apical media (Figure 20). Experimental data shown in Figure 19 represent one experiment done in two/three replicates. As the baseline production of RANTES differed in each of the two experiments, data from each treatment was normalized to its internal experimental control and expressed as a percentage. Combined, normalized data from the two separate experiments confirm that MPA is the primary factor significantly altering production of RANTES (Figure 21). Data shown in Figure 21 represents data gathered from two separate experiments with two-three experimental replicates and normalized to each respective control.

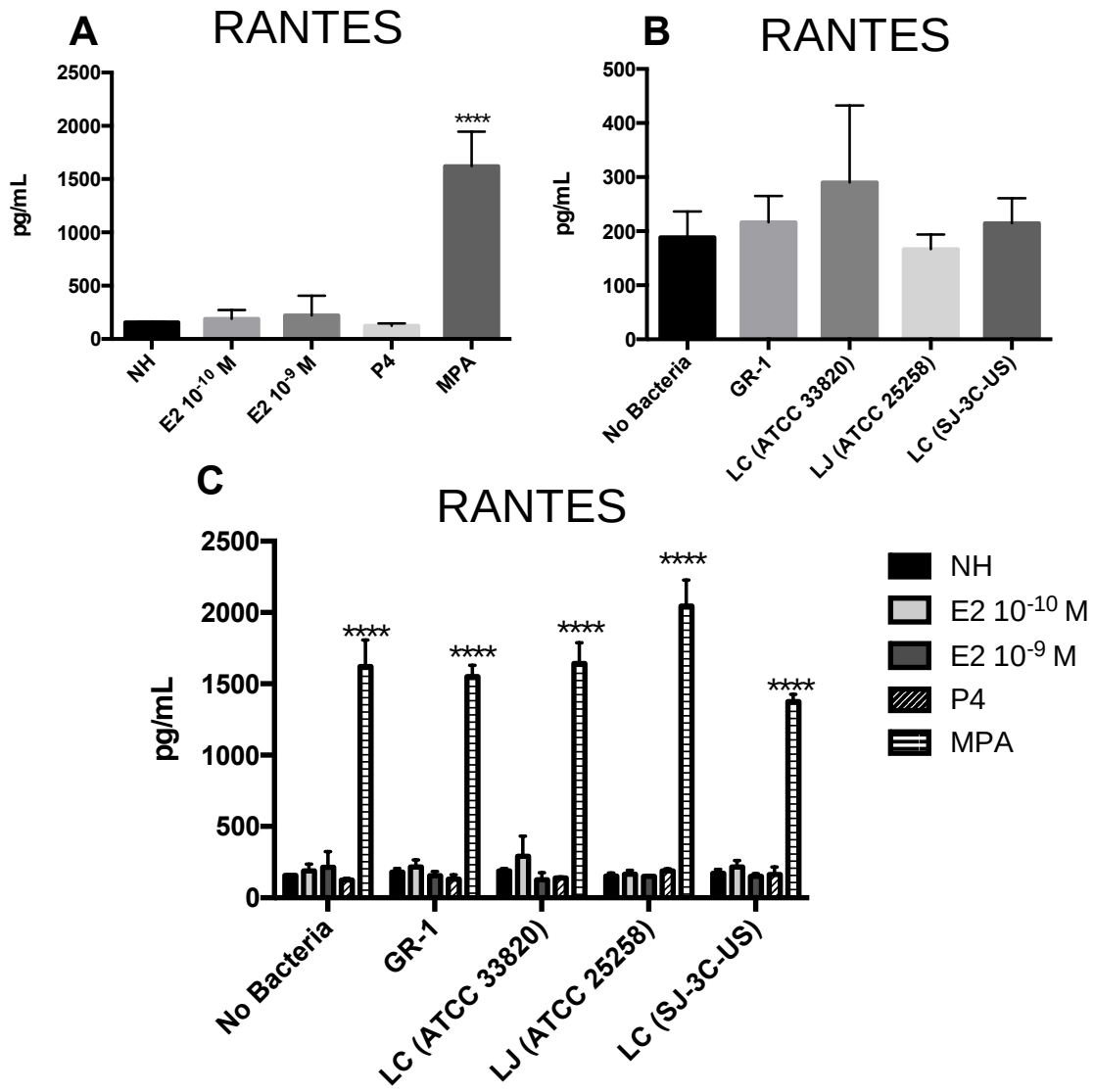

Figure 19: MPA treated Vk2 cells have elevated levels of RANTES in apical media, regardless of Lactobacilli treatment. **(A)** Level of RANTES in hormonal treated, and control Vk2 cells **(B)** Level of RANTES in Lactobacilli treated, and control Vk2 cells. **(C)** Level of RANTES in hormonal treated, and/or Lactobacilli treated Vk2 cells. Data shown represents one representative experiment done in two/three replicates. Data was analyzed by two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. * represents significance compared to control (no hormone, no Lactobacilli). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

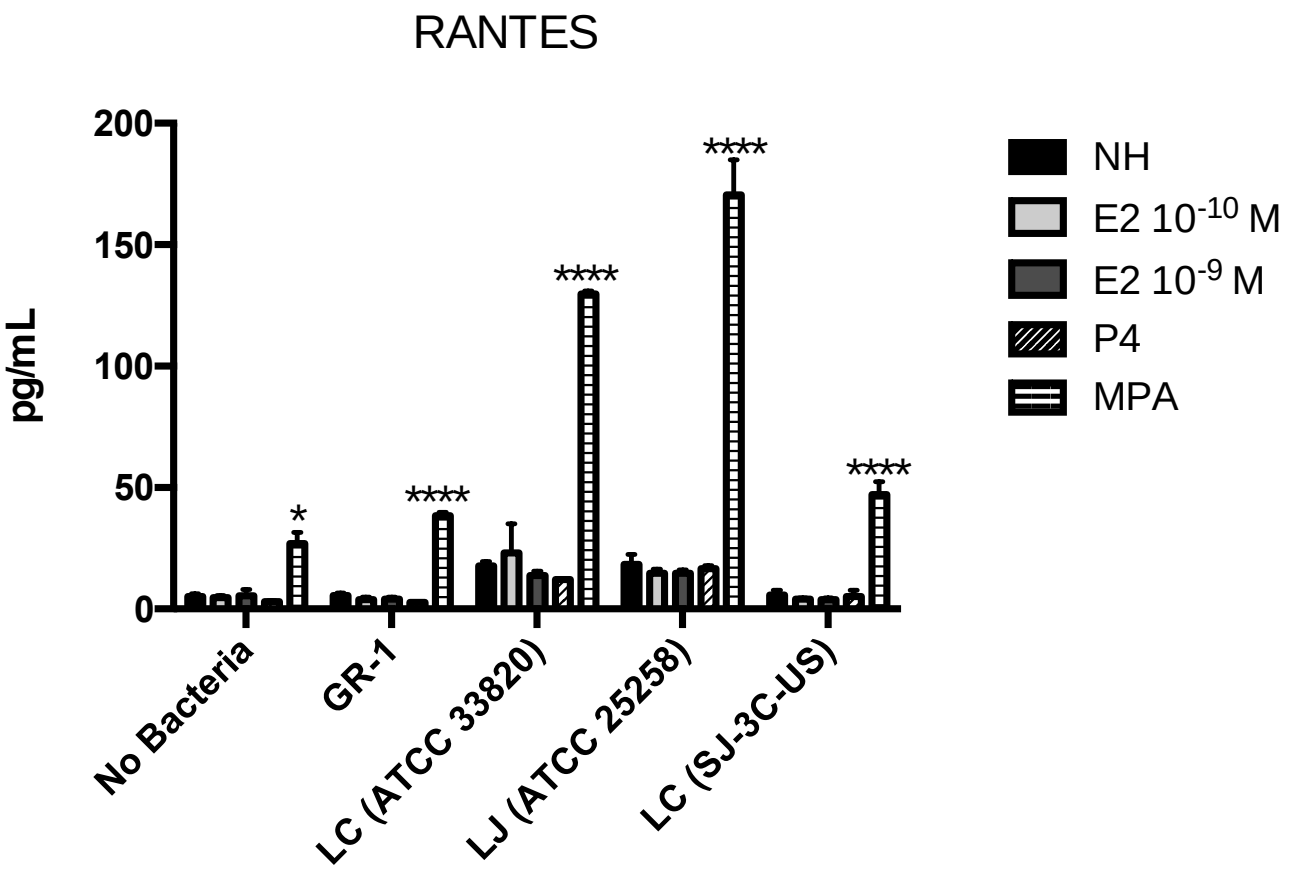

Figure 20: Levels of RANTES in the basolateral MEDIA of Vk2 cells is increased with MPA treatment, regardless of Lactobacilli. * represents significance compared to control (no hormone, no bacteria). Data shown represents one experiment, done in two/three replicates. Data was analyzed using a two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

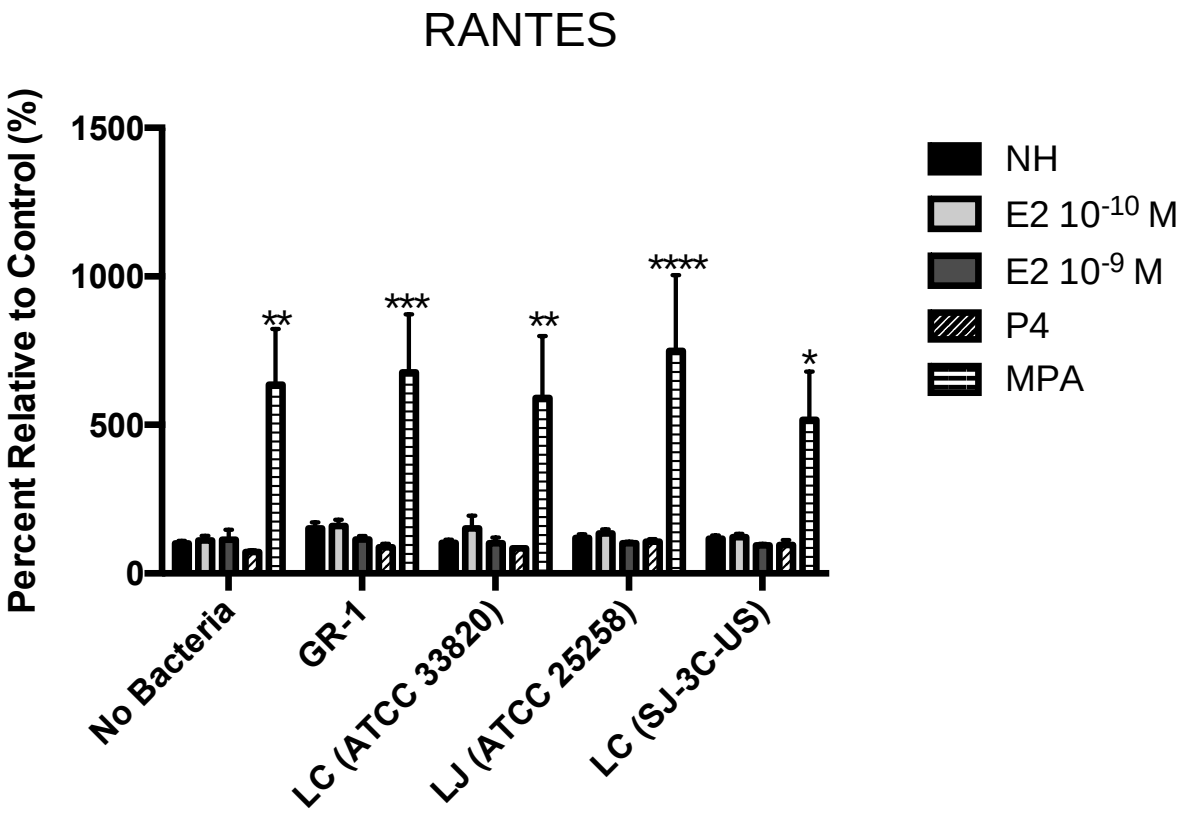

Figure 21. Normalized, and combined data show confirm that MPA is the main driver in increasing the level of RANTES in the apical media of Vk2 cell cultures. Data shown represents data normalized to control from each respective experiment (no hormone, no bacteria). Data shown represents two separate experiments, each done in two/three replicates. * represents significance compared to control (no hormone, no bacteria). 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, ****p<0.0001. Error bars shown represent SEM.

IL-1RA levels in the basolateral media of Vk2 cells was not significantly altered by either lactobacilli or hormonal treatments (Figure 22). In apical samples MPA appears to increase IL-1RA production in Vk2 cells in the absence of Lactobacilli (Figure 23). Further, MPA was able to significantly increase IL-1RA production in Vk2 cells in the presence of each of the Lactobacilli spp. studied (GR-1, LC, LJ, LC (SJ-3C-US)) when compared to control (Figure 23).When combined Lactobacilli and MPA treatments were compared to Lactobacilli treatments alone, three of four (GR-1, LJ, and LC (SJ-3C-US)) were significantly higher than control, indicating that the increase in IL1-RA is an MPA effect (Figures 23 B, 19D, 19E). It is however, interesting to note that there was no significant difference in IL-1RA production in Vk2 cells between LC (ATCC 33820) alone and LC (ATCC 33820) combined with MPA, but there is a trend towards increased IL-1RA with MPA, (Figure 23).

Data shown in Figure 23 represents one representative experiment done in two/three replicates. To verify these findings, an additional separate experiment was performed using the apical media of Lactobacilli and hormone treated Vk2 cells. Data from both experiments were normalized to their respective controls, expressed as a percentage and combined. Normalized data from two separate experiments show that MPA increases the production of IL-1RA in Vk2 cells when compared to control ($p=0.065$), whereas other hormonal conditions do not (Figure 24). In addition, normalized data show that Lactobacilli treatment alone does not alter IL-1RA production in Vk2 cells (Figure 24). Once again, regardless of the

presence of any *Lactobacillus spp.* tested, MPA treatment significantly increased the production of IL-1RA in Vk2 cells compared to control (Figure 24). Similar to previous results, there was a significant difference in IL-1RA production in the apical between Lactobacilli treatment alone versus Lactobacilli in combination with MPA (Figure 24), with the exception of *L. crispatus* (ATCC 33820) (Figure 24). Data shown in Figure 24 represents two separate experiments, each with two/three experimental replicates, and normalized to their own respective controls.

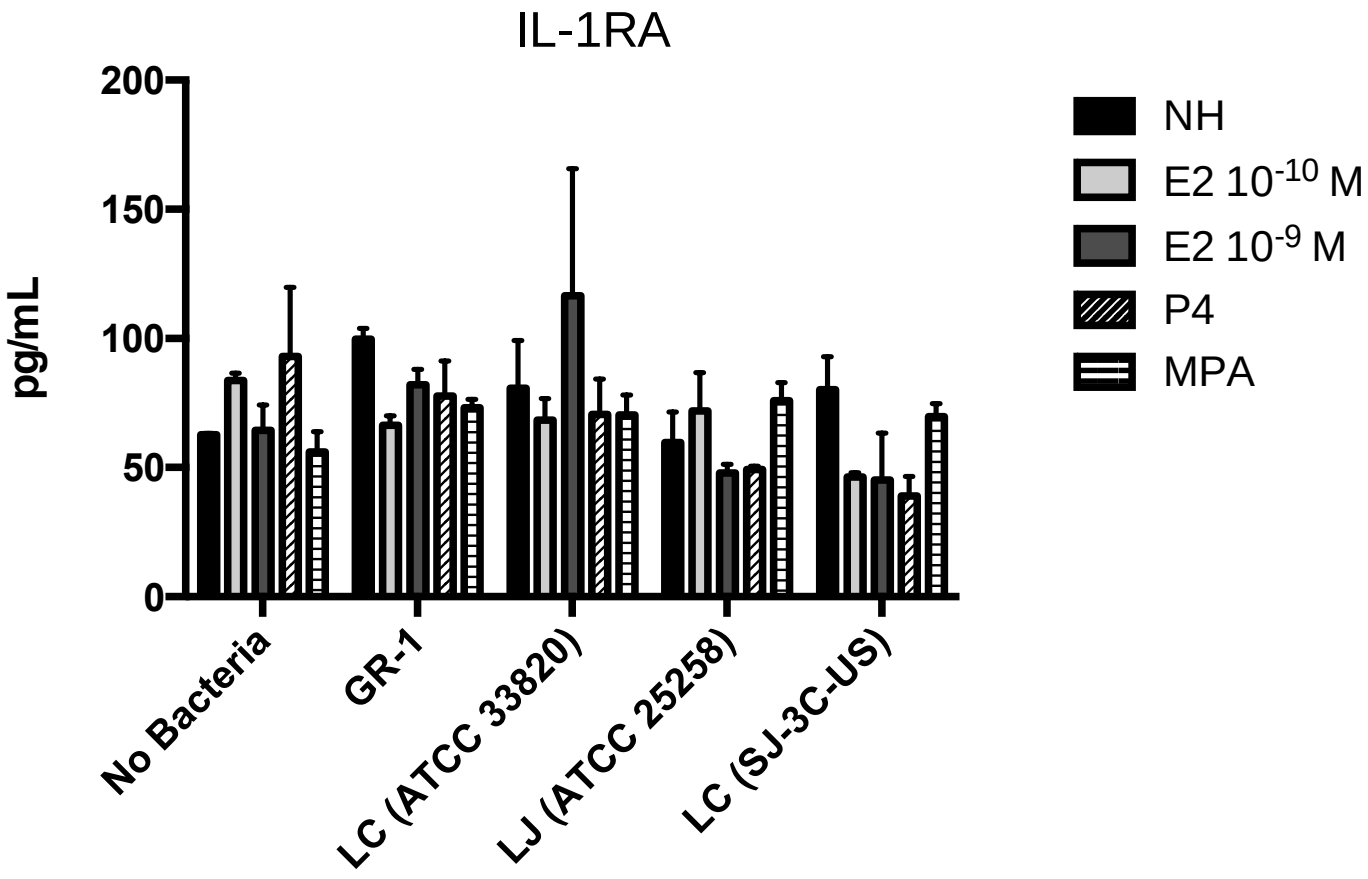

Figure 22: Level of IL-1RA in basolateral media of Vk2 cells are not affected by hormonal, and/or Lactobacilli treatment. Data shown represents one experiment, done in two/three replicates. Data was analyzed using a two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. Error bars shown represent SEM.

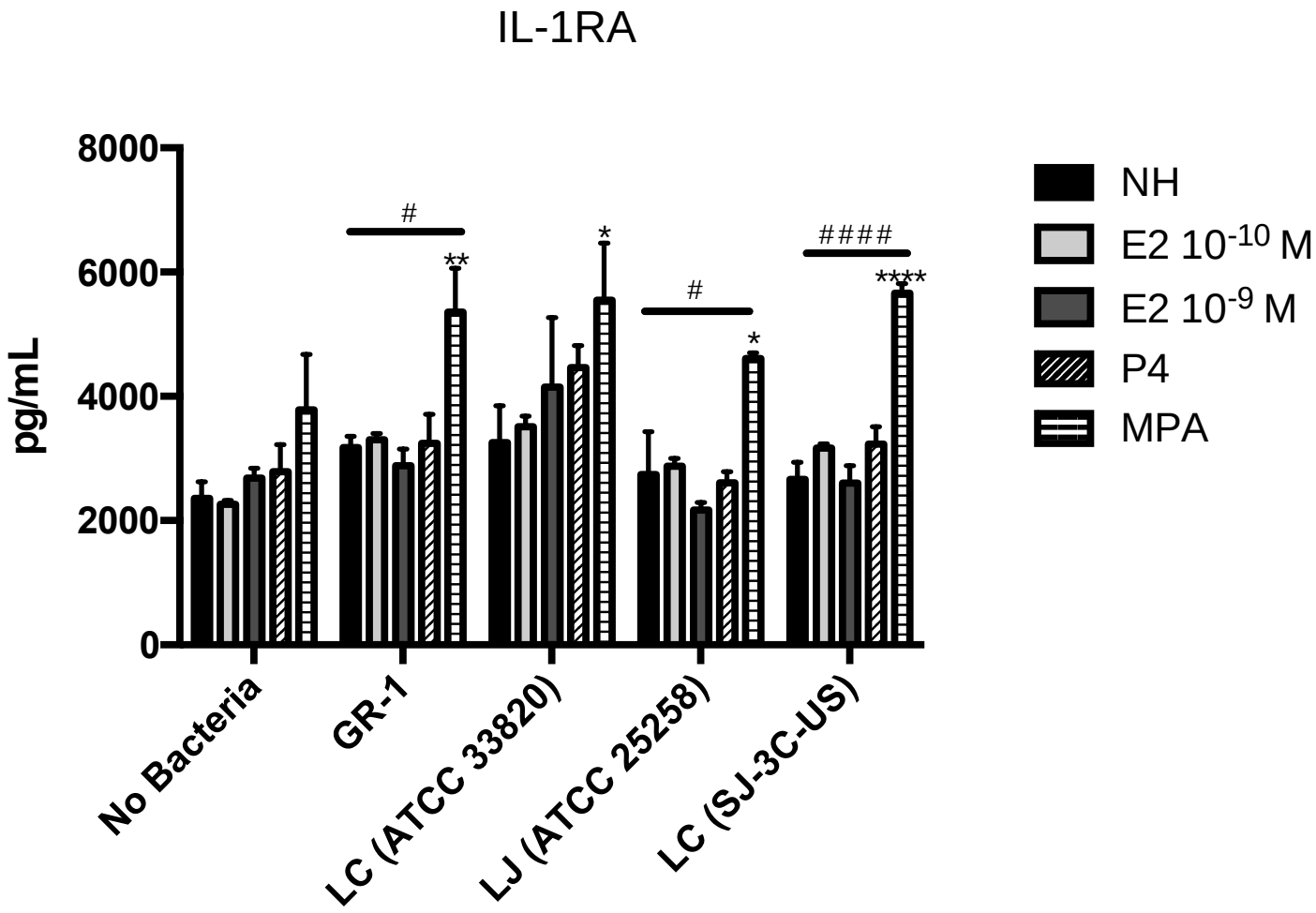

Figure 23: Levels of IL-1RA in the apical media of Vk2 cells treated with different hormones, and/or different Lactobacilli. Data shown represents one representative experiment, done in two/three replicates. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. * indicates significance relative to no bacteria, no hormone control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. # indicates significance relative to respective bacteria treatment (no hormone, bacteria treated) . #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001. Error bars shown represent SEM.

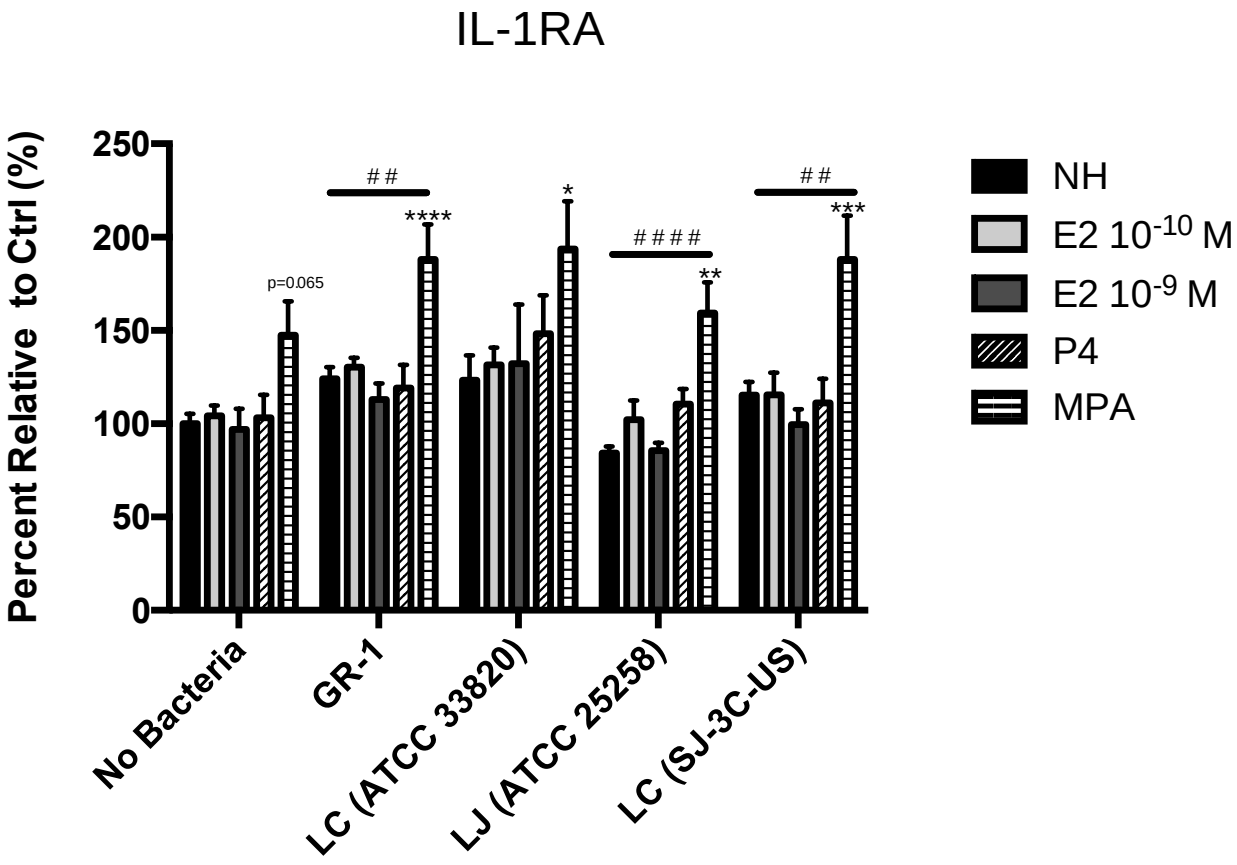

Figure 24: Levels of IL-1RA in the apical media of Vk2 cultures is increased in the presence of MPA, regardless of the Lactobacilli treatment. Data shown represents combined, and normalized data from two separate experiments done in two/three replicates. Data was analyzed using two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. * indicates significance relative to no bacteria, no hormone control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. # indicates significance relative to respective bacteria only treatment (no hormone, bacteria treated). #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001. Error bars shown represent SEM.

Data gathered from one representative experiment show that MPA treatment led to decreased production of IL-8 in the apical media of Vk2 cells nearly significantly (p=0.054), whereas other hormonal treatments had no significant effect (Figure 25A). *L. rhamnosus* GR-1 co-culture significantly decreased production of IL-8 in Vk2 cells, whereas *L. crispatus* (SJ-3C-US) treatment led to significantly increased levels of IL-8 (Figure 25B). When examining the combined effect of Lactobacilli and hormones, we found that GR-1 with E2 (10^{-10} M), and GR-1 with MPA significantly diminished levels of IL-8 in Vk2 cells compared to control (Figure 25C). Similarly, *L. crispatus* (ATCC 33820) with MPA also significantly lowered levels of IL-8 when compared to control (Figure 25D). Although not significant, an effect of MPA treatment was also observed with *L. jensenii* (ATCC 25258), where levels of IL-8 was the lowest when Vk2 cells were treated with MPA (Figure 25E). Interestingly, *L. crispatus* (SJ-3C-US) was the only treatment which led to increased levels of IL-8 in Vk2 cells (Figure 26A). We observed a significant increase in levels of IL-8 compared to control when cells were treated with *L. crispatus* (SJ-3C-US) alone, or *L. crispatus* (SJ-3C-US) with P4 (Figure 26A). In both the E2 conditions (10^{-10} M, 10^{-9} M) with *L. crispatus* (SJ-3C-US), the IL-8 levels did not significantly differ from control (Figure 26A) suggesting that that E2 treatment (10^{-10} M, 10^{-9} M) may somewhat abrogate the effect of *L. crispatus* (SJ-3C-US) on increased IL-8 levels There was also a significant difference in IL-8 levels between MPA + *L. crispatus* (SJ-3C-US) , *L. crispatus* (SJ-3C-US) alone,

and *L. crispatus* (SJ-3C-US) + P4, indicating that MPA is able to attenuate enhanced IL-8 production observed with *L. crispatus* (SJ-3C-US) (Figure 26B). Data shown in these experiments represent one single experiment done in two/three experimental replicates.

To verify findings from this initial experiment, an additional experiment was performed, data from both experiments were normalized to their respective controls and combined. Data from these combined experiments confirm many observations that were seen discussed above. MPA treatment leads to decreased IL-8 levels (Figure 27A), whereas co-culturing with *L. crispatus* (SJ-3C-US) increased IL-8 levels in the apical media of Vk2 cells (Figure 27B). With regard to combined hormonal and lactobacilli treatments, MPA treatment, regardless of the presence or absence of Lactobacilli reduced IL-8 in Vk2 cells (Figure 28 A,B). Although not significant, MPA in combination with *L. jensenii* also led to a decrease in IL-8 compared to control, or *L. jensenii* alone (Figure 28C). MPA was confirmed to be able to attenuate the effect of *L. crispatus* (SJ-3C-US) increased IL-8 production in Vk2 cells (Figure 29B). In addition, E2 (10^{-9} M) treatment also seemed to be able to abrogate the effect of *L. crispatus* (SJ-3C-US) on IL-8 production, as E2 (10^{-9} M) + *L. crispatus* (SJ-3C-US) led to decreased IL-8 production ($p=0.07$) when compared to *L. crispatus* (SJ-3C-US) alone (Figure 29C).

Overall, this data suggests that MPA is able to reduce the level of IL-8 below basal levels in Vk2 cells while *L. crispatus* (SJ-3C-US) was able to increase the levels of IL-8 in Vk2 cells. MPA was also able to reduce the levels of IL-8 in Vk2 cells regardless of any Lactobacilli treatment. In addition, when there is stimulus which causes increased IL-8 production (*L. crispatus* (SJ-3C-US)), E2 (10^{-9}M) was able to attenuate IL-8 production, although not to the same extent as MPA. Levels of IL-8 in basolateral media treated with hormones and Lactobacilli were also examined. It was found that the levels of IL-8 in the basolateral supernatants resembled those found in the apical compartment of the respective experiment (Figure 30).

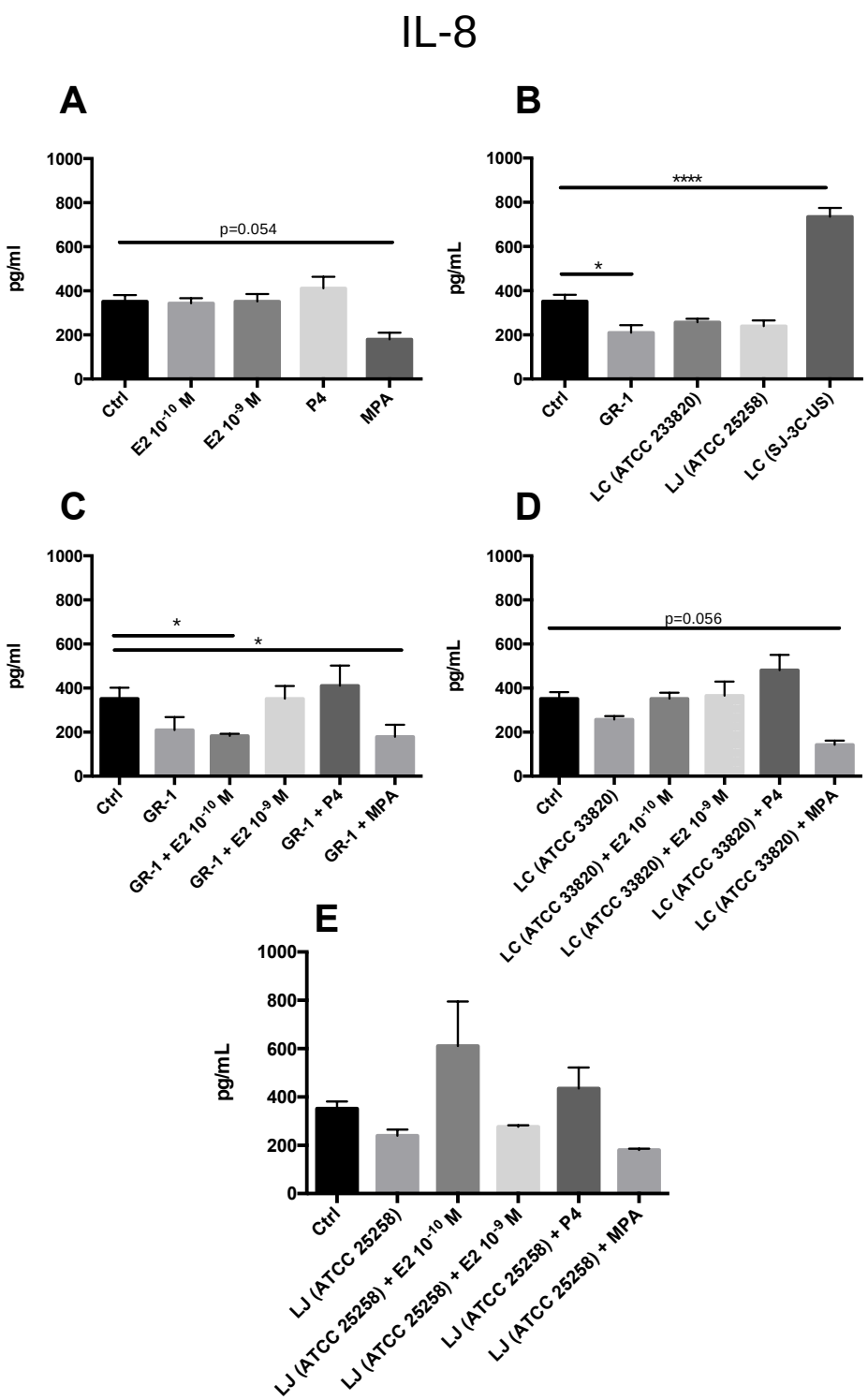

Figure 25: Levels of IL-8 in the apical media of Vk2 cells treated with different hormones, and/or different Lactobacilli. **(A)** Hormone effect **(B)** Lactobacilli effect **(C)** Effect of GR-1 + different hormones. **(D)** Effect of LC (ATCC 33820) + different hormones. **(E)** Effect of LJ (ATCC 25258) + different hormones. Data shown represents one representative experiment, done in two/three replicates. Data was analyzed using one-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

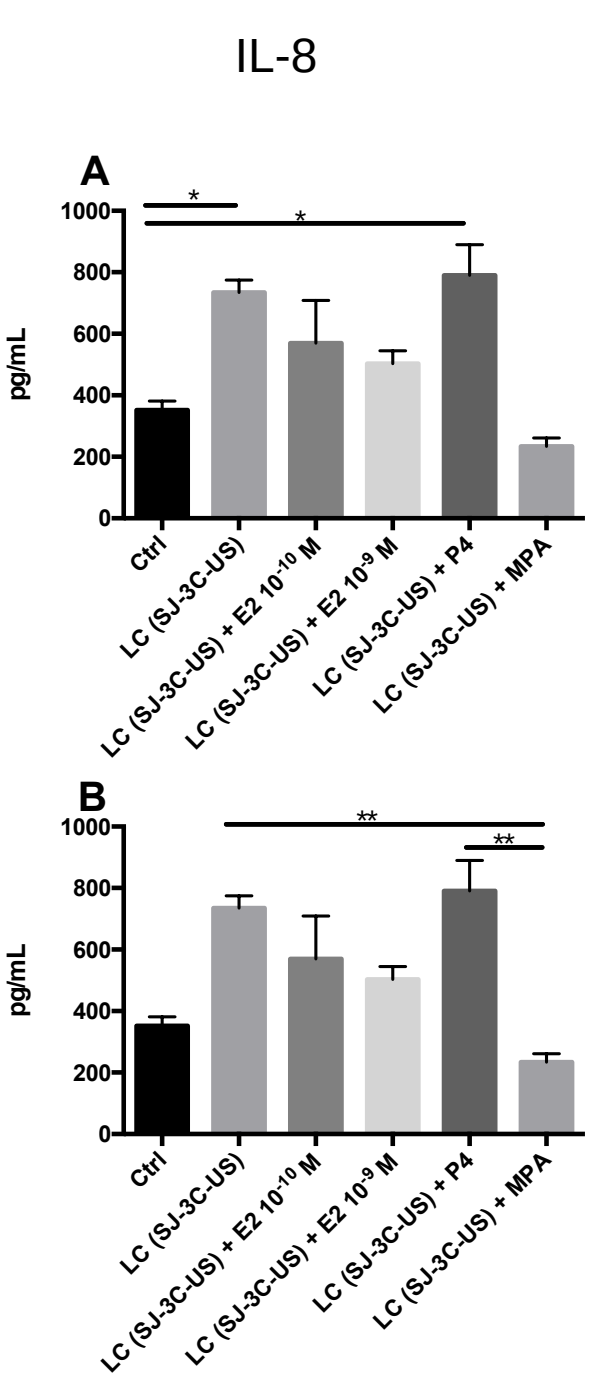

Figure 26: Level of IL-8 in the apical media of Vk2 cells treated with different hormones, and/or *L. crispatus* (SJ-3C-US). **(A)** Effect of LC (SJ-3C-US) + different hormones compared to control (no hormone/no Lactobacilli) (**B)** Effect of LC (SJ-3C-US) + different hormones compared to LC (SJ-3C-US). Data shown represents one representative experiment, done in two/three replicates. Data was analyzed using one-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

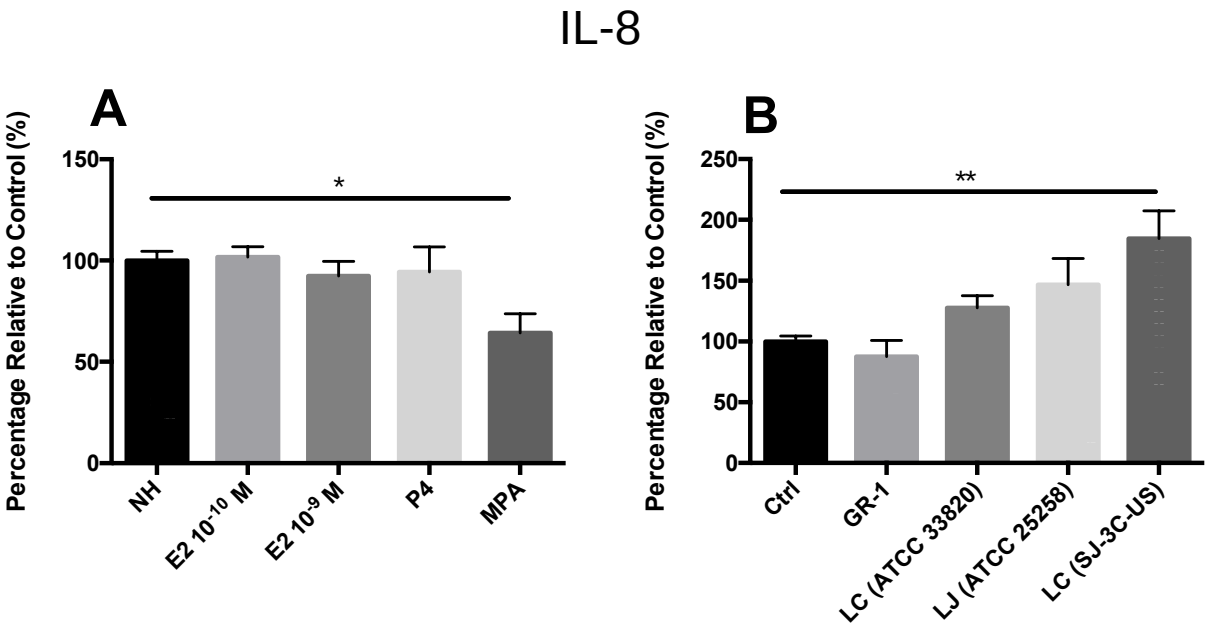

Figure 27. Levels of IL-8 in the apical media of Vk2 cells is decreased with MPA treatment and increased with *L. crispatus* (SJ-3C-US) treatment. (**A)** Hormone Effect. (**B)** Lactobacilli Effect Data shown represents combined, and normalized data from two separate experiments, each with two/three experimental replicates. Data was analyzed by one-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

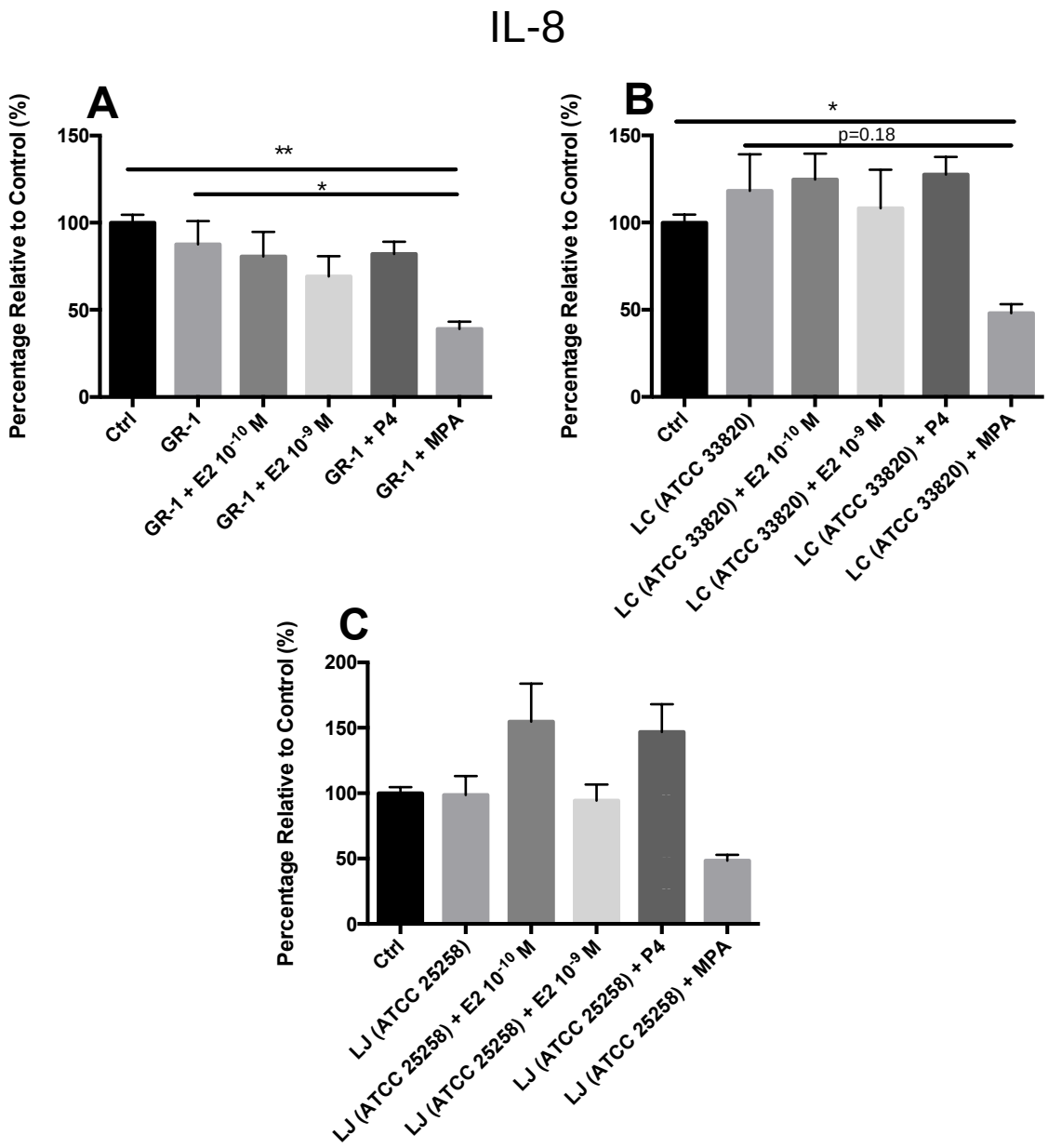

Figure 28: Levels of IL-8 is in the apical media of Vk2 cells is decreased in the presence of MPA regardless of the different Lactobacilli treatment. (**A)** GR-1 + different hormones. (**B)** LC (ATCC 33820) + different hormones. (**C)** LJ (ATCC 25258) + different hormones. Data shown represents combined, and normalized data from two separate experiments, each with two/three experimental replicates. Data was analyzed by one-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. *p<0.05, **p<0.01. Error bars shown represent SEM.

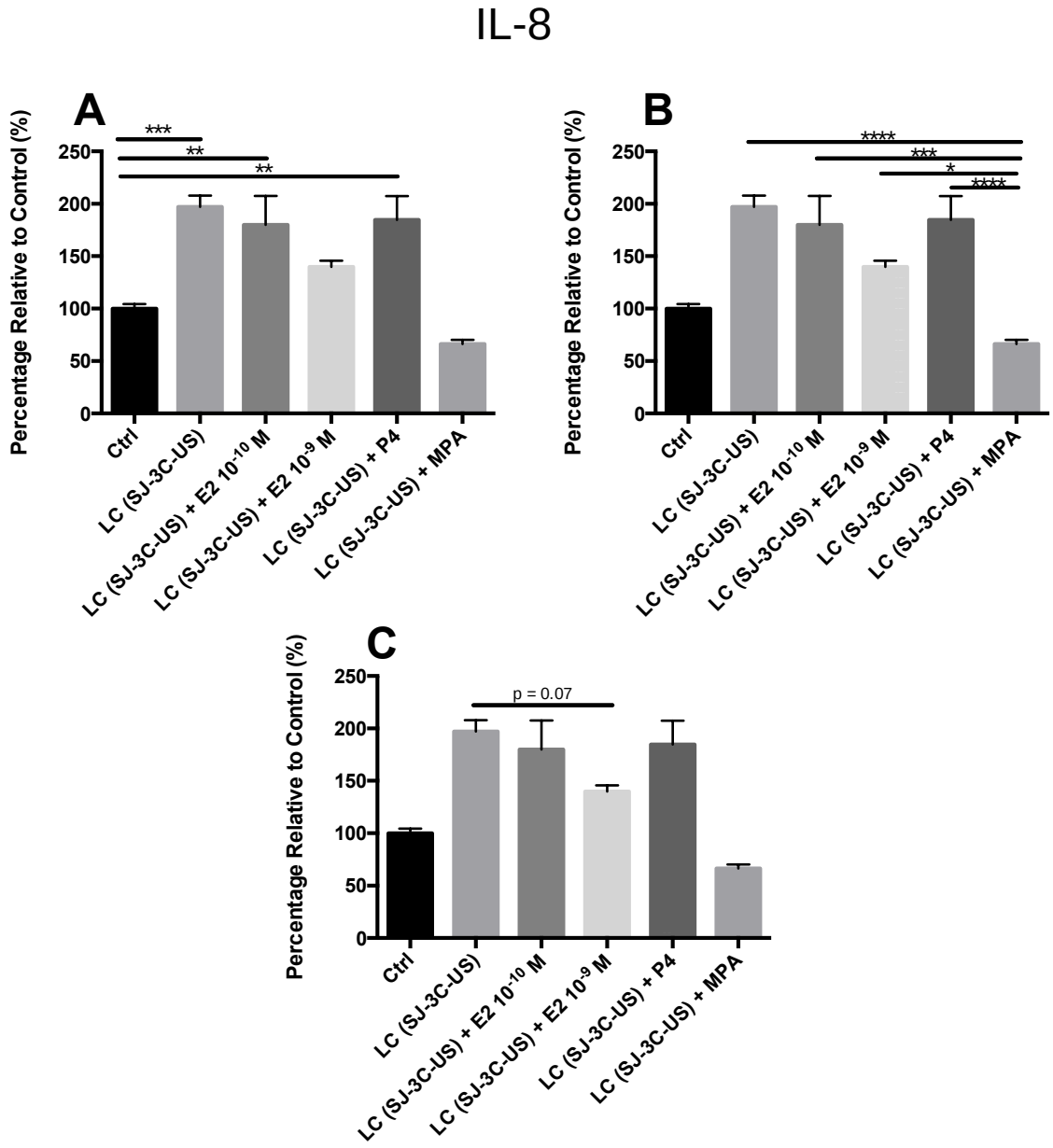

Figure 29: MPA, and E2 (10^{-9} M) is able significantly abrogate *L. crispatus* (SJ-3C-US) induced increased IL-8 in the apical media of Vk2 cells. **(A)** Levels of IL-8 in the apical media of Vk2 cells of *L. crispatus* (SJ-3C-US), and hormone + *L. crispatus* (SJ-3C-US) treatments. Comparisons were made to the control. **(B)** Levels of IL-8 in the apical media of Vk2 cells of *L. crispatus* (SJ-3C-US), and hormone + *L. crispatus* (SJ-3C-US) treatments. All other treatment combinations were compared to *L. crispatus* (SJ-3C-US) + MPA treated Vk2 cells. **(C)** Levels of IL-8 in the apical media of Vk2 cells of *L. crispatus* (SJ-3C-US), and hormone + *L. crispatus* (SJ-3C-US) treatments. All treatments were compared to *L. crispatus* (SJ-3C-US) + E2 (10^{-9} M). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars shown represent SEM.

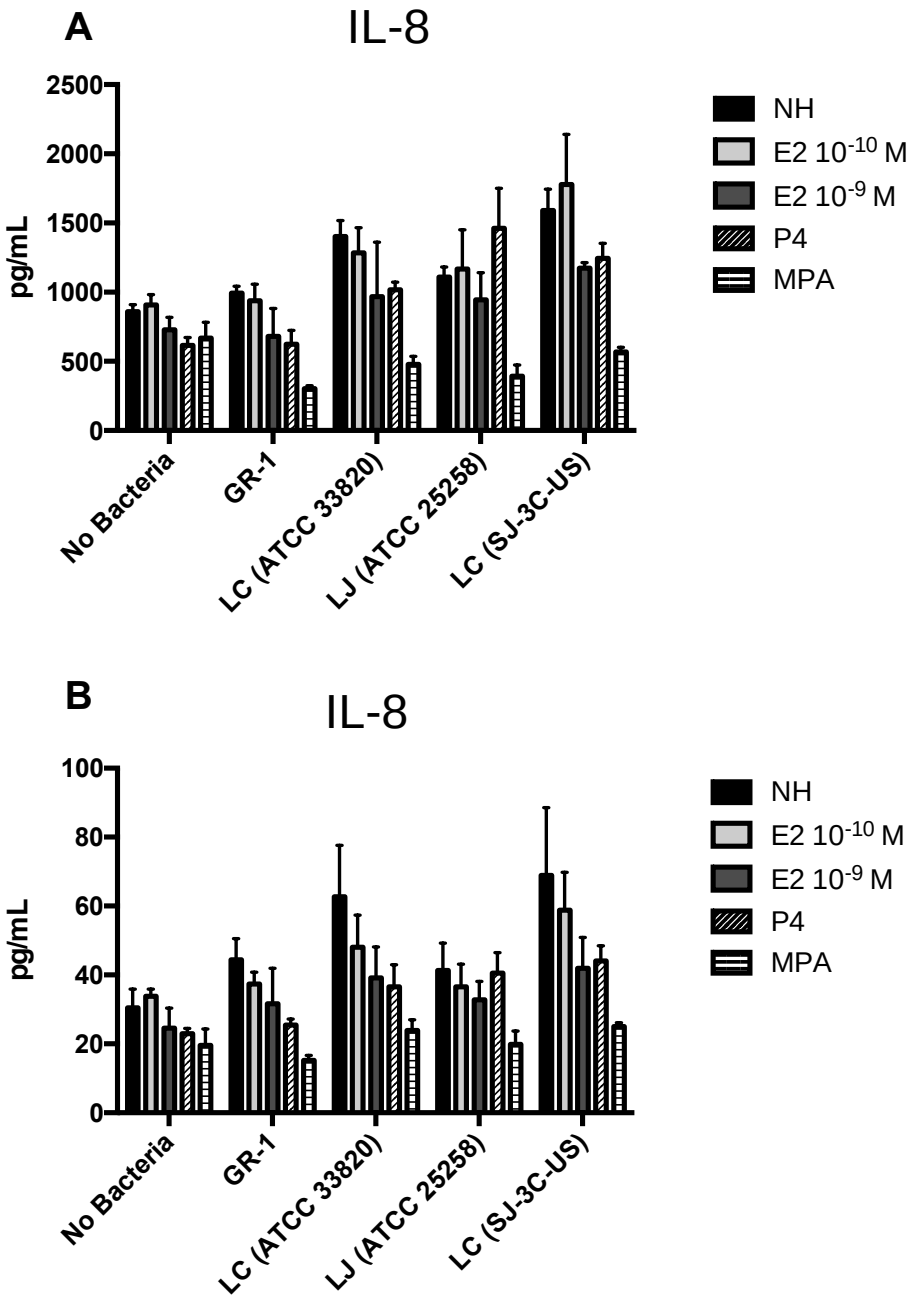

Figure 30: Level of IL-8 in apical **(A)**, and basolateral **(B)** media of Vk2 cells are similar in different hormonal, and Lactobacilli treatments. Data shown represents one experiment, done in two/three replicates. Data was analyzed using Two-way ANOVA, with Holm-Sidak Test to correct for multiple comparisons. Error bars shown represent SEM.

3.3 Aim 3: Identifying the effect of different Lactobacilli species on the Transcriptome of ALI Cultured Vk2 Cells

Although endogenous vaginal Lactobacilli have been known to play a key role in protection against STIs, such as HIV, in the vaginal tract, there have not been any studies which examined how endogenous Lactobacilli is able to accomplish this. Therefore, in the third aim of our study, we sought to examine any changes in gene expression when vaginal epithelial cells were co-cultured with different species of Lactobacilli. To do this, Vk2 cells were grown in ALI culture, and co-cultured with different *Lactobacillus spp.* (*Lactobacillus rhamnosus* GR-1, *Lactobacillus crispatus* (ATCC 33820), *Lactobacillus jensenii* (ATCC 25258), *Lactobacillus crispatus* (SJ-3C-US)) for 24 hours. Subsequently, RNA of Vk2 cells were extracted and sent for microarray analysis. In order to ensure that the data gathered from the transcriptomics analysis is consistent, several separate experiments were done. Four separate experiments were done for *L. rhamnosus* GR-1, *L. crispatus* (ATCC 33820), and *L. jensenii* (ATCC 25258), and three for *L. crispatus* (SJ-3C-US).

To ensure that different Lactobacilli species had consistent effects on Vk2 cells throughout separate experiments, a multidimensional (MDS) scaling on the 1000 most variable probe sets was done (Figure 31A). The MDS plot confirms that there is clustering of different Lactobacilli species throughout separate experiments, indicating that it is indeed a Lactobacilli effect (Figure 31).

Interestingly, it was also observed that that certain Lactobacilli treatments had tighter clustering than others (Figure 31A). Next, hierarchical clustering analysis was done on differentially expressed genes to visualize similarities, and differences of different Lactobacilli treatments. Interestingly, hierarchy clustering analysis of differentially expressed genes reveal that all four of the Lactobacilli species had similar effects on Vk2 cells, as most upregulated and downregulated genes are similar across different Lactobacilli treatments (Figure 31B). In addition, the plot also show that *L. jensenii* seemed to have the pronounced effect as seen by the higher intensity of downregulated/upregulated genes (Figure 31B).

To identify which gene pathways were being differentially regulated in the presence of Lactobacilli, genes which were differentially expressed compared to control due to Lactobacilli treatment and met the adjusted p value of 0.05 were subjected to DAVID (the database for annotation, visualization and integrated discovery) annotation clustering. As we are analyzing a large number of genes, an adjusted p value was utilized to ensure that there is a higher significance threshold that genes which are required to meet decrease false positives.

To ensure that all conclusions being drawn from this dataset are valid, only gene clusters which had an enrichment score of 1.3 or higher were utilized (Huang da, Sherman, & Lempicki, 2009). An enrichment score of 1.3 or higher corresponds to a p value of 0.05 or lower in the positive log scale (Huang da, Sherman, & Lempicki, 2009). Annotated clusters of differentially expressed genes of different

Lactobacilli treatments of Vk2 cells are shown in Tables 2,3,4,5,6,7,8. The full list of differentially regulated genes of Lactobacilli treated Vk2 cells are available in a separate supplementary excel document attached at the end of the thesis. Upregulated and downregulated genes which met the adjusted p value of 0.05 were differentiated and inserted into the DAVID functional annotation tool. To compare and contrast the effects of different Lactobacilli on Vk2 cells, Venn diagrams (Figure 32, 33, 34) were constructed from the data gathered from Tables 2,3,4,5,6,7,8. As the four variable Venn diagram cannot identify differences which are only shared between groups which are across from each other (Figure 32), additional Venn diagrams were constructed to compensate for this (Figure 33).

Interestingly, it was observed that all Lactobacilli treatments led to increased expression of cell-cell junctional associated transcripts by Vk2 cells, as seen in Figure 32. In addition, it was observed that Lactobacilli treatment often led to upregulation of gene clusters relating to protein biosynthesis, and/or transcription (Figure 32). Notably, *L. crispatus* (SJ-3C-US) treatment led to upregulation of a unique cluster of genes relating to inflammation in Vk2 cells. In addition, vaginal *L. crispatus* (SJ-3C-US), and *L. jensenii* (ATCC 25258) also commonly upregulated gene clusters relating to adhesion and diapedesis of lymphocytes/granulocytes in Vk2 cells (Figure 33). There were no downregulated gene clusters for vaginal *L. crispatus* (SJ-3C-US) treated Vk2 cells which met the filter parameters, therefore, only the effect of the other three different Lactobacilli are seen in Figure 34. Cell cycle associated genes are all downregulated in *L. rhamnosus* (GR-1), *L. crispatus*

(ATCC 33820), and *L. jensenii* (ATCC 25258) treated Vk2 cells. Interestingly, *L. crispatus* (ATCC 33820), and *L. jensenii* (ATCC 25258) uniquely downregulated genes associated with interferon responses and/or interferon signalling pathways.

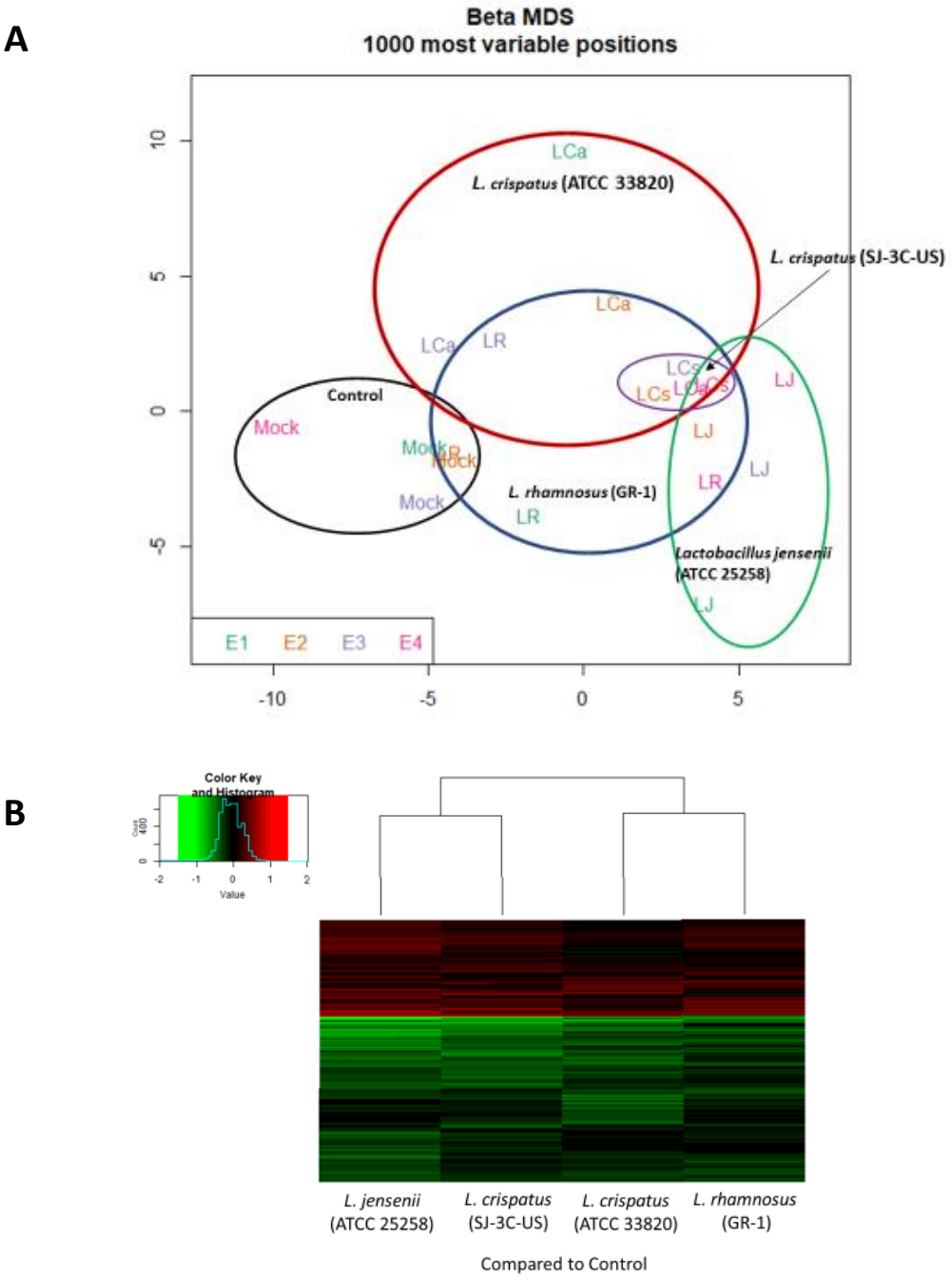

Figure 31. Differences seen in the transcriptomes of Lactobacilli treated Vk2 cells cluster based on treatment. The four different Lactobacilli have a similar effect on Vk2 cells. **(A)** MDS plot of 1000 most variable probes. Clustering was done manually and only for visual reference. **(B)** hierarchy clustering analysis of differentially expressed genes of different Lactobacilli co-cultured Vk2 cells.

Table 2: Upregulated gene clusters of *L. rhamnosus* GR-1 co-cultured Vk2 cells

***Lactobacillus rhamnosus* (GR-1)**		**Upregulated Genes**
Cluster Number	**Annotated Cluster Description**	**Enrichment Score**
1	**Nucleotide-binding**	**7.68**
2	**Nucleus**	**6.73**
3	**Protein biosynthesis**	**5.66**
4	**rRNA processing**	**3.67**
5	**focal adhesion**	**2.77**
6	**Cell-cell adherens junction**	**1.73**
7	**Translation**	**1.50**

Table 3: Downregulated gene clusters of *L. rhamnosus* GR-1 co-cultured Vk2 cells

***Lactobacillus rhamnosus* (GR-1)**		**Downregulated Genes**
Cluster Number	**Annotated Cluster Description**	**Enrichment Score**
1	**Cell cycle**	**2.01**
2	**Chromosome**	**1.73**
3	**GTPase activation**	**1.67**

Table 4: Upregulated gene clusters of *L. crispatus* (ATCC 33820) co-cultured Vk2 cells.

***L. crispatus* (ATCC 33820)**		**Upregulated Genes**
Cluster Number	**Annotated Cluster Description**	**Enrichment Score**
1	**cell-cell adherens junction**	**3.18**
2	**mitochondrion**	**2.22**
3	**endoplasmic reticulum lumen**	**2.12**
4	**Actin-binding**	**1.88**

Table 5: Downregulated gene clusters of *L. crispatus* (ATCC 33820) co-cultured Vk2 cells.

***L. crispatus* (ATCC 33820)**		**Downregulated Genes**
Cluster Number	**Annotated Cluster Description**	**Enrichment Score**
1	**Centromere**	**3.47**
2	**DNA damage**	**2.96**
3	**DNA replication**	**2.47**
4	**positive regulation of interferon-beta production**	**1.71**
5	**DNA repair**	**1.42**

Table 6: Upregulated gene clusters of *L. jensenii* (ATCC 25258) co-cultured Vk2 cells.

***L. jensenii* (ATCC 25258)**		**Upregulated Genes**
Cluster Number	**Annotated Cluster Description**	**Enrichment Score**
1	**Protein biosynthesis**	**7.98**
2	**Cell-Cell Adherens Junction**	**7.37**
3	**rRNA Processing**	**5.97**
4	**Nucleotide Binding**	**5.88**
5	**Mitochondrion**	**3.81**
6	**rRNA Processing**	**3.76**
7	**Chaperone**	**3.58**
8	**Stress Response**	**2.38**
9	**Protein Stabilization**	**2.06**
10	**Initiation Factor**	**1.91**
11	**ATP-dependent RNA helicase activity**	**1.70**
12	**Adhesion and Diapedesis of Lymphocytes/Granulocytes**	**1.52**
13	**WD Repeat**	**1.41**

Table 7: Downregulated gene clusters of *L. jensenii* (ATCC 25258) co-cultured Vk2 cells

***L. jensenii* (ATCC 25258)**		**Downregulated Genes**
Cluster Number	**Annotated Cluster Description**	**Enrichment Score**
1	**Cell Cycle**	**3.53**
2	**Serine Protease**	**2.51**
3	**Chromosome**	**2.50**
4	**Interferon Signaling Pathway**	**2.26**
5	**Autophagy**	**1.92**
6	**ATP-binding**	**1.78**
7	**Transcription**	**1.77**
8	**Cell cycle**	**1.75**

Table 8: Upregulated gene clusters of *L. crispatus* (SJ-3C-US) co-cultured Vk2 cells.

***L. crispatus* (SJ-3C-US)**		**Upregulated Genes**
Cluster Number	**Annotated Cluster Description**	**Enrichment Score**
1	**Protein biosynthesis**	**3.76**
2	**ATP-binding**	**3.04**
3	**Inflammatory response**	**2.98**
4	**Mitochondrion**	**2.93**
5	**cell-cell adherens junction**	**2.80**
6	**nucleolus**	**1.97**
7	**Adhesion and Diapedesis of Lymphocytes/Granulocytes**	**1.73**

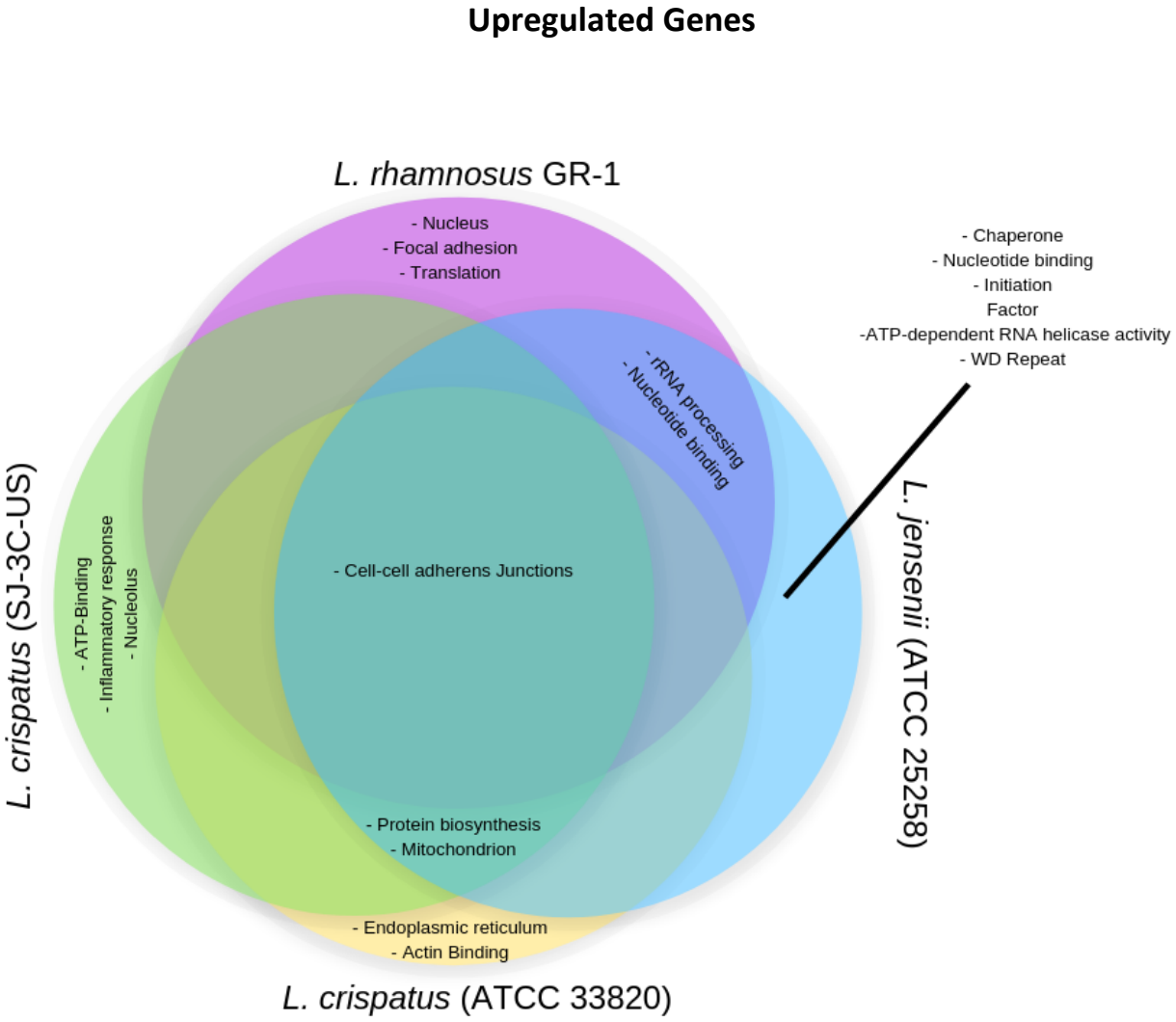

Figure 32: Similar and different upregulated gene clusters of different Lactobacilli treated Vk2 cells shown in a Venn diagram. Genes which are uniquely shared across (e.g. *L rhamnosus* GR-1, and *L. crispatus* (ATCC 33820) could not be shown in this Venn diagram, and is shown in the next figure.

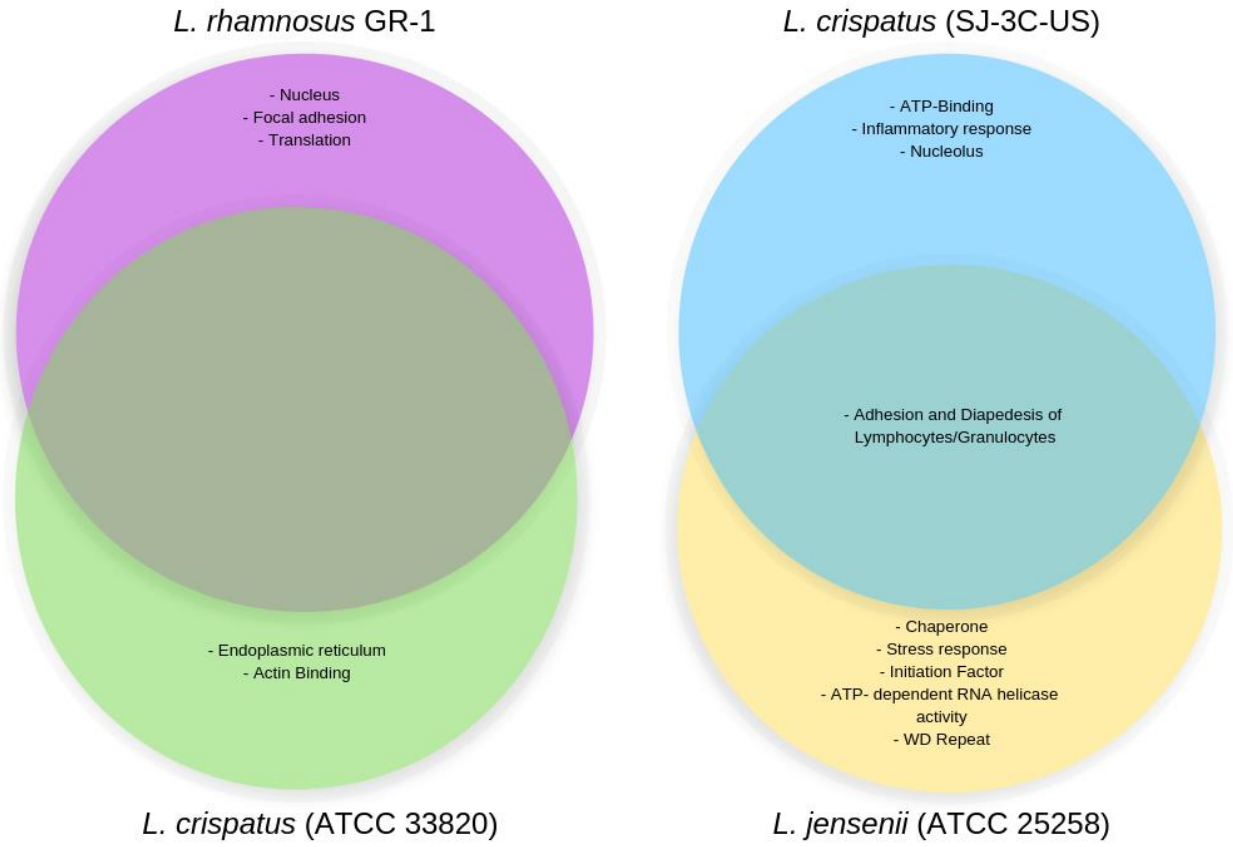

Figure 33: Upregulated gene clusters which are only shared between *L. rhamnosus* GR-1 and *L. crispatus* (ATCC 33820) and *L. crispatus* (SJ-3C-US) and *L. jensenii* (ATCC 25258).

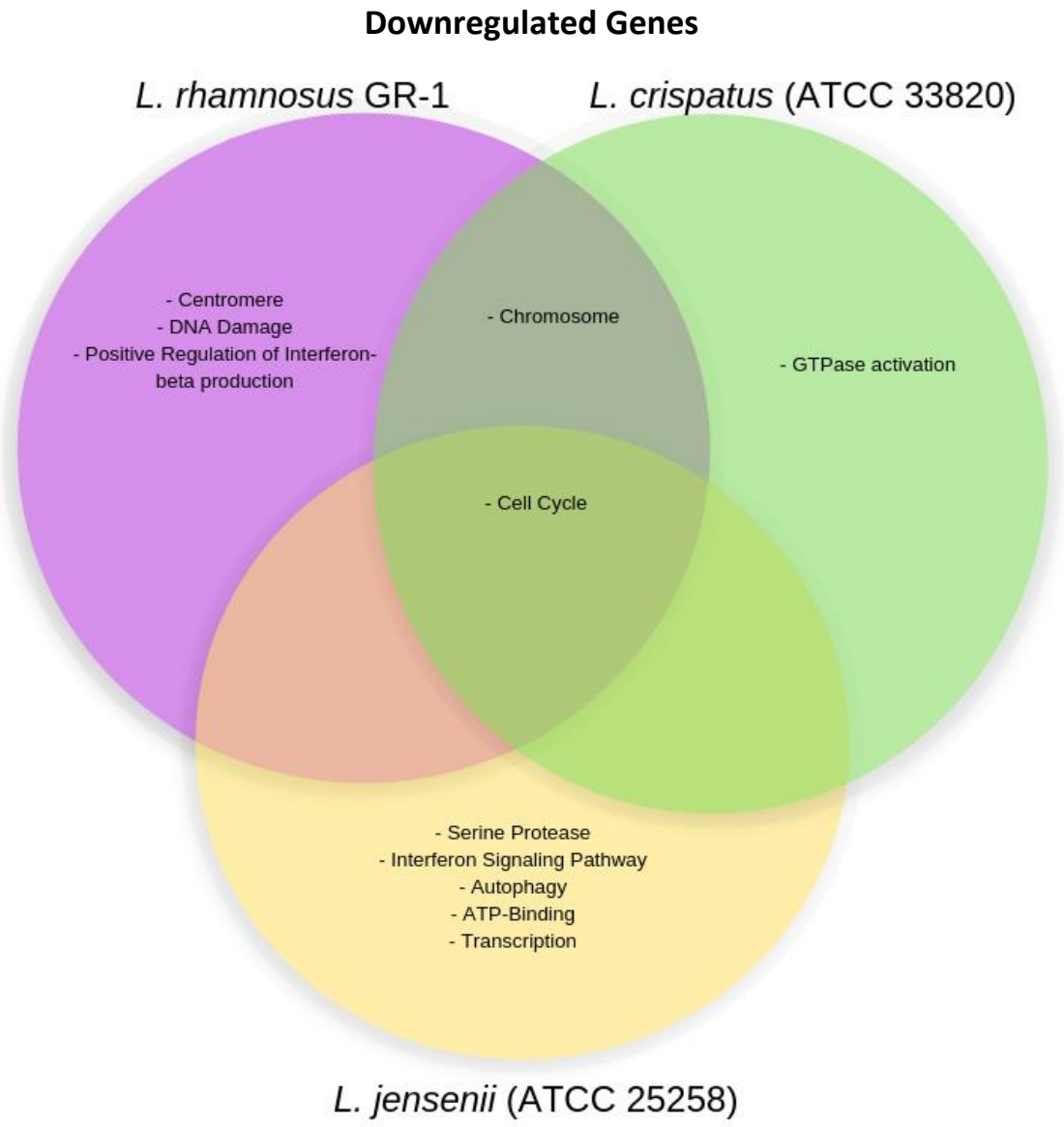

Figure 34: Similar and different downregulated gene clusters of different Lactobacilli co-cultured Vk2 cells.

CHAPTER 4: DISCUSSION

Two key factors that are consistently shown to alter HIV susceptibility in the FRT are the presence of female sex hormone

s and the vaginal microbiota (Vitali, Wessels, & Kaushic, 2017; Wessels et al., 2018). Women with a *Lactobacillus* dominant vaginal microbiota are less susceptible to STIs, including HIV (Petrova et al., 2013; Vitali et al., 2017; Wessels et al., 2018). Although there are large epidemiological studies demonstrating this, there are limited focused studies investigating the direct protective effects of *Lactobacillus* on vaginal epithelial cells. In addition, female sex hormones and contraceptives, such as E2 and MPA, play pivotal roles in altering susceptibility to HIV in the FRT. E2 has consistently been shown to be protective in many studies (Dupont et al., 2018; Hel et al., 2010; Wessels et al., 2018). A recent meta-analysis study also showed that MPA usage in women is associated with increased susceptibility to HIV (Ralph et al., 2015). In addition, there is substantial evidence which support that MPA usage in women leads to increased HIV susceptibility (Hapgood et al., 2018). Therefore, the aim of our study was to establish an *in vitro* vaginal cell culture system to examine these factors independently and in the context of HIV susceptibility. To do this, we expanded on an *in vitro* vaginal (ALI) cell culture system which we recently developed. Cells grown in this system display characteristics that are more physiologically relevant to vaginal cells *in vivo* (Lee et al., 2016). In order to examine how these factors may impact HIV susceptibility

in the FRT, we chose to examine readouts that are associated with HIV susceptibility *in vitro*. These readouts for testing barrier functions included tests to measure permeability/membrane integrity of Vk2 cells in ALI culture, levels of pro-inflammatory/anti-inflammatory cytokines and chemokines, and comparative transcriptomic analysis.

We first performed preliminary experiments in order to establish an experimental timeline (Figure 1). Frozen stock Lactobacilli were inoculated until they reached log phase, where they were harvested and used for subsequent co-culture experiments. The OD reading was correlated with bacterial count (CFU/mL) to ensure that a consistent amount of Lactobacilli were utilized for each experiment (Figure 2). The full experimental schematic is shown in Figure 3. We next performed experiments to ensure that the experimental treatments (Lactobacilli/hormone) were not negatively impacting the cell viability of our ALI grown Vk2 cells (Figure 4,5).

After ensuring that these parameters remained unaltered by our treatment conditions, we next examined whether the bacterial replication of our various Lactobacilli strains would differ when they were grown in KSFM alone or co-cultured with Vk2 cells.

Out of all four Lactobacilli strains we tested, *L. rhamnosus* GR-1 grew the best in both KSFM and in Vk2 co-culture (Table 1). The results indicate that out of all the Lactobacilli strains tested, *L. rhamnosus* GR-1 was able to thrive the best

due to efficient utilization of nutrients present in KSFM. The bacterial count of vaginal *L. jensenii* (ATCC 25258) was not altered after 24 hours in KSFM alone for both starting concentrations, however, the load significantly increased after co-culturing with Vk2 cells for 24 hours (Table 1). These results show that *L. jensenii* was not able to thrive on KSFM alone, as seen in a lack of replication. However, interaction with vaginal epithelial cells led to higher bacterial count after 24 hours (Table 1), indicating that *L. jensenii* was able to thrive better when in the presence of Vk2 cells. Similar to vaginal *L. jensenii* (ATCC 25258), ocular *L. crispatus* (33820) was also able to replicate better when in the presence of Vk2 cells (Table 1). In contrast, vaginal *L. crispatus* (SJ-3C-US) bacterial replication was not different when grown in KSFM alone, or co-cultured with Vk2 cells (Table 1). These results suggest that the presence of Vk2 cells does not benefit this strain of Lactobacilli nutritionally.

The bacterial ecosystem within the human body is complex. The gut microbiome alone is comprised of multiple bacterial genus (Hillman, Lu, Yao, & Nakatsu, 2017). Metabolites produced by one bacteria could then be utilized by another and vice versa, showcasing the complexity of a microbial ecosystem as occurs in the gut (Wrzosek et al., 2013) and, to a lesser extent, the vagina (Ravel et al., 2011).

In our study however, we are utilizing an *in vitro* system where bacteria are cultured in isolation with Vk2 cells and are not exposed to other bacterial strains.

Therefore, our system does not account for potential nutrients that may be produced by other endogenous vaginal bacteria, only nutrients from KSFM or Vk2 cells are available for utilization.

In gut related studies, the gut microbiota is known to be able to utilize host derived nutrients (Sonnenburg et al., 2005). For instance, previous studies have shown that a prominent gut bacterium, *Bacteroides thetaiotaomicron* was capable of utilizing host derived carbohydrate sources when polysaccharides were not present in diet, indicating the presence of host-bacterial metabolic interaction (Sonnenburg et al., 2005). Although there have not been any studies which showcase host-bacterial metabolic interaction in the vaginal tract, this is highly likely as the vaginal tract does not receive any external nutritional sources, unlike the gut. In agreement with this, vaginal Lactobacilli have been shown to have smaller genome sizes compared to intestinal Lactobacilli, which indicates that vaginal Lactobacilli are more reliant on host cells for nutrition, rather than external sources (Mendes-Soares, Suzuki, Hickey, & Forney, 2014). In fact, the niche where bacteria reside will often dictate which functional genes are expressed. Genes which code for products which other bacteria, or the host cells provides will often undergo genomic reduction through evolution, as stated by the Black Queen Hypothesis (Mendes-Soares et al., 2014). For instance, when plant associated Lactobacilli evolved to adapt to the intestinal environment, genes responsible for transporting plant associated carbohydrates such as cellobiose, and sucrose were lost in transition (Mendes-Soares et al., 2014). In our results, it was observed that

certain Lactobacilli strains (vaginal *L. jensenii* (ATCC 25258), ocular *L. crispatus* (ATCC 33820)) replicated better in the presence of Vk2 cells. Therefore, it could be speculated that these Lactobacilli strains may be able to better utilize nutrients in our *in vitro* system when in conjunction with Vk2 cells due to possible metabolic interactions. However, more studies will need to be done in examine these potential metabolic interactions between vaginal epithelial cells and Lactobacilli.

One possible reason as to why *L. rhamnosus* GR-1 was able to replicate best in KSFM, and co-culture may be due to its large genome size relative to the other Lactobacilli used in this study. The genome of *L. rhamnosus* GR-1 is 2.89 Mbp (Petrova et al., 2018), whereas the genome size of vaginal *L. crispatus*, and vaginal *L. jensenii* ranged from 1.73 - 2.35 Mbp, and 1.59 Mbp - 1.76 Mbp respectively (Mendes-Soares et al., 2014). Therefore, the observation that *L. rhamnosus* GR-1 optimally utilized nutrients available in our *in vitro* system is fitting as a larger genome size likely means it has the capacity to utilize more diverse array of nutrients. As mentioned previously, previous Lactobacilli studies found that a smaller genome size was associated with a phenotype which was more host-dependent. *Lactobacillus* species with smaller genome sizes are more likely to rely on host cells, and/or surrounding bacteria for nutrients. For instance, *L. iners* which has a genome size of 1.3 Mbp rely heavily on host cells, and surrounding bacteria for nutrients (Macklaim, Gloor, Anukam, Cribby, & Reid, 2011; Mendes-Soares et al., 2014). Due to its small genome size, *L. iners* lacked the ability to synthesize amino acids *de novo* (Macklaim et al., 2011), in addition, a large percentage of the

L. iners genome was found to code for genes which were responsible for transport of metabolites, indicating its dependence on host cells, or surrounding microorganisms for nutrients, and its lack of metabolic capacity (Mendes-Soares et al., 2014). In contrast, a larger genome size was associated with a phenotype which resembled more of a free-living organism, which depended less on host cells for nutrients and survival (Mendes-Soares et al., 2014)..

Since vaginal *L. jensenii* has an overall smaller genome size than vaginal *L. crispatus*, it is possible that it may rely more on host cells for nutrients, rather than *de novo* synthesis. This could be a possible explanation to why we observed that our vaginal *L. jensenii* counts were higher with Vk2 cells, whereas this was not observed with vaginal *L. crispatus*. Although this is a possible explanation, more studies will need to be conducted to explore this further.

When examining the adherence of different Lactobacilli species in our co-culture system, it was observed that vaginal *L. crispatus* (SJ-3C-US) had the highest adherence to Vk2 cells in cocultures compared to the three other Lactobacilli species tested (Figure 9). However, we did not see any differences in bacterial replication in Vk2 co-culture when Vk2 cells were grown in the presence of hormones (Figure 10). In literature, estrogen is associated with increased Lactobacilli populations in the vaginal tract (Shen et al., 2016), whereas MPA usage was associated with decreased Lactobacilli (Mitchell et al., 2014). Although, the exact mechanism of how E2, and MPA could affect vaginal Lactobacilli

bacterial load *in vivo* is uncertain, it is speculated that these hormones are able to alter the availability of carbohydrates in the vaginal microenvironment, which ultimately impacts Lactobacilli bacterial load in the vaginal tract. In particular, E2 has been thought to increase glycogen content in the vaginal tract (Petrova et al., 2013), whereas MPA usage has been associated with decreased glycogen and amylase in the vaginal tract of women (Wessels et al, 2018). The only source of carbohydrate present in KSFM is glucose, therefore, the carbohydrate source is likely not a nutritional bottle neck for Lactobacilli replication in our system. It is therefore likely the bacterial counts will not change whether Vk2 cells are grown under the presence of E2, or MPA in our system, as the mechanism of how these hormones alter Lactobacilli bacterial load *in vivo* is through changing the abundance of carbohydrates present in the vaginal microenvironment.

To address the first aim outlined in my thesis, I have optimized a Vk2 and Lactobacilli co-culture model. Specifically, we have optimized an experimental timeline for our *in vitro* system, and we have shown that cell viability of Vk2 cells were not affected by Lactobacilli and/or hormone treatment. In addition, we have also shown that certain Lactobacilli strains are able to better replicate with Vk2 cells, which may indicate possible metabolic interaction between the two.

We next examined whether hormonal treatments or Lactobacilli co-culturing could affect the barrier function of ALI grown Vk2 cells. It was observed that ALI cultured Vk2 cells grown in the presence of MPA had significantly lower TERs

when compared to no hormone treated Vk2 cells on days 6 and 7 of culture (Figure 11). Similarly, FITC-dextran leakage assays performed in Vk2 cells treated with MPA also showed significantly higher leakage compared to control (Figure 15A). MPA was the main factor contributing to decreased Vk2 cell barrier function in our system, as any treatment combination that included MPA, regardless of the presence of Lactobacilli, led to increased FITC-dextran leakage in Vk2 cells (Figure 16). As discussed previously, the barrier function of the vaginal epithelial is integral in decreasing susceptibility to HIV, as the virus needs to cross the epithelial barrier to reach underlying target cells. Although, MPA usage in women is associated with decreased barrier function in the vaginal epithelium (Hapgood et al., 2018), this evidence was gathered primarily from animal models such as mice and macaques (Butler et al., 2015; Butler et al., 2016; Quispe Calla et al., 2016). The vaginal epithelium of these animals is structurally different from humans, as it is keratinized in contrast to the human vaginal epithelium which undergoes cornification (D. J. Anderson et al., 2014; Poonia et al., 2006). The effect of MPA on the barrier function vaginal epithelium in humans remain inconclusive, as there are multiple studies with contradicting results (L. Bahamondes et al., 2000; M. V. Bahamondes et al., 2014; Miller et al., 2000). However, observations gathered from our study are the first piece of experimental evidence which demonstrates that MPA does indeed directly impact the barrier function of the human vaginal epithelium in a negative manner. This may translate to increased HIV susceptibility *in vivo*, as the virus is able to cross the

compromised epithelium easier. Our group has also gathered transcriptomic data regarding ALI grown Vk2 cells treated with MPA (Woods et al, unpublished). In agreement with previous results, we found that Vk2 cells treated with MPA had an overall decreased expression of genes associated with cell-cell adhesion and cell cycle (Woods et al, unpublished). Thus, in the *in vitro* ALI vaginal epithelial cell system, MPA treatment leads to decreased expression of cell-cell adhesion gene clusters, which could result in impaired barrier function, as seen in the form of increased FITC-dextran leakage and decreased TERs seen in this study.

Although estrogen, and progesterone has been shown to thicken and thin the vaginal epithelium, respectively, these conclusions were chiefly gathered from macaque studies (Poonia et al., 2006; Smith et al., 2004). In one study which examined the thickness of the vaginal epithelium between the progesterone high and estrogen high phases of the menstrual cycle in humans, the authors found a small but significant difference in thickness, although it is likely that it is not clinically relevant (Patton et al., 2000). There has been one study which found that estradiol treatment led to increased TERs in Vk2 cells (Wagner & Johnson, 2012), however, this study was performed using an *in vitro* Vk2 monolayer system which may not accurately represent what occurs *in vivo*. In our study, we did not see any effect of estradiol in increasing barrier function (Figure 15A). In agreement with this, transcriptomics data gathered from estradiol treated ALI grown Vk2 cells did not reveal any differential expression of gene clusters related to barrier function (Woods et al, unpublished). Although it is unclear why progesterone did not have

an effect on barrier function in our system (Figure 15A), there are studies which show that the expression of the progesterone receptor (PR) in the vaginal epithelium of mice is dependent on the presence of estradiol (Kurita et al., 2000). As Vk2 cells are passaged, they are grown in media lacking hormones. Therefore, it may be that Vk2 cells lose expression of PRs due to lack of estradiol, and therefore are minimally responsive to progesterone. Interestingly, although MPA is a progestin-based contraceptive, which exerts its effects through the PR, it has been shown previously to also bind to the GR which is ubiquitously expressed in Vk2 cells (Bamberger et al., 1999; Govender et al., 2014). This is likely the reason why MPA exerted such a potent effect in our system, while progesterone did not.

Although one of the postulated mechanisms of Lactobacilli mediated protection against HIV in the vaginal tract is through enhancement of the vaginal epithelial barrier (Petrova et al., 2013), there have not been any studies which demonstrate this. We have previously shown that the barrier function of primary endometrial genital epithelial cells was enhanced when they were co-cultured with probiotic Lactobacilli (Dizzell et al, unpublished). Therefore, we sought to examine whether Lactobacilli co-cultured Vk2 cells would have enhanced barrier function. It was observed that there was no difference in FITC-dextran leakage or TERs of Vk2 cells co-cultured with Lactobacilli (Figure 15A, 12A). Although we did not find any functional differences in cell integrity through FITC-dextran leakage or TER, transcriptomic data gathered from Vk2 cells co-cultured with Lactobacilli revealed that Vk2 cells did indeed upregulate cell-cell adhesion related gene clusters

(Figure 32). This will be discussed more in-depth in a subsequent section. Functional and transcriptomic data regarding the barrier function of MPA treated Vk2 cells were in agreement as mentioned previously. However, it was interesting that transcriptomic, and functional data regarding Lactobacilli treated Vk2 cells did not correlate with each other. This could be possibly explained by the fact that our vaginal epithelial cells were only co-cultured with Lactobacilli for only 24 hours, whereas hormonal treatments lasted for eight days prior to analysis. Therefore, it can be postulated that Lactobacilli could enhance barrier function of Vk2 cells, if given enough time.

It is well established that higher levels of pro-inflammatory cytokines/chemokines (IL-1α, IL-1β, IL-6, IL-8, TNF-α, MIP-1α, MIP-1b, IP-10) in the FGT increases susceptibility to HIV (Masson et al., 2015). We have previously shown that elevated pro-inflammatory levels could lead to impairment of barrier function in primary genital endometrial cells (Nazli et al., 2010; Nazli et al., 2013). In addition to this, higher levels of pro-inflammatory cytokines in the FGT is associated with increased numbers of HIV target cells (Kaul et al., 2015). Previous studies also show that higher levels of chemokine RANTES was associated with increased target cells in the FGT (Kaul et al., 2008). In contrast, anti-inflammatory cytokines such as IL-10, and IL-1RA have been with decreased HIV susceptibility as they counteract the effects of pro-inflammatory cytokines (Corley, 2000; Kwon & Kaufmann, 2010).

Therefore, we next examined the effects of Lactobacilli and hormones on the cytokine production of ALI grown Vk2 cells. Out of the six cytokines which were examined, only IL-1RA, IL-8, and RANTES were affected by our treatments. We observed that MPA was the main cause of elevated RANTES levels in our Vk2 cell cultures and this effect was observed regardless of Lactobacilli treatment (Figure 21). Similarly, MPA was the main factor increasing IL-1RA production in Vk2 cells (Figure 24). Any treatment condition which had MPA led to higher production of IL-1RA in Vk2 cells, regardless of the presence of Lactobacilli (Figure 24). Interestingly, when examining the levels of pro-inflammatory cytokine IL-8 in Vk2 cells, it was observed that treatment with the vaginal strain of *Lactobacillus crispatus* (SJ-3C-US) led to significantly higher levels (Figure 27B). When examining the effect of hormonal treatments alone, MPA treatment led to significantly decreased levels of IL-8 in Vk2 cells, even compared to baseline control (Figure 29A). It was also observed that MPA was able to attenuate the effect of vaginal *L. crispatus* (SJ-3C-US) induced increased IL-8 levels (Figure 29B). Interestingly, E2 (10^{-9} M) was also able to attenuate the effect of *L. crispatus* (SJ-3C-US) (Figure 29C). This was interesting, as E2 (10^{-9} M) alone was not able to decrease the levels of IL-8 below the control baseline, however, it was able to attenuate the effect of *L. crispatus* (SJ-3C-US).

It is clear that increased levels of pro-inflammatory cytokines in the FGT leads to increased susceptibility to HIV (Masson et al., 2015). Similar to the observations gathered from leakage experiments, it was observed in our study that

MPA was once again the main factor altering cytokine production in Vk2 cells. MPA exerted an effect regardless of the presence of Lactobacilli in all cases. In literature, the effect of MPA on the cytokine production of epithelial cells remains controversial, as there are many conflicting reports on whether MPA has an overall pro-inflammatory or anti-inflammatory effect on these cells (Hapgood et al., 2018). For instance, certain clinical studies show that MPA have an overall pro-inflammatory effect (Francis et al., 2016; Deese et al., 2015). There are also epithelial cell culture studies present which show that MPA exerts a pro-inflammatory response (increased IL-8, RANTES, TNF-a) (Ferreira et al., 2015). Whereas other epithelial cell studies report that MPA has an anti-inflammatory effect (decreased IL-8, IL-6) (Govender et al., 2014). Therefore, we sought to examine its effect on vaginal epithelial cells. Although there are previous studies that examined the effect of MPA on the cytokine production of vaginal epithelial cells, the findings from these studies may not accurately represent what is actually occurring, as these studies utilized concentrations well beyond the physiological concentration (Africander, Louw, Verhoog, Noeth, & Hapgood, 2011; Irvin & Herold, 2015). MPA levels range from 0.8 – 1 ng/mL (2.1 nM – 2.6 nM) 30 days after administration in women (Hapgood et al., 2018). The study conducted by Africander et al utilized 1uM MPA (1000 nM), which is approximately 500 times higher than the MPA serum concentration 30 days after administration (Africander, Louw, et al., 2011). Similarly, the study conducted by Irvin et al utilized concentrations which ranged from 15ng/mL – 15,000ng/mL (Irvin & Herold, 2015).

Therefore, there is a definite need to elucidate the effect of physiological levels of MPA on the cytokine production of vaginal epithelial cells. In this study, we utilized a physiological concentration of MPA measured 60 days after administration (1nM) in our studies (Mishell, 1996).

Overall, our results indicate that physiological levels of MPA are able to suppress pro-inflammatory cytokine IL-8, increase anti-inflammatory cytokine IL-1RA, and increase chemokine RANTES production in vaginal epithelial cells. We have previously shown similar observations in the primary endometrial genital epithelial cells of the URT, where physiological levels of MPA upregulated chemokines, but not pro-inflammatory cytokines (Ferreira et al., 2015). From the literature, it is clear that MPA has a varied role in altering the cytokine profile of epithelial cells and it is inconclusive whether MPA exerts an overall pro-inflammatory or anti-inflammatory effect (Hapgood et al., 2018). However, in clinical studies, it has been consistently shown that CVLs collected from women using MPA have elevated RANTES levels (Deese et al., 2015; Fichorova et al., 2015; Morrison et al., 2014). There are also several studies that show that women utilizing MPA have increased $CCR5^{+}$ target cells in vaginal tissues and samples collected from cytobrush (Byrne et al., 2016; Chandra et al., 2013). Therefore, there is solid evidence that MPA increases RANTES levels in the FGT. RANTES is an chemokine that has a chemotactic effect on HIV target cells, however, it is also able to prevent HIV entry by binding onto the CCR5 co-receptor (Cocchi et al., 1995; Dupont et al., 2018). Although RANTES have been shown to inhibit HIV

entry through receptor competition, it is likely that an increased level of RANTES in the FGT will have an overall detrimental effect, as increased number of target cells at the initial site of exposure will increase the chance of productive HIV infection (Dupont et al., 2018). The data gathered in our study indicates that vaginal epithelial cells may be one of the major contributors to the elevated levels of RANTES observed in the FRT of women using MPA, similar to what was observed in primary endometrial genital epithelial cells (Ferreira et al., 2015). Consistent with literature, we observed that MPA did not exert a uniform pro-inflammatory effect, as we also observed an anti-inflammatory effect of MPA through the down-regulation of IL-8 and upregulation of IL-1RA.

Interestingly, vaginal *L. crispatus* (SJ-3C-US) treatment led to a significant increase in IL-8 in Vk2 cells (Figure 27B). This was a surprising finding as vaginal Lactobacilli are not known to exert any pro-inflammatory effect on vaginal epithelial cells (Petrova et al., 2013; Rose et al., 2012). However, this could possibly be explained by the fact that vaginal *L. crispatus* (SJ-3C-US) does not fare well in our co-culture system, as seen in the significant decrease in bacterial count after 24 hours (Figure 7D). It is known that when bacteria experience nutritional stress, they can express certain genes to aid in their survival. For instance, when *E. coli* are grown in nutrient poor conditions, adherence related factors are expressed to ensure survival (Alverdy, Zaborina, & Wu, 2005). Due to the inability to utilize the nutrients in available in our system, vaginal *L. crispatus* (SJ-3C-US) may be expressing factors that enhance survival but may induce inflammation in vaginal

epithelial cells. Further studies are required to confirm this. Another interesting observation seen in our study was the anti-inflammatory effect of E2. When Vk2 cells were stimulated with IL-8 inducing *L. crispatus* (SJ-3C-US), E2 (10^{-9} M) was able to abrogate this effect (Figure 29C). In literature, estradiol is generally associated with an overall anti-inflammatory effect in the FGT (Hel et al., 2010; Wessels et al., 2018; Wagner & Johnson, 2012). Therefore, the finding that E2 exerts an anti-inflammatory effect in our study is consistent with literature. The anti-inflammatory effect of E2 was also observed in another study involving vaginal epithelial cells (Wagner & Johnson, 2012). The authors in this study observed that IL-8 upregulation induced by a combination of vaginal probiotic bacteria (*L. rhamnosus* GR-1, *L. reuteri* RC-14) could be attenuated if cells were treated with E2 (10^{-9} M). Altogether, these results indicate that E2 is able to dampen the inflammatory response of vaginal epithelial cells when they encounter pro-inflammatory stimuli.

When examining the cytokine levels between the apical, and basolateral side of the cell cultures we observed that basolateral cytokine levels often resembled apical cytokine levels but was lower concentration between different treatments. A possible reason as to why we observe this could be that there is no differential secretion of these cytokines to either side, however, cytokine contents from sloughed off apical superficial cells would result in a higher concentration of cytokines in the apical side of our culture system. In agreement with this hypothesis, clinical studies were able to correlate the inflammatory cytokine levels

collected from CVLs, which would be equivalent to cytokines present in the apical side of the epithelium, to increased HIV susceptibility (Masson et al., 2015).

Although we have previously investigated the effects of hormonal treatments on the transcriptome of Vk2 cells (Woods et al, unpublished), there is still little information regarding the effect of Lactobacilli on transcriptome of Vk2 cells. Therefore, in our final aim, we examined the transcriptomic changes in Vk2 cells after 24 hour co-culture with Lactobacilli. We found that all four Lactobacilli strains used in this study upregulated cell-cell adhesion gene clusters according to DAVID (the database for annotation, visualization and integrated discovery) (Figure 32). These results indicate that Lactobacilli are capable of enhancing the barrier function of vaginal epithelial cells. Interestingly, when examining the individual genes within these clusters, many of them were associated with focal adhesions.

Focal adhesions (FAs) are cellular structures that connect an epithelial cell to the underlying extracellular matrix (ECM) in the basement membrane (Epifano & Perez-Moreno, 2012). The FA consists of two segments, the cytoplasmic and transmembrane segments both anchor the cell to the underlying basement ECM. The cytoplasmic segment consists of FA associated proteins such as talin and vinculin whereas the transmembrane segment consists of integrins responsible for anchoring to the basement ECM. As of now, focal adhesions have been mainly characterized as junctional proteins which is responsible for the adherence of the

basal cell layer to the underlying basement ECM. There is currently no evidence that focal adhesions exist within the upper layer of cells in the vaginal epithelium, however, if they do exist, they would be able to further strengthen the barrier integrity of the vaginal epithelium. FAs do not only play a structural role in ensuring that the epithelial layer is firmly attached onto the underlying ECM, but they also play key roles in regulating cell-cell adhesion and wound healing (Epifano & Perez-Moreno, 2012). For instance, it has been shown that knockdown of certain integrins led to decreased cell-cell adhesion in HeLa cells (Yano et al., 2004). In mice, kidney epithelial cells which were deficient in the integrin α3β1 showed decreased cadherin to catenin binding, indicating decreased cell-cell adhesion (Wang et al., 1999). In addition, it has been shown that when focal adhesion signaling is disrupted in mice, and human epithelial cells, wound healing was impaired due to increased expression of matrix metalloproteinase 9 (MMP-9) (Wong et al., 2014). Altogether, these studies indicate that focal adhesions in epithelial cells are not only crucial structurally but are also key players in regulating cell-cell adhesion and wound healing.

In our dataset, it was observed that the four different *Lactobacillus spp*. which we tested upregulated certain key genes associated to focal adhesions such as vinculin, zyxin, and/or PDLIM1. Vinculin is an adapter protein which is present in both cell-ECM, and cell-cell adhesion complexes (Blaskewicz et al., 2011; Carisey & Ballestrem, 2011). It plays a critical role in the formation of FAs and it has been shown that mutation of its binding site resulted in failure to form FAs

(Humphries et al., 2007). The focal adhesion associated protein zyxin plays a critical role in wound healing and cell-cell adhesion (T. N. Nguyen, Uemura, Shih, & Yamada, 2010). Zyxin deficient canine kidney epithelial cells had weaker cell-cell adhesion, as they were easily separated in a clustering assay compared to wild type cells (T. N. Nguyen et al., 2010). In addition, wound healing was also negatively affected in zyxin deficient cells (T. N. Nguyen et al., 2010). The PDLIM1 gene is another critical gene responsible for the formation of FAs (Tamura, Ohno, Katayama, Kanayama, & Sato, 2007). A recent study showed that the PDLIM1 gene plays a critical role in the stabilization of adherens junctions, PDLIM1 knock-down cells had decreased E-cadherin expression, resulting in decreased cell-cell adhesion (Chen et al., 2016). It is known that cell-cell adhesions can determine the cell integrity of the epithelial layer. In our study, all four different *Lactobacillus spp.* tested upregulated gene clusters relating to cell-cell adhesion according to the DAVID database. This indicates that Lactobacilli is indeed able to enhance the barrier function of vaginal epithelial cells. Upon further inspection, the four different Lactobacilli tested also all upregulated genes related to FAs, which is surprising, as there was no prior literature that directly associated FAs and HIV susceptibility. However, according to results gathered from other epithelial cell studies discussed above, it is evident that FAs do not only serve a structural purpose, but also regulate the strength of cell-cell adhesions and wound healing. Therefore, based on the results gathered from this study, it is highly possible that Lactobacilli are also able to enhance the strength of the epithelial layer through cell-ECM

adhesion, cell-cell adhesion, and perhaps also enhance wound healing in the vaginal tract. In agreement with this, it has been previously shown through proteomic analysis that vaginal samples gathered from women with a *Gardnerella vaginalis* dominant vaginal microbiota had higher levels of proteins relating to decreased barrier integrity and wound healing compared to a *L. iners* dominant vaginal microbiota (Zevin et al., 2016). However, functional studies will need to be performed to determine whether this is indeed an accurate finding in our system.

Another finding in our Lactobacilli-vaginal cell transcriptomic study is that gene pathways relating to protein synthesis (endoplasmic reticulum, translation, rRNA, tRNA chaperone), energy synthesis (nucleotide binding, mitochondria), and nucleus related genes were upregulated. It is known that when cells need to produce an abundance of proteins, genes relating to endoplasmic reticulum stress are upregulated (Pluquet, Pourtier, & Abbadie, 2015). For instance, when B cells differentiate into antibody secreting plasma cells, endoplasmic reticulum stress genes are upregulated as an essential part of differentiation (Gass, Gifford, & Brewer, 2002). It is also well known that protein synthesis is an energy exhaustive procedure, as ATP is utilized in many steps during protein synthesis (Pontes, Sevostyanova, & Groisman, 2015). As discussed above, the different Lactobacilli in our system are able to exert a clear effect on vaginal epithelial cells in a manner that enables them to upregulate cell-cell/cell-ECM associated genes. Therefore, when vaginal cells exhibit a change in their phenotype due to the surrounding

Lactobacilli in our system, it is likely that the metabolic pathways relating to energy and protein synthesis will also be affected as seen in our results.

It was also observed that vaginally isolated endogenous vaginal Lactobacilli (*L. jensenii* (ATCC 25258) and *L. crispatus* (SJ-3C-US)) also upregulated gene clusters associated with adhesion and diapedesis of lymphocytes/granulocytes in the Vk2 cells. However, these gene clusters were both relatively less significant compared to the ones discussed above and consisted of only of 3-4 genes each (Supplementary Files). In addition, in the case of *L. jensenii*, the three genes in the clusters were IL-8, IL-1A, and I-CAM1 (Supplementary Files). In our cytokine analysis, we did examine whether vaginal *L. jensenii* (ATCC 25258) altered the products of these genes (IL- 8, IL-1α), but we did not see any significant changes (Figure 18B, Figure 27B). On the contrary, as discussed previously, we observed that vaginal *L. crispatus* (SJ-3C-US) co-cultured Vk2 cells had significantly higher levels of IL-8 (Figure 27B). Transcriptomic data also reflect this, as there was an upregulated gene cluster associated with the inflammatory response according to the DAVID database (Table 8).

When examining the downregulated gene clusters, it was observed that both ocular *L. crispatus* (ATCC 33820) and vaginal *L. jensenii* (ATCC 25258) downregulated genes associated with interferon responses in vaginal epithelial cells (Figure 34). In particular, the data gathered from *L. jensenii* treated vaginal cells is relatively significant, as it had a high enrichment score (2.6) and the gene

cluster comprised of seven genes (Supplementary Table). The interferon response is associated with protection against HIV, particularly in the context of restriction factors, which are a part of the interferon stimulating genes (ISGs) (Soper et al., 2017). In addition, we have recently shown that interferon beta is protective against HIV-mediated barrier impairment in primary endometrial genital epithelial cells (Nazli et al., 2018). These studies often examine the role interferon in the context of acute infection. For instance, administering interferon receptor blocking antibodies to rhesus macaques challenged with SIV resulted in decreased expression of restriction factors, leading to increased depletion of T-cells (Sandler et al., 2014). Although the interferon response has been shown to be protective during acute infection, there are not many studies which examine whether chronic heightened sensitivity to interferon prior to exposure may alter susceptibility. It could be argued that dampened sensitivity to interferon may provide a beneficial effect in vaginal epithelial cells in the context of HIV. Many PRRs, including TLRs, utilize adapter proteins, which are ISGs, in their signaling pathways (Takaoka & Yanai, 2006). For instance, the family of interferon regulatory factors (IRFs) are ISGs (Takaoka & Yanai, 2006) and are also common adapter proteins in the signaling pathways of various TLRs (Watters, Kenny, & O'Neill, 2007). Therefore, when the interferon signaling pathway is dampened due to downregulation of interferon associated genes, it could also lead to downregulation of ISGs, which are involved in downstream signaling of TLRs. Ultimately, this could result in dampened TLR signaling, leading to a less inflammatory microenvironment. This

was interesting, as the vaginal *L. jensenii* strain had the most polarizing effect in downregulating this pathway next to the *L. crispatus* strain which was isolated from the eye. However, it could be speculated that the vaginal *L. crispatus* strain would also have the same effect, if it was able to maintain a higher bacterial count in our co-culture system after 24 hours (Figure 7D).

We also observed that vaginal *L. jensenii* (ATCC 25258) uniquely downregulated genes associated with serine proteases. Increased levels of proteases are present in women with an elevated pro-inflammatory cytokine profile in the FGT (Arnold et al., 2016). In our study, vaginal *L. jensenii* (ATCC 25258) primarily downregulated the family of Kallikrein-related peptidases (KLKs) (Supplementary Table). KLKs play a major role in degradation of the ECM (Sotiropoulou & Pampalakis, 2010). In particular, they have been shown to degrade the ECM of skin cells and thus play a key role in wound healing (Sotiropoulou & Pampalakis, 2010). Aberrant upregulation of KLKs is also associated with increased inflammation in skin diseases, such as psoriasis (Sotiropoulou & Pampalakis, 2010). Subcutaneous administration of KLK5 in mice led to increased infiltration of neutrophils and increased inflammation at the site of injection (Yamasaki et al., 2007). Therefore, the finding the *L. jensenii* uniquely downregulated serine proteases such as KLKs was interesting, as this could be another mechanism by which vaginal Lactobacilli are able ensure the integrity of the epithelial layer and reduce inflammation.

Finally, we observed that *L. rhamnosus* (GR-1), ocular *L. crispatus* (ATCC 33820), and vaginal *L. jensenii* (ATCC 25258) all downregulated genes associated with cell cycle in vaginal epithelial cells. This could be explained by an overall change in phenotype of vaginal cells when in co-culture with Lactobacilli (Figure 32). For instance, upregulation of cell-cell adhesion/cell-ECM associated genes may induce the cell to divide less frequently. However, it should be noted that vaginal *L. crispatus* (SJ-3C-US) also upregulated gene clusters related to cell-cell adhesion but did not downregulate any gene clusters which met the stringent criteria we utilized to filter our data. Therefore, another possible scenario as to why this was observed is that vaginal *L. crispatus* (SJ-3C-US) was not as capable of utilizing nutrients available in the media compared to the other Lactobacilli, as discussed previously (Figure 7). If the other Lactobacilli were able to utilize some of the nutrients available, this may limit the replication of vaginal epithelial cells due to decreased nutrient availability in culture.

CHAPTER 5: ADVANTAGES AND DISADVANTAGES

5.1 Strengths of the study

A major strength of this study is the use of the ALI Vk2 culture system. As discussed previously, the ALI Vk2 cell culture system is more physiologically relevant than other studies which utilize the liquid-liquid interface culture system (Lee et al., 2016). For instance, ALI grown Vk2 cells form of multiple layers, which is more representative of squamous epithelial cells of the vaginal tract. As a result, this study is likely to provide more accurate insight when compared to other studies. When examining the role of hormones, we utilized physiological level of hormones in our study, which many other similar studies fail to take into consideration. We utilized the concentration of MPA found in the serum of women 60 days after administration (Mishell, 1996). Whereas other similar studies which examine the effect of MPA on vaginal cells utilize concentrations well beyond the MPA serum level 30 days after administration (Africander, Louw, et al., 2011; Hapgood et al., 2018; Irvin & Herold, 2015). Therefore, the results gathered from our study offer valuable insight regarding the effect of physiological levels of MPA on vaginal epithelial cells, where as other studies may not. From literature, it is also clear that the origin of the bacterial strain plays a role in its ability to affect host cells (Rose et al., 2012). We therefore utilized four different Lactobacilli strains in our study in order to compare and contrast their effects on vaginal epithelial cells. Specifically, we utilized two *L. crispatus* strains of differing origins, vaginal *L.*

jensenii, and probiotic *L. rhamnosus* (GR-1) in our studies. Finally, we also examined the transcriptomic changes that vaginal epithelial cells undergo when they were co-cultured with different Lactobacilli. This was particularly informing, as this was the first study which did this. As a result, the data provided insights into several possible protective mechanisms of how Lactobacilli may exert its protective effect on vaginal epithelial cells *in vivo* against HIV.

5.2 Limitations and Future Directions

Although we used an in vitro system that is more physiologically relevant, findings from this study may not directly correlate to what is occurring *in vivo.* The vaginal microbiota consists of multiple genus of bacteria (Ravel et al., 2011), a major limitation within our study is that we only examined the effects of one Lactobacilli at a time on vaginal epithelial cells. Therefore, possible nutritional interactions between different vaginal bacteria was not accounted for in our study. In addition, if bacteria are under stress they may produce bacterial products which aid in survival (Alverdy et al., 2005). This may in turn lead to aberrant reactions from host cells which may not accurate represent what is occurring *in vivo*. Therefore, future studies should aim to address this by using multiple genus of bacteria. *In vivo*, the vagina is lined by mucus which is secreted by glands in the cervix (Gipson, 2001). Therefore, in future studies the addition of mucus should also be considered to make the system more physiologically relevant.

CHAPTER 6: CONCLUSION

The vaginal epithelial barrier represents an essential barrier against STIs, such as HIV. The goal of this project was to investigate the effects of Lactobacilli, and female sex hormones on vaginal epithelial cells in an *in vitro* system, in the context of HIV susceptibility. To do this, we first optimized an *in vitro* Vk2 and Lactobacilli cell co-culture system. We then examined the barrier function, and cytokine profiles of Vk2 cells when they were treated with different Lactobacilli, and/or hormones. Finally, we examined the transcriptomic changes in Vk2 cells after co-culture with Lactobacilli.

The main findings in this study were that MPA was able to 1) decrease barrier function of ALI Vk2 cell cultures as demonstrated through decreased TERs, and increased FITC-dextran leakage. 2) increase chemokine production of RANTES in ALI Vk2 cell cultures. Overall, this suggests these may be two contributing mechanisms of how MPA increases HIV susceptibility *in vivo*. In addition, in our study, certain Lactobacilli species tested had increased replication when co-cultured with Vk2 cells, which may indicate metabolic interaction. We also show that *Lactobacillus spp.* treated Vk2 cells differentially express certain gene clusters which may have implications in decreasing HIV susceptibility. This study successfully established an *in vitro* model where the effect of Lactobacilli and/or female sex hormones on vaginal epithelial cells was studied. In addition, this study provided insights into several possible mechanisms of how vaginal Lactobacilli are

able to exert its protective effects on vaginal epithelial cells against HIV through transcriptomic analysis.

This study will lay the foundation for the establishment of *in vitro* models which aims to better resemble *in vivo* conditions. In addition, we have also identified several possible novel mechanisms of how Lactobacilli exert its protective effect on vaginal epithelial cells against HIV. This lays the groundwork for future functional studies which will be aimed to verify and assess the weight of these mechanisms.

CHAPTER 7: MATERIALS AND METHODS

7.1 Vk2/E6E7 Air-Liquid Interface Cell Culture

Vk2 cells (ATCC® CRL-2616™) were cultured in antibiotic free keratinocyte serum free media (antibiotic free KSFM) (Thermofisher, Cat.17005042) until 80% confluency. The flask containing the Vk2 cells was washed with phosphate buffered saline (PBS), and trypsinized using trypsin-EDTA. DMEM media containing 10% FBS was added to the trypsinized cells to deactivate the trypsin. The cell containing solution was then vortexed at 1,500 rpm for 5 minutes. Subsequently, the 10% DMEM was decanted, and the cell pellet was resuspended in PBS, and vortexed 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 using a hemocytometer (via trypan blue). 60,000 cells were 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 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. Cells were then incubated at 37 degrees Celsius, 5% carbon dioxide. One day after seeding, the apical media was aspirated to induce air-liquid interface conditions. Basolateral media was replaced with either no hormone, E2 (10^{-10} M), E2 (10^{-9} M), P4 (10^{-7}M), or MPA (10^{-9} M) containing antibiotic free SFM media. Basolateral media was changed every two days.

7.2 Keratinocyte Serum Free Media Preparation and Hormone Media Preparation

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. E2 (10^{-9}M, 10^{-10}M), P4 (10^{-7}M) and MPA (10^{-9}) KSFM containing media was prepared. All prepared media was stored at 4^{o}C.

7.3 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^{o}C, 5% CO_2 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 hormones on TERs of Vk2 cells, TER values measured on day one (prior to the addition of any hormone containing media) was utilized as a reference point. To evaluate the effect of Lactobacilli on TERs of Vk2 cells, day 7 (prior to the addition of Lactobacilli) was utilized as a reference point.) TER was shown as Percent Pre-treatment of TER

The calculations are as follows:

Effect of Hormones:

TER at specific timepoint/TER at day one (prior to addition of any hormones) x 100%

Effect of Lactobacilli + Hormones

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

7.4 Lactobacilli Stock Preparation

Lactobacillus rhamnosus (GR-1), and *Lactobacillus crispatus* (ATCC 33820) were generously provided by Dr. Gregor Reid (Western University). *Lactobacillus jensenii* (ATCC 25258) was purchased from ATCC. *Lactobacillus crispatus* SJ-3C-US (PTA-10138) from ATCC was generously provided by Dr. Nuch Tanphaichitr (University of Ottawa).

All Lactobacilli bacteria was grown in Man, Rogosa & Sharpe (MRS) broth (VWR, Cat. 90004-082) for 24 hours 50mL Falcon tubes at 37^{o}C. Cyrotubes containing 200uL of glycerol was prepared. After 24 hours, the bacterial suspension was vortexed and aliquoted into the appropriately labeled cyrotubes (800uL). To ensure that the glycerol/bacterial suspension was mixed evenly, the cryotubes were

inverted several times before storage. The Lactobacilli stock was stored at -80^{o}C until further use.

The bacterial stock concentration was enumerated by serial dilution (MRS media), then subsequently plated on MRS Agar (VWR, Cat. 90004-084).

7.5 Lactobacilli Growth Curve in MRS Broth

Frozen stock Lactobacilli was added into 10mL of MRS broth in 15mL Falcon tubes. To correlate optical density (OD) with bacterial count (CFU/mL), OD readings were taken using a cell density meter and bacterial count was enumerated by serial dilution and plating. MRS agar plates were incubated in 37^{o}C, anaerobically, using gas packs (VWR, Cat. CA90010--202) for 24 hours. Number of colonies were enumerated by counting. OD values and bacterial count (CFU/mL) were plotted to a linear equation.

7.6 Vk2/E6E7 and Lactobacilli Co-culture

Frozen stock Lactobacilli was inoculated in 15mL polypropylene tubes containing 10mL of MRS broth to reach the following initial bacterial concentration: *Lactobacillus rhamnosus* (GR-1), *Lactobacillus crispatus* (ATCC 33820), *Lactobacillus crispatus* SJ-3C-US: 5.1 x 10^{6} CFU/mL, and *Lactobacillus jensenii*

(ATCC 25258) 7.5 x 10^6 CFU/mL. Optical density (OD) readings were measured using a cell density meter every hour. Once the Lactobacilli has entered log 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 CFU/mL of 6,000,000 CFU/mL, or 600,000 CFU/mL depending on the experimental condition. 100uL of the KSFM bacterial suspension was added onto the apical side of 7 day old ALI grown Vk2 cells, 100uL of antibiotic free KSFM containing no bacteria was added to any experimental conditions containing no bacteria.

7.7 Lactobacilli Growth in KSFM

Frozen stock Lactobacilli was inoculated and grown until log phase in MRS broth, as mentioned above. Once Lactobacilli reached log phase, it was harvested as previously mentioned, and resuspended to a final bacterial concentration of either 6,000,000 CFU/mL or 600,000 CFU/mL in antibiotic free KSFM according to the experimental condition. During selected timepoints, antibiotic free KSFM containing Lactobacilli was vortexed, and 100uL of the solution was serially diluted and plated on MRS agar plates in order to enumerate bacterial count at different timepoints. After plates were inoculated at 37^{o}C for 24 hours, bacterial colonies were enumerated to obtain the bacterial count.

7.8 Lactobacilli enumeration in Vk2 Co-culture

On day 8 of cell culture, after 24 hours of bacterial co-culturing the apical antibiotic free KSFM (100uL) containing Lactobacilli was serially diluted and plated on MRS agar to determine the non-adherent Lactobacilli count (Apical). Subsequently, the apical side of each Vk2 cell culture transwell was washed with PBS. 100uL of 1% Triton-X 100 was added onto the apical side. After 20 minutes of incubation in 37^{o}C, 5% CO_2, the 100uL of 1% Triton-X solution in the apical side of the transwells were serially diluted and plated on MRS agar in order to quantify the adhered Lactobacilli count. After 24 hours, the bacterial colonies were counted. The total Lactobacilli count is the sum of non-adherent Lactobacilli count, and adhered Lactobacilli count. The ratio of adhered bacteria is calculated by the number of adhered bacteria divided by the total bacterial count.

7.9 Vk2 Cell Viability Via Trypan Blue

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 Lactobacilli), the apical media was removed. 100uL of trypsin-EDTA was added onto the apical side of each transwell and incubated at 37^{o}C, 5% CO_2 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.

7.10 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 Lactobacilli), the apical media was collected. The apical media collected was utilized to run a lactate dehydrogenase assay (LDH) (Thermofisher, Cat. 88953) according to the manufacturer's instructions. A maximum lysis control was obtained by adding in 50uL of lysis buffer provided in the kit of one day 8 old ALI Vk2 culture, diluted with antibiotic free KSFM to a final volume of 100uL before being assayed for LDH. LDH activity is represented by absorbance at 490nm subtracted by absorbance at 680nm (background). % cell viability was calculated by dividing the LDH activity of each experimental condition by the LDH activity of the maximum lysis control x 100%.

7.11 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 Lactobacilli), the apical media was removed. 300uL of antibiotic free KSFM containing 10kDa FITC-Dextran

(2.5mg/mL) (Sigma Aldrich, Cat. FD10S250MG) was added onto the apical side of each transwell. After specific timepoints (4, 8, 24 hours), the basolateral media (25uL) 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.

N-9 (Cedarlane Labs) was diluted in KSFM containing 2.5mg/mL of FITC-dextran to a final concentration of 1%, and 100uL of the 1% N-9/FITC-dextran solution was added to the apical side of the 8 day old ALI Vk2 cells. After 24 hours, basolateral media was sampled, and the concentration of FITC-dextran was read as described above.

7.12 Cytokine Quantification of Vk2 Cell Culture 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 Lactobacilli), the apical (100uL) and basolateral (700uL) media was collected. Apical, and basolateral collected Vk2 supernatants were assayed for IL-1α, IL-6, IL-8, IL-10, TNF-α, and RANTES using

a Magpix multiplex kit (Millipore, Cat. HCYTOMAG-60K) according to manufacturer's instructions.

7.13 RNA Extraction and Microarray

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 Lactobacilli), the apical media was removed. RNA was extracted using a RNeasy Plus Mini Kit (Qiagen, Cat. 74134) according to manufacturer's instructions. RNA samples were subsequently sent to the Centre of Applied Genomics, Peter Gilgan Centre for Research and Learning for Microarray analysis using the Affymetrix Human Gene 2.0 ST gene chip.

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