THE ROLE OF FEMALE SEX HORMONES AND LACTOBACILLI ON
GENITAL EPITHELIAL CELL BARRIER FUNCTIONS AND INNATE IMMUNE
RESPONSES IN THE PRESENCE AND ABSENCE OF HIV

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BY

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TITLE: The role of female sex hormones and lactobacilli on genital epithelial cell barrier functions and innate immune responses in the presence and absence of HIV

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Abstract

Background: Approximately 40% of global human immunodeficiency virus-1 (HIV) transmission occurs in the female genital tract (FGT). Epithelial cells lining the FGT comprise the first barrier to HIV-1 entry. The functions of these cells are influenced by female sex hormones and the mucosal microbiota. Studies have suggested that hormonal environment and a dysbiosis of the FGT microbiota may lead to inflammation in the genital mucosa and enhance HIV acquisition. A *Lactobacillus* dominant microenvironment in the FGT is considered to have protective functions against sexually transmitted pathogens, however the interaction between sex hormones and lactobacilli and their effect on epithelial cell functions remains to be determined.

Methods of Study: For these studies, primary genital epithelial cells (GECs) were isolated from hysterectomy tissues obtained following patient consent. GEC cultures were grown to confluence on cell culture inserts in the presence or absence of the female sex hormones estrogen (E2), progesterone (P4), or medroxyprogesterone acetate (MPA). Polarized monolayers were exposed to two probiotic strains of *Lactobacillus*: *L. reuteri* (RC-14) or *L. rhamnosus* (GR-1), or the most common strain of bacteria found in the FGT, *L. crispatus* in the presence or absence of HIV-1. Cell viability, barrier integrity, and innate inflammatory factors were among the primary measures performed.

Results: In our system, cell viability was unaltered in the presence of *Lactobacillus* species and/or female sex hormones. All three strains of bacteria (*L. crispatus* and probiotic lactobacilli GR-1 and RC-14) significantly increased GEC barrier integrity, as measured by transepithelial electrical resistance (TER). Both GR-1 and RC-14 significantly reduced GEC barrier permeability as measured by a dextran dye leakage assay, whereas *L. crispatus* did not. Conversely, hormones did not alter barrier integrity nor barrier permeability. However, hormones did alter secretion of cytokines and chemokins by GECs. GECs grown in the presence of estrogen decreased TNF-α, IL-1α, IL-1β and IL-8 secretion in comparison to no hormone treatment, while GECs grown in the presence of MPA significantly decreased MIP-1α and TNF-α secretion. In the presence of HIV both GR-1 and RC-14 were able to confer an increase in barrier integrity similar to that observed with GR-1 and RC-14 treatment alone. Addionally, GECs grown in the presence of E2 and MPA displayed a less inflammatory (TNF-α, IL-1α, and IL-1β) environment when exposed to HIV compared to no hormone and P4. Interstingly, the decrease in inflammation was not observed when measuring chemokines such as IL-8 and RANTES. Furthermore, probiotic bacteria were able to significantly reduce HIV mediated increases in TNF-α when grown in the presence of no hormone, P4, and MPA. A similar trend was observed for GECs grown in the presence of E2 however, given that E2 reduced the TNF-α response mediated by HIV, results were not significant. Overall, probiotic

lactobacilii GR-1 and RC-14 enhanced GEC barrier functions while E2 and MPA appeared to exert an anti-inflammatory effect on epithelial cell innate responses in both the presence and absence of HIV.

Conclusions: In our system, probiotic lactobacilli enhanced GEC barrier functions and estrogen appeared to exert an anti-inflammatory effect on epithelial innate responses. Enhanced barrier function and decreased inflammation correlate with decreased in HIV acquisition and replication. These studies provide an insight into how factors in the genital microenvironment can affect HIV acquisition in the FGT, and will subsequently assist in the development of prophylactic strategies to reduce HIV transmission.

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<u>Declaration of Academic Achievement</u>

All experiments were conceived and designed by Sara Dizzell and Dr. Charu Kaushic. Dr. Gregor Reid provided the bacterial strains *L. rhamnosus* (GR-1), *L. reuteri* (RC-14), and *L. crispatus.* Sara Dizzell performed all experiments. Sara Dizzell wrote this dissertation with contributions from Dr. Charu Kaushic.

Preface

This thesis is original, unpublished, independent work by Sara Dizzell. It is prepared in the format outlined in the "Guide for the preparation of Master's and Doctoral Theses" available through McMaster University's School of Graduate Studies. Chapter 1 of this thesis serves as a general introduction. The body of this thesis consists of 3 chapters (Chapters 2-4). Chapter 2 describes the rational, hypothesis, and aims. Chapter 3 includes all results for this thesis. Results are organized by each specific aim. The discussion of this thesis (Chapter 4), is divided by aim and summarizes the conclusions, how the results fit in to the current literature and discusses overall implications of the study. The future directions for this work and limitations of the study are described in Chapter 5. Finally, the overarching conclusions of this thesis are summarized in Chapter 6. Lastly, the methodologies for this study are described in Chapter 7. Throughout this thesis, HIV refers to HIV-1 unless otherwise.

Table of Contents

List of Figures

3.1 Aim 2: Examine how the presence of female sex hormones influences barrier function and innate immune responses in genital epithelial cell cultures

3.3 Aim 3: Examine how interactions between hormones and lactobacilli modulate female genital tract epithelial cell barrier function and innate immune responses

3.4 Aim 4: Examine how interactions between hormones and lactobacilli modulate barrier function and innate immune responses of female genital tract epithelial cells in the presence of HIV

Figure 31. Primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14 and exposed to HIV-1 increase in TER measurements.

Figure 32. Production of TNF-α by primary genital epithelial cells grown in hormones and exposed to HIV.

Figure 33. Primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV decrease TNF-α production compared to HIV.

Figure 34. Production of IL-α by primary genital epithelial cells grown in hormones and exposed to HIV.

Figure 35. Production of IL-1α by primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV.

Figure 36. Production of IL-1β by primary genital epithelial cells grown in hormones and exposed to HIV.

Figure 37. Production of IL-1β by primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV.

Figure 38. Production of IL-8 by primary genital epithelial cells grown in hormones and exposed to HIV.

Figure 39. Production of IL-8 by primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV.

Figure 40. Production of RANTES by primary genital epithelial cells grown in hormones and exposed to HIV.

Figure 41. Production of RANTES by primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV.

List of Abbreviations

α	Alpha
β	Beta
Y	Gamma
κ	Kappa
λ	Lambda
ε	Epsilon
Ab	Antibody
AIDS	Acquired immune deficiency syndrome
Akt	Activator protein B
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
APOBEC3G	Apolipoprotein B mRNA gene-editing enzyme-25 catalytic polypeptide-like 3G
BST2	Bone marrow stromal antigen
BV	Bacterial vaginosis
CDC	Centers for Disease Control and Prevention
CFUs	Colony forming units
CVL	Cervico-vaginal lavage
CVM	Cervico-vaginal mucus
DMEM	Dulbecco's modified essential media
DMPA	Depot medroxyprogesterone acetate
E2	(17)β-estradiol
EB	Elementary body
EC	Epithelial cell
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
ERs	Estrogen receptors
ETEC	enterotoxigenic *Escherichia coli*
FBS	Fetal bovine serum
FGT	Female genital tract
FITC	Fluorescein isothiocyanate
FPV	Fowlpox virus
G-CSF	Granulocyte colony-stimulating factor
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GECs	Genital epithelial cells
gp120	HIV glycoprotein 120
GR-1	*Lactobacillus rhamnosus*
GR	Glucocorticoid receptor

HAART	Highly active antiretroviral therapy
HBDs	Human beta defensins
HC	Hormonal contraceptive
HDs	Human defensins (alpha)
HNDs	Human neutrophil defensins (alpha)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HESN	Highly exposed persistently seronegative
HIV-1	Human immunodeficiency virus -1
HS	Heparan sulphate
HSPGs	Heparan sulphate proteoglycans
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
IBD	Inflammatory bowl disease
IECs	Intestinal epithelial cells
IFN	Interferon
IFNAR1	Interferon (alpha and beta) receptor 1
IFNAR2	Interferon (alpha and beta) receptor 2
IL	Interleukin
IL-1RA	Interleukin 1 receptor antagonist
IP-10	Inflammatory protein-10
IRF3	Interferon regulatory factor-3
ISGs	Interferon stimulated genes
IVUs	Infectious viral units
JAK-STAT	Janus kinase-signal transducer and activator of transcription
L-glu	L-glutamine
LGT	Lower genital tract
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MAP kinase	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MDA5	Melanoma differentiation-associated gene 5
MDP	Muramyl dipeptide
MEM	Minimum essential media
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
MIP-1α	Macrophage inflammatory protein-1 Alpha
MIP-1β	Macrophage inflammatory protein-1 Beta
MIP3α/CCL20	Macrophage inflammation protein 3-alpha
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MOI	Multiplicity of infection

MPA	Medroxyprogesterone acetate
MxA	Myxovirus resistance gene A
MX2/MxB	Myxovirus resistance gene 2
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor-kappa B
NOD	Nucleotide oligomerization domain
NLRs	NOD-like receptors
NK	Natural kill cells
NS	Not significant
OAS	2',5'-oligoadenlylate synthetase
OD	Optical density
OCPs	Oral contraceptive pills
P4	Progesterone
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pen/strep	Penicillin/streptomycin
PFA	Paraformaldehyde
PFUs	Plaque-forming units
PG	Peptidoglycan
PID	Pelvic inflammatory disease
Poly (I:C)	Polyinosinic-polycytidylic acid
PRs	Progesterone receptors
PRR	Pattern recognition receptors
PSK3	Pam$_3$-Cys-SK4
RANTES	Regulated on activation, normal T cell expressed and secreted
RC-14	*Lactobacillus reuteri*
RIG-1	Retinoic acid inducible gene 1
RLR	RIG-I like receptors
RPM	Revolutions per minute
RPMI	Royal Park Memorial Institute media
SAMHD1	SAM domain and HD domain 1
SDF-1	Stromal cell derived factor
SEM	Standard error of the mean
SIV	Simian immunodeficiency virus
SLPI	Secretory leukocyte protease inhibitor
STIs	Sexually transmitted infections
TER	Trans-epithelial resistance
TGF-β	Tumor growth factor-β
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α

TRIM5α	Tripartite motif 5a
UNAIDS	United Nations joint program on HIV/AIDS
UGT	Upper genital tract
VV	Vaccinia Virus
WHO	World Health Organization
ZO-1	Zona occludens-1

CHAPTER 1: INTRODUCTION

1.1   Increased Susceptibility to HIV in women

Currently, women account for more than 50% of HIV (human immunodeficiency virus-1) infected people worldwide (UNAIDS, 2014). The number of new HIV infections reported in women has continuously increased globally over the past few decades and women have been shown to be at an increased risk of HIV infection compared to men (UNAIDS, 2014). Infection in women occurs primarily through heterosexual transmission in the female genital tract (FGT) and the early events of heterosexual transmission of HIV are still not well understood (Hladik & Hope, 2009). Further knowledge regarding the early events of infection is necessary for designing better strategies for prevention. Although the etiology of increased acquisition within the FGT is not fully understood, increased inflammation in the FGT has been suggested as a common theme associated with increased transmission (Ferreira, Kafka, & Kaushic, 2014; Firoz Mian & Ashkar, 2011; Kaul et al., 2015; Nguyen, Kafka, Ferreira, Roth, & Kaushic, 2014; Passmore, Jaspan, & Masson, 2016; Reis Machado et al., 2014).

A significant body of research has suggested that female sex hormones, progesterone (P4) and estrogen (E2), play a role in HIV infection in women, as they have been shown to regulate innate immunity and inflammation in the FGT (Ferreira, Kafka, et al., 2014; Kaushic, Roth, Anipindi, & Xiu, 2011; Wira et al., 2010; Wira, Rodriguez-Garcia, & Patel,

2015; Wira, Rodriguez-Garcia, Shen, Patel, & Fahey, 2014). Given the importance of female sex hormones, recent research has focused on hormonal contraception methods associated with increased HIV acquisition (Brind, Condly, Mosher, Morse, & Kimball, 2015; Heffron et al., 2012; C. S. Morrison et al., 2015). Furthermore, studies have shown that sex hormones are also involved in the regulation of the FGT microbiota (Achilles & Hillier, 2013; Brotman, Ravel, Bavoil, Gravitt, & Ghanem, 2014). Therefore, for my research project, I have been studying the interactions between sex hormones and microbiota, in order to address how these factors affect susceptibility and epithelial barrier functions in the FGT.

1.2 Transmission of HIV in the Female Genital Tract and the Epithelial Barrier

The FGT can be divided into upper and lower parts. The upper genital tract (UGT) encompasses the uterus, fallopian tubes, ovaries and endocervix, and the lower genital tract (LGT) consists of the ectocervix and vagina. Two different cell types define the epithelium of the FGT: the UGT is lined by a single layer of columnar epithelium and the LGT is lined by multiple layers of stratified squamous epithelium (Nguyen, et al., 2014). Both types of epithelium demonstrate features which contribute to the ability of these cells to prevent entry of pathogens (Kaushic, 2011). This includes the formation of tight junctions in between the columnar epithelial cells of the UGT which

prevent entry of microorganisms and pathogens (Wira, Grant-Tschudy, & Crane-Godreau, 2005). In addition, the multiple layers of squamous epithelium in the LGT contain adherens and desmosomal junctions that decrease the ability of pathogens to pass through the epithelium and establish infection (Nguyen, et al., 2014).

The transformation zone between the epithelial cells of the UGT and the LGT has been proposed as the primary site for HIV acquisition (Carias et al., 2013; Li et al., 2009). However, HIV has been shown to traverse through both the UGT and LGT (Hladik & Hope, 2009). A comprehensive study using a non-human primate model of intravaginal simian immunodeficiency virus (SIV) infection indicated that the primary site for SIV acquisition is through the columnar epithelium of the UGT (Li, et al., 2009). In further support of this idea, it has been observed that the cervix has increased T cells and antigen presenting cells, offering HIV a plentiful source of target cells (Li, et al., 2009; Pudney, Quayle, & Anderson, 2005). This suggests that the epithelium of the UGT is a relevant and important location to study HIV infection.

As explained above, the epithelial cells of the FGT form a barrier against the entry of pathogens. In order for HIV to establish successful infection, the virus must cross this barrier in order to access target cells (Haase, 2010). This epithelial cell barrier has been shown to have increased permeability following exposure to HIV, allowing the translocation of HIV across the epithelium as well as potential translocation of other microbes

3

(Nazli et al., 2010). Understanding the mechanism of barrier permeability is critical to preventing HIV infection. Previous work in our lab has demonstrated that the HIV surface glycoprotein-120 (gp120) directly interacts with primary human genital epithelial cells, which leads to the production of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α). It was further shown that the production of TNF-α by GECs induced a decrease in GEC barrier integrity, leading to increased barrier permeability and subsequent microbial translocation of HIV across the epithelium. The increase in barrier permeability was mediated by a disruption of tight junction proteins (ZO-1, claudin 1, 2, 4, and occludin), which normally function to maintain a tight contact between adjacent cells and prevent the passage of pathogens from the lumen to the lamina propria (Nazli, et al., 2010). Thus, our studies suggest that HIV directly impairs the epithelial barrier through the production of TNF-α, causing increased epithelial barrier permeability. More recently, we observed that gp-120 induced production of pro-inflammatory cytokines from GECs occurs as a result of gp120 binding to TLR-2, TLR-4 and heparin sulfate moieties on GECs (Nazli et al., 2013).

The presence of inflammation in the FGT is associated with increased HIV susceptibility and disease progression (Ferreira, Kafka, et al., 2014; Kaul, et al., 2015; Nkwanyana et al., 2009). This has been attributed to the increase in HIV target cells observed within an inflammatory environment in the FGT (Arnold et al., 2016; Kaul et al., 2008; Pudney, et al., 2005). Inflammation in

the FGT has also been associated with increased HIV replication in activated target cells (Chun, Engel, Mizell, Ehler, & Fauci, 1998; Folks et al., 1989; Poli et al., 1990). Furthermore, our lab has associated inflammation as a mediator of increased permeability of the UGT epithelial barrier (Nazli, et al., 2010). Local factors such as semen from HIV uninfected and infected men as well as presence of bacterial and viral co-infections have been shown to induce genital epithelial inflammation and indirectly enhance HIV replication (Ferreira et al., 2011; Kafka et al., 2012). These studies support the premise that inflammation in the mucosal environment increases the susceptibility to HIV in women. The current research proposal which focuses on the roles of female sex hormones, hormonal contraceptives and the microbiota will examine the ability of these factors to influence local pro- and/or anti-inflammatory environments, which may affect epithelial barrier functions.

1.3   The epithelial innate immune response to HIV

The innate immune system plays a pivotal role in host defense as it is the first response to pathogens and responds rapidly after encountering infectious agents (Medzhitov & Janeway). Within the FGT the innate immune system is composed of mechanical, chemical, and cellular barriers. Mucosal epithelial cells of the FGT act not only as a physical barrier but also secrete mucus, anti-microbial peptides (AMPs), cytokines and chemokines to assist in normal regulation as well as combat pathogens (Amjadi, Salehi, Mehdizadeh,

& Aflatoonian, 2014; Wira, Fahey, Sentman, Pioli, & Shen, 2005; Wira, Grant-Tschudy, et al., 2005).

1.3.1 Mucus

The epithelial cells and the contiguous mucus layer that line the FGT are considered to be a mechanical barrier against various pathogens. The apical surface of polarized GECs are covered by the glycocalyx, consisting of oligosaccharides of glycolipids and transmembrane glycoproteins. Immediately on top of the glycocalyx are mucins, which are glycosylated O-linked proteins (glycoproteins) that form the structural component of the mucus layer (Carson et al., 1998; DeSouza et al., 1999; Radtke, Quayle, & Herbst-Kralovetz, 2012). Mucus functions as a physical barrier to pathogens and has the ability to retain immunologic mediators such as AMPs and immunoglobulins that bind and neutralize bacteria (Ganz, 2003b; Hickey, Patel, Fahey, & Wira, 2011; Kutteh, Moldoveanu, & Mestecky, 1998; McGuckin, Linden, Sutton, & Florin, 2011; Olmsted et al., 2001). Surfactant proteins are also contained within the FGT mucus layer and enhance the phagocytosis of bacteria (Wira, Patel, Ghosh, Mukura, & Fahey, 2011). Additionally, mucus serves to keep the epithelial surface lubricated and hydrated (DeSouza, Lagow, & Carson, 1998; Lagow, DeSouza, & Carson, 1999). Mucin glycoproteins can be categorized into two types: secreted and membrane bound. The secreted mucins are further divided into insoluble gel forming mucins and soluble mucins (Gipson et al., 1997). Within the FGT, all

epithelial areas including the ovaries, fallopian tubes, endometrium, and vagina contain mucins, however the cervix is the predominant location of mucin expression (Gipson, et al., 1997). The fallopian tubes, uterus, and cervix contain secreted mucins, while the vagina contains only membrane bound mucins. Therefore, the vagina is bathed in mucous secreted from the upper reproductive tract. The secretion of mucus by the cervix is able to prevent the interaction and penetration of luminal pathogens. A study by Lai et al., 2009, showed that CVM (cervico vaginal mucus) obtained from healthy donors diffused efficiently trapped HIV, as it caused the virus to diffuse 1000-fold more slowly in comparison to water (Lai et al., 2009). Furthermore, it has been shown that mucins are able to directly bind to pathogens through frucosylated glycans to compete with binding sites on epithelial cells (Domino et al., 2009; Lai, et al., 2009).

1.3.2 Pattern Recognition Receptors

Additionally, these epithelial cells express pattern recognition receptors (PRRs), specifically Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide oligomerization domain (NOD)-like receptors (NLRs) (Hart et al., 2009; Nguyen, et al., 2014; Pioli et al., 2004; Wira, Grant-Tschudy, et al., 2005). The role of PRRs is to recognize structurally conserved molecular patterns that are broadly shared by pathogens, also known as pathogen associated molecular patterns (PAMPs). The columnar epithelial cells of the UGT express numerous PRRs in both the

endometrium and endocervix. Endometrial epithelial cells express NLRs, RLRs, as well as TLRs 1-9, while endocervical epithelial cells express NLRs, RLRs, and TLRs 1-3, 5 and 6 (Ghosh et al., 2013; King, Horne, Hombach-Klonisch, Mason, & Critchley, 2009; Wira, Fahey, et al., 2005). TLR 1, 2, 4, 5, and 6 detect pathogen membrane components as they are located on the plasma membrane of cells while the cytoplasmic organelles express TLR 3, 7, 8, and 9 to detect the nucleic acids of pathogens (Kaisho & Akira, 2006; Thompson, Kaminski, Kurt-Jones, & Fitzgerald, 2011). Bacterial and fungal PAMPs are recognized by TLRs 1, 2, and 4, while viral double-stranded RNA (dsRNA) is recognized by TLR 3. TLR7 and 8 bind to imiquidizalones and single-stranded RNA (ssRNA). Furthermore, TLR 9 binds to unmethylated CpG DNA (Kawai & Akira, 2010). Both NLRs and RLRs detect microbial components within the cytosol. NLRs have been shown to recognize peptidoglycan motifs located on bacterial cell walls. More specifically, NOD1 binds to gram-negative bacteria, and NOD2 detects the intracellular molecule, muramyl dipeptide (MDP) a constituent of certain gram positive and negative bacterial stains (G. Chen, Shaw, Kim, & Nunez, 2009; Franchi, Warner, Viani, & Nunez, 2009). Lastly, the RLRs, specifically RIG-I and melanoma differentiation-associated gene 5 (MDA5) are intracellular cytoplasmic detectors of viral dsRNA (Ghosh, et al., 2013; Loo & Gale, 2011). Upon recognition of PAMPs, PRRs initiate an intra-cellular signaling cascade, which activates transcription factors such as NF-κB and ultimately results in the

production of pro-inflammatory cytokines and chemokines, and anti-viral cytokines such as interferons (IFNs) (Schaefer, Desouza, Fahey, Beagley, & Wira, 2004). Stimulation of the innate immune system through PRRs creates an inhospitable pro-inflammatory environment for pathogens, recruits other innate immune cells, and serves as a bridge to the adaptive arm of the immune system.

Chemokines are characterized for their chemotactic ability to attract immune cells, namely leukocytes, to sites of inflammation. Cytokines on the other hand, activate and differentiate the immune cells recruited by chemokines (Amjadi, et al., 2014; Fahey, Schaefer, Channon, & Wira, 2005). Cytokines and chemokines are produced constitutively by a number of immune and non-immune cells, including genital epithelial cells and in response to pathogens sensed by PRRs within the FGT (Fahey, et al., 2005; Schaefer, et al., 2004; Schaefer, Fahey, Wright, & Wira, 2005; Wira, Fahey, et al., 2005; Wira, Grant-Tschudy, et al., 2005). Some of the cytokines and chemokines constitutively secreted by polarized columnar epithelial cells of the cervix, uterus and fallopian tubes include granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), tumor necrosis factor- α (TNF-α), IL-1, IL-6, leukemia inhibitory factor (LIF), stromal cell derived factor (SDF-1), transforming growth factor-β (TGF-β), macrophage inflammatory protein-1β (MIP-1β), macrophage inflammatory protein-1α (MIP-1α) monocyte chemoattractant protein-1 (MCP-1), regulated on

activation, normal T cell expressed and secreted (RANTES) and IL-8 (Fahey, et al., 2005; Kayisli, Mahutte, & Arici, 2002; Ochiel, Fahey, Ghosh, Haddad, & Wira, 2008; Wira, Fahey, et al., 2005; Wira, Grant-Tschudy, et al., 2005). Cytokine and chemokine secretion can occur apically into the lumen and basolaterally into the lamina propria (Carolan, Mower, & Casale, 1997; Fahey, et al., 2005). During a PRR mediated response, the majority of cytokines and chemokines are secreted apically into the luminal compartment to attract immune cells to the epithelial surface where the pathogen has been detected (Schaefer, et al., 2004; Schaefer, et al., 2005). Our lab has demonstrated that HIV glycoprotein120 binds to PRRs such as TLR-2 and -4, as well as heparin sulfate moieties (Nazli, et al., 2013). This resulted in the upregulation of the NF-κB pathway and the induction of several pro-inflammatory cytokines and chemokines both apically and basolaterally, including TNF-α, IL-1β, IL-6, and MCP-1.

FGT associated cytokines and chemokines have been shown to both block and enhance HIV infection. Agace *et al.,* demonstrated that stromal derived factor-1 (SDF-1) is able to competitively inhibit HIV (Agace et al., 2000). Additionally, MIP-1α, MIP-1β and RANTES are able to bind to the CCR5 receptor and thus prevent HIV binding (Abdelwahab et al., 2003; Cocchi et al., 1995). Conversely, in the presence of latently infected target cells pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α enhance HIV replication through activation of HIV- long terminal repeat (LTR) (Chun, et al., 1998; Folks, et

al., 1989; Poli, et al., 1990). Ferriera et al., showed that primary GECs up-regulated IL-6, IL-8, MCP-1, and TNF-α in response to HSV-1, HSV-2, and *Nisseria gonorrhoeae*, which indirectly induced the HIV-LTR promoter in T cells (Ferreira, et al., 2011). Furthermore, the inflammatory associated increase in HIV replication through HIV-LTR can be abrogated by blocking the production of inflammatory cytokines through broad spectrum anti-inflammatory compounds such as curcumin (Ferreira, Nazli, Dizzell, Mueller, & Kaushic, 2015). Lastly, chemokine induced recruitment of activated immune cells in the FGT increases the amount of target cells available for HIV (Plummer, 1998). This suggests that pro-inflammatory cytokines and chemokines play a role in facilitating the acquisition and spread of HIV.

1.3.4 Interferons

Interferons (IFNs), a group of signaling proteins, also belong to the cytokine family. They are well known for their ability to 'interfere' with viral replication and are secreted by GECs in response to pathogens. A broad repertoire of IFN genes and proteins have been identified in humans. IFNs are divided into three main categories: Type I IFN, Type II IFN, and Type III IFN, all with a wide variety of immunomodulatory and anti-viral effects. In humans, type I IFNs consist of several subtypes, including single forms of IFN-β, IFN-κ, IFN-ω, IFN-ε, and IFN-α (13 isoforms).   All Type I IFNs bind to the type I IFN receptor (IFNAR) which activates several intracellular signaling cascades including janus kinase-signal transducer and activator of transcription

(JAK/STAT), p38 (mitogen activated protein kinase) MAPK and (Crk-like) CrkL pathways (Noppert, Fitzgerald, & Hertzog, 2007; Thomas et al., 2011). These pathways induce the expression of several IFN stimulated genes (ISGs) that in turn up-regulate virucidal and restriction factors that block HIV-1 replication. Myxovirus resistance gene A (MxA) and 2',5'-oligoadenlylate synthetase (OAS) are ISGs involved in inhibiting the viral lifecycle (Le Bon & Tough, 2002). Both MxA and OAS are expressed by primary uterine and fallopian tube epithelial cells and exhibit inhibition of several virus families (Ghosh, Schaefer, Fahey, Wright, & Wira, 2008; Haller, Staeheli, & Kochs, 2007; Schaefer, et al., 2005). MxA is able to bind viral nucleocapsid proteins, which prevents mature virion formation and release by the formation of aggregates while OAS inhibits protein synthesis by degrading viral and cellular RNAs (Haller, et al., 2007). The importance of the anti-viral immune response induced by IFN-β was previously observed in IFN-β knock-out animals. When mice lacking the ability to induce an IFN-β response were exposed to viral infection the host showed increased susceptibility compared to wild type animals (Deonarain et al., 2000). Within the human FGT, IFN-β shows a protective role against HIV through the secretion of anti-HIV molecules such as macrophage inflammation protein three-alpha (MIP3α/CCL20) and human beta defensin-2 (HBD2) (Ghosh et al., 2009). Type 1 IFNs also induce the up-regulation of several other restriction factors that impede HIV replication such as SAM domain and HD domain 1

(SAMHD1) (Hrecka et al., 2011), myxovirus resistance 2 (MX2/MxB) (Kane et al., 2013), tripartite motif 5a (TRIM5α) (Stremlau et al., 2004), apolipoprotein B mRNA gene-editing enzyme-25 catalytic polypeptide-like 3G (APOBEC3G) (K. Chen et al., 2006; Holmes, Malim, & Bishop, 2007) and bone marrow stromal antigen or tetherin (BST2) (Neil, Zang, & Bieniasz, 2008).

Unlike other type I IFNs which are induced via PRR pathways, IFN-ε is constitutively expressed by epithelial cells of the FRT and other mucosal tissues (Demers et al., 2014; Fung et al., 2013; Hermant, Francius, Clotman, & Michiels, 2013). IFN-ε binds to IFNAR1 and IFNAR2 receptors and induces the expression of viral restriction factors to exert its anti-viral activity (Eid, Mangan, Hertzog, & Mak, 2015). An *in vivo* study by Fung et al., showed that IFN-ε deficient mice are more susceptible to HSV-2 or Chlamydia infection compared to wild-type mice. IFN-ε expression was not altered in wild-type mice exposed to HSV-2 or Chlamydia, further supporting that it is not pathogen induced (Fung, et al., 2013). An additional study by Matsumiya et al., demonstrated that IFN-ε is the only type 1 IFN induced by HeLa cells, and that TNF-α stimulation up-regulated the expression of IFN-ε (Matsumiya, Prescott, & Stafforini, 2007). Similar to other type 1 IFNs, IFN-ε has been shown to possess anti-HIV activity. A recent study used multiple cell lines to show the impairment of HIV function by IFN-ε at multiple stages of the replication cycle after viral entry. This was observed by the up-regulation of host cell restriction factors known to inactivate HIV (Garcia-Minambres et al.,

2017). The adjuvant activity of IFN-ε was evaluated in a study by co-expressing IFN-ε on the encoded vaccine antigen HIV gag/pol, and using a recombinant fowlpox virus (FPV) and vaccinia virus (VV) prime-boost strategy. Mice were primed with an intranasal immunization (FPV-HIV- IFN-ε) and boosted with an intramuscular immunization (VV-HIV-IFN-ε). Increased HIV-specific CD8+ effector T cells were observed in peyers patches, spleen, lung and the genito-rectal tract of mice co-expressing IFN-ε, compared to prime boost vaccination control with no IFN-ε co-expression (Xi, Day, Jackson, & Ranasinghe, 2012). Furthermore, the up-regulation of IFN-ε was also observed in cervico-vaginal tissues in response to seminal plasma, further suggesting that within mucosal tissues, especially the FGT, IFN-ε plays a unique role in mucosal immunity.

Newly identified members of the IFN family of cytokines are type III IFNs (IFN-λ also known as IL28/29) consisting of three subfamily members, IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL28B) (Ank & Paludan, 2009; Wack, Terczynska-Dyla, & Hartmann, 2015). Similar to type 1 IFNs, type III IFN responses are induced by viral infections or TLR stimulation and subsequently suppress viral replication by the induction of anti-viral factors that suppress viral replication (Lazear, Nice, & Diamond, 2015). Furthermore, type III IFN induced anti-viral protection has been demonstrated with both *in vivo* and *in vitro* models (Ank et al., 2008; Iversen, Ank, Melchjorsen, & Paludan, 2010; Liu et al., 2012). Type III IFNs inhibit HIV integration and post-

14

translational events in macrophages resulting in the abrogation of HIV infection *in vitro (Hou et al., 2009; Liu, et al., 2012; Tian et al., 2012).* Although type I and type III IFNs share several similarities, the important item of note is how they are different. Unlike type I IFNs, where receptor expression is highly abundant in a multitude of locations, type III IFN receptors are only expressed on a narrow subset of cells, primarily epithelial cells (Ank & Paludan, 2009; Kotenko et al., 2003; Z. Zhou et al., 2007). Type I, and III IFNs have also shown differences in their ability to respond to pathogens and the cellular signaling pathways involved. Iverson et al., demonstrated that in the vaginal tract of mice, mucosal administration of different PAMPs differentially induced expression of type I, and type III IFN. High levels of activation of the NF-κb pathway were correlated with a strong type III IFN response. Interestingly, when the NF-κb pathway was blocked, expression of type III IFN was abrogated as expected, but no changes were seen with type I IFNs. Furthermore, administration of a TLR 9 agonist in the FGT induced a stronger type III IFN response than a treatment targeting cytoplasmic PRRs such as nucleotide PAMPs (Iversen, et al., 2010). Although both the NF-κb and interferon regulatory factor (IRF) pathways involved in IFN induction, it is hypothesized that distinct requirements are necessary for the induction of type I and type II IFNs by the two pathways. These results suggest that type III IFNs and IFN-ε mediate antiviral defenses mainly at mucosal epithelial surfaces while type I IFNs have a more systemic effect.

1.3.5 Antimicrobial Peptides

AMPs are often referred to as the 'endogenous microbicides' within the FGT. They are small molecules secreted by GECs and other innate immune cells as a natural form of protection against bacterial, viral, parasitic and fungal pathogens (Amjadi, et al., 2014; Bahar & Ren, 2013). AMPs are positively charged, and are therefore able to target pathogens based on the surface charge of their membrane (Harris, Dennison, & Phoenix, 2009). Many microbial membranes contain a negative net charge and therefore electrostatically attract AMPs allowing the microbe-AMP binding interaction (Matsuzaki, 1999; Yeaman & Yount, 2003). In particular, bacterial cell membranes contain negatively charged phospholipids and a peptidoglycan cell wall with additional negatively charged components making them an attractive target for AMPs. They primarily exert their neutralization effect on microbes by integrating into the microbial cell membrane or viral envelope. By increasing permeability or directly forming pores in the pathogen cell membrane, the pH and ionic concentration gradients of the target cell become unstable, thus rendering the target cell inactive. AMPs are able to reduce the binding of pathogens to host cells. Heparin sulfate is a negatively charged cell surface receptor for some viruses such as HSV and HIV. Given that AMPs are positively charged, they are able to block virus-receptor interactions by binding to heparin receptors (Hazrati et al., 2006; Selsted & Ouellette, 2005). In addition to binding host cell receptors, AMPs are able to block viral-host

cell interactions by binding to viral glycoproteins (Hazrati, et al., 2006; Pazgier, Li, Lu, & Lubkowski, 2007; Selsted & Ouellette, 2005; Wang et al., 2004). By crossing the cell membrane, AMPs are also able to exert anti-pathogenic effects through intracellular mechanisms. These includes the following: inhibition of protein and cell wall synthesis, inhibition of DNA and RNA replication, cytoplasmic membrane alteration, activation of autolysin, inhibition of certain enzymes, and iron deprivation (Amjadi, et al., 2014; Bahar & Ren, 2013; Ganz, 2003a; Klotman & Chang, 2006).

Epithelial cells of both the upper and lower FGT have been shown to secrete AMPs using both *in vitro* and *in vivo* models. AMPs are constitutively produced by GECs, however secretion can be up regulated in response to environmental stimuli. GECs are able to produce a broad repertoire of AMPs. The major AMPs produced by epithelial cells include: defensin, elafin, cathelicidin, secretory leukocyte protease inhibitor (SLPI), lysozyme, and lactoferrin (Amjadi, et al., 2014; Ochiel, et al., 2008; Yarbrough, Winkle, & Herbst-Kralovetz, 2015).

The most prominent AMPs produced by GECs are defensins of which two sub-families exist, each containing six isoforms. α-definsins consist of human neutrophil peptide (HNP) 1-4 and human defensin (HD) 5-6 while β-defensins consist of human-β defensins (HBD) 1-6 (Yarbrough, et al., 2015). α-defensins are cleaved by proteases to create an active peptide. The propeptide form of HD5 and -6 have both been isolated in the FGT, while

HNP1-4 are primarily excreted by neutrophils. HBD1-4 are also expressed throughout the FGT (Horne, Stock, & King, 2008; Valore et al., 1998; Wira, et al., 2011). HBD1 is constitutively expressed and induced by TLR3 agonists while HBD2, -3 and -4 are mainly expressed upon stimulation (King, et al., 2009; Radtke, et al., 2012). Both α- and β-defensins have shown anti-viral activity, including the ability to inhibit HIV (Sun et al., 2005; Weinberg, Quinones-Mateu, & Lederman, 2006; Wira, et al., 2011). Quinones et al, demonstrated that exogenous HBD2 and -3 inhibit HIV viral replication *in vitro* at concentrations that did not affect cell viability (Quinones-Mateu et al., 2003). Additionally, in peripheral blood mononuclear cells and T lymphocytes HBD2 and -3 down-regulated the level of CCR4, an HIV co-receptor (Quinones-Mateu, et al., 2003). Interestingly, HBD3 has also been used to increase epithelial tight junction barrier function in human keratinocytes (Kiatsurayanon et al., 2014).

Protease inhibitors belong to the whey acid protein family and are a group of enzymes involved in the digestion of proteins. SLPI and Elfain are protease inhibitors found within the FGT. SLPI is expressed constitutively, and is inducible by pathogens in the cervix, fallopian tube, and endometrium (Horne, et al., 2008; Wira, et al., 2011). SLPI has several immunomodulatory roles in epithelial cells including the promotion of wound healing, cell proliferation, and inhibition of LPS induced inflammation and inhibition of NF-κB expression (Ashcroft & Mills, 2002; Jin, Nathan, Radzioch, & Ding, 1997;

Lentsch et al., 1999; Zhu et al., 2002). Similar to SLPI, elafin is constitutively produced by GECs, while inducible expression occurs within the endometrium (Yarbrough, et al., 2015). Elafin is synthesized by the proteolytic cleavage of trappin-2 and thus is able to inhibit proteases and bind transglutaminase (Schalkwijk, Wiedow, & Hirose, 1999). By binding to transglutaminase, elafin is able to assist in the maintenance of barrier integrity in mucosal tissue by cross-linking extracellular matrix proteins (Guyot, Zani, Maurel, Dallet-Choisy, & Moreau, 2005). Previous studies have shown that elafin is associated with protection of tight the junction proteins zona-occludens in respiratory and intestinal epithelial cells (Galipeau et al., 2014; Li, Zhou, Xu, & Yang, 2010). Protease inhibitors, such as SLPI and elafin have both been shown to exert anti-HIV effects (Cole & Cole, 2014). Protease inhibitors block proteolytic cleavage of protein precursors necessary for viral replication by selectively binding to viral proteases. More specifically, SLPI suppresses elastase and cathepsin G while elafin suppresses elastase and proteinase3 (Moreau et al., 2008). *In vitro*, SLPI is able to inhibit HIV activity independent from its inhibition of proteases by competing for anexin binding sites on the cell membrane and thus reducing viral entry (G. Ma et al., 2004; McNeely et al., 1997). Furthermore, a positive correlation was observed between increased SLPI concentration in genital tract secretions and reduced risk of perinatal HIV transmission (Pillay et al., 2001). An *in vitro* study by Ghosh et al., showed that elafin had a dose dependent effect on anti-HIV activity (Ghosh et

al., 2010). Furthermore, elevated levels of trappin/elfin were observed in HIV highly exposed seronegative women (HESN) when compared with HIV uninfected women, suggesting a role for trappin/elafin in HIV protection (Iqbal et al., 2009). In addition to their anti-viral effects, SLPI and elafin also possess anti-bacterial and anti-fungal activity (Baranger, Zani, Chandenier, Dallet-Choisy, & Moreau, 2008).

Other AMPs such as calthelicidin (LL-37), lactoferrin and lysozyme have also been shown to exert anti-HIV properties *in vitro* (Bergman, Walter-Jallow, Broliden, Agerberth, & Soderlund, 2007; Harmsen et al., 1995; Lee-Huang et al., 2005). Together, AMPs provide a variety of mechanism by which they exert their anti- viral, bacterial, fungal, and parasitic effects thus allowing them to respond to a broad array of pathogens to facilitate protection within the FGT.

1.4 Female Sex Hormones, Hormonal Contraceptives, and Altered Susceptibility to HIV

The ovaries are the predominant source of female sex hormones that maintain the reproductive cycle. Endogenous levels of these hormones fluctuate through a woman's lifetime, and women of reproductive age undergo cyclic changes in levels of estradiol (E2) and progesterone (P4) during the monthly menstrual cycle. 17-β estradiol is the dominant estrogen found in women during the proliferative phase and it fluctuates at levels between 10^{-10}

M to 10^{-11} M, during a normal menstrual cycle (Stricker et al., 2006). In comparison, P4 fluctuates between 10^{-8} M and 10^{-9} M during the menstrual cycle and is the dominant hormone during the secretory phase (Stricker, et al., 2006).

When considering the increased prevalence of HIV in women compared to men, an important biological factor to study is the influence of female sex hormones on HIV infection. Numerous studies have suggested that female sex hormones are able to modulate HIV susceptibility in the FGT (Hel, Stringer, & Mestecky, 2010; Kaushic, Roth, et al., 2011; Marx et al., 1996; Wira & Fahey, 2008; Wira, et al., 2015). An study by Smith *et al.* (2000) reported that in ovariectomized Rhesus macaque monkeys treated with implants of E2 or P4, E2 treated macaques demonstrated significantly increased protection against SIV infection compared to P4 treated macaques (Smith, Baskin, & Marx, 2000). More recently, a study utilized human cervical tissue explants from women at different stages of the menstrual cycle to assess HIV susceptibility in relation to female sex hormones. An *ex vivo* HIV challenge of the cervical explants revealed that all productively infected tissues were from women in the progesterone high phase of the menstrual cycle thereby suggesting that progesterone may increase susceptibility to HIV (Saba et al., 2013). The underlying mechanism behind altered HIV susceptibility in the presence of female sex hormones remains unclear. However, it has been suggested that E2 is associated with inhibition of a pro-

inflammatory environment, while P4 is associated with inflammation (Taggart et al., 2005; von Wolff et al., 2000; Wira, et al., 2015). Therefore, potentially, the protection observed in E2 associated conditions may be a result of the anti-inflammatory environment in the FGT during the proliferative phase of the menstrual cycle. An anti-inflammatory environment decreases both the potential for inflammation induced genital barrier disruption, and the number of target cells present. (Rodriguez-Garcia et al., 2013). It has been hypothesized that increased HIV susceptibility observed in the presence of P4 could potentially be attributed to the pro-inflammatory environment during the secretory phase, leading to increased barrier permeability and increased number of target cells (Marx, et al., 1996); however, this has not been shown. Details regarding the regulation of epithelial barrier functions in the presence of female sex hormones remains understudied in the literature. More work in this area is necessary as the epithelial barrier is the first line of defense against HIV acquisition. A further understanding of how female sex hormones modulate barrier functions may help elucidate differences in HIV susceptibility regulated by hormonal contraceptives (HCs).

Globally, over 100 million women use hormonal contraceptives (HCs) (Bureau, 2002). A growing body of literature has suggested that there may be an increased risk of HIV acquisition caused by use of injectable HCs (Huijbregts et al., 2013; C. S. Morrison, et al., 2015; Polis et al., 2014). Of particular interest is the progestin-based HC, Depot Medroxy-progesterone

acetate (DMPA or Depo-Provera). The low cost, long-term effectiveness of DMPA as a contraceptive makes it an attractive choice for women. Notably, DMPA is most often used in the developing world, especially in regions such as sub-Saharan Africa, where HIV prevalence is at its highest. Studies in non-human primates have demonstrated that risk of SIV acquisition is increased when animals are administered DMPA (Marx, et al., 1996; Mascola et al., 2000; Trunova et al., 2006). Human epidemiologic studies support this by showing an association between DMPA use and increased HIV infection, disease progression and mortality (Baeten et al., 2007; Crook et al., 2014; Polis & Curtis, 2013). Furthermore, a prospective cohort study of 3800 discordant couples examining injectable HC use and HIV susceptibility recently gained ample media attention (Heffron, et al., 2012). The findings from this study indicated that HIV-uninfected women using injectable HCs were twice as likely to acquire HIV from an infected male partner compared to women using no HC, and HIV-infected women in the same contraceptive group were twice as likely to infect their male partner compared to women not using HC (Heffron, et al., 2012). Since then several meta-analysis have examined the strength of the evidence as there are several studies that have shown conflicting evidence regarding the use of HCs and HIV acquisition (C. S. Morrison, et al., 2015; Polis & Curtis, 2013; Polis, et al., 2014; Ralph, Gollub, & Jones, 2015). A recent meta-analysis by Morrison et al., found that DMPA increased women's risk of contracting HIV

by 50 percent compared to women not using HC (C. S. Morrison, et al., 2015). This was confirmed in two further meta-analysis by Brind *et al.,* and Ralph *et al.,* showing a 49 and 40 percent increased risk of HIV acquisition with DMPA use respectively (Brind, et al., 2015; Ralph, et al., 2015). Although associations have been observed between the use of injectable HCs and increased HIV susceptibility, the pathways involved in these outcomes remain unclear and under investigated. Based on the controversy of DMPA use, and unresolved issues around the mechanism of increased HIV acquisition, in 2012 UNAIDS released an urgent call for analysis and more research in this area in order to understand the possible influence of HC use on HIV infection (UNAIDS, February 16, 2012). Since then, a number of studies have been published that examine biological mechanisms of DMPA, suggesting that an alteration in immune response and changes in the genital tract microenvironment may be associated elevated risk of HIV (Byrne et al., 2016; Carias et al., 2016; Chandra et al., 2013; C. M. Mitchell et al., 2014). Recent work in our lab has demonstrated that in the presence of MPA (DMPA is the long acting injectable form of MPA), primary GECs have increased HIV uptake, trancytosis, and infection of target cells (Ferreira et al., 2014).

Other studies have shown that the use of DMPA may decrease levels of E2 (Torgrimson, Meendering, Kaplan, & Minson, 2011). Since E2 has been suggested to have anti-inflammatory and protective effects, decrease in E2 levels could also contribute to increased susceptibility (Torgrimson, et al.,

2011). Given the fact that DMPA is a progestin-based contraceptive, it is possible that the increase in HIV susceptibility observed with DMPA use is similar to that seen under the influence of P4. However, there is little reported evidence suggesting DMPA use is associated with increased pro-inflammatory cytokine production and increase in target. Even less information is available regarding mucosal barrier functions in the genital tract associated with DMPA use, and this is an area of research that needs to be further investigated.

1.5 The Female Genital Tract Microbiota

In addition to hormones, the microbiota of the FGT has also been correlated with alterations in HIV susceptibility. The composition of the microbiota within the FGT has been shown to influence HIV susceptibility and may also be regulated by female sex hormones (Wira, et al., 2015). Molecular studies have identified more than 250 microbial species in the vaginal tract (Ravel et al., 2012). Interestingly, contrary to the common belief that the upper genital tract is a sterile environment, a smaller population of microbial species has also been identified in this region (C. M. Mitchell et al., 2015). Factors such as age, race, female sex hormones and use of HC have been shown to affect the composition and relative abundance of these species in the FGT of women (Petrova, van den Broek, Balzarini, Vanderleyden, & Lebeer, 2013). In majority of women, a 'healthy' FGT microbiota is

characterized by the abundance of *Lactobacillus* species, mainly, *L. crispatus, L. jensenii, L. gasseri* and/or *L. iners* (Cowling, McCoy, Marshall, Padfield, & Reeves, 1992; C. M. Mitchell, et al., 2015; Moller, Kristiansen, Thorsen, Frost, & Mogensen, 1995; Verstraelen et al., 2016). However, when the composition of the microbiota is shifted toward a more polymicrobial environment, which is dominated by anaerobic bacteria, it is associated with bacterial vaginosis (BV) (Ravel, et al., 2012). The dysbiosis of the 'healthy' flora is commonly associated with increased susceptibility to STIs including HIV, HSV-2, gonorrhea, and Chlamydia (Doerflinger, Throop, & Herbst-Kralovetz, 2014; Schwebke & Weiss, 2002). Furthermore, studies have observed that there is increased HIV and HSV-2 replication and viral shedding in women with clinical symptoms of BV (Cu-Uvin et al., 2001; Nagot et al., 2007).

A *Lactobacillus* rich environment has been shown to exert protective effects against urogenital diseases, including, BV, yeast infections, HSV-2, and HIV (B. Ma, Forney, & Ravel, 2012). Direct beneficial effects produced by lactobacilli in the FGT involve the production of lactic acid, hydrogen peroxide, and bacteriocins (Petrova, Lievens, Malik, Imholz, & Lebeer, 2015). Lactic acid accumulates in the environment as a product of fermentation and assists to lower the pH of the surrounding environment (Boskey, Telsch, Whaley, Moench, & Cone, 1999). In general, the low pH of the FGT mucosa is considered to be one of the main strategies to prevent bacterial and viral infections (Boskey, Cone, Whaley, & Moench, 2001). Interestingly, the acidic

environment has been shown to decrease activation of target cells and thus decrease target cell susceptibility to HIV infection (Aldunate et al., 2013). Indirect mechanisms involved in the protection associated with lactobacilli include competition with BV associated pathogens for colonization of the epithelial cell surface, immune system stimulation by epithelial cells, and increased epithelial barrier function (Petrova, et al., 2015). Although several mechanisms have been proposed, the details of these mechanisms and potential immune modulation by lactobacilli remains a major gap in the literature.

1.6 Probiotic *Lactobacillus* species in the Female Genital Tract

1.6.1 General Overview of Probiotics

The definition of a probiotic microbial isolate was created in 2002 by the Food and Agriculture Organization of the United Nations and World Health Organization. According to their guidelines, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". The beneficial effects of probiotic lactobacilli have been extensively studied in inflammatory bowel diseases (IBD), and to a lesser extent, in the FGT (Garcia-Lafuente, Antolin, Guarner, Crespo, & Malagelada, 2001). While the full details regarding the mechanisms of how these bacteria exert their protective effects are still being investigated, a few effects have been well documented. In general *Lactobacillus* species in the

gut have been shown to a) inhibit growth of microbial pathogens through competitive exclusion (Bernet, Brassart, Neeser, & Servin, 1994), b) modify intestinal permeability by increasing tight junction protein expression (Eun et al., 2011; Seth, Yan, Polk, & Rao, 2008), c) compete for pathogen binding sites through adherence to epithelial cells (Bernet, et al., 1994), d) secrete antimicrobial products such as bacteriocins (Flynn et al., 2002), e) modify immune responses by down regulating inflammatory responses (Ulisse et al., 2001), and f) increase mucin production to reduce pathogen interactions with epithelial cell surface (Mack, Michail, Wei, McDougall, & Hollingsworth, 1999). These well-demonstrated and functional characteristics of *Lactobacillus* species within are gut are also important in the FGT. Few studies have assessed the mechanisms of the beneficial effects of probiotic lactobacilli in the FGT, however they have been shown to adhere to urogenital epithelial cells *in vivo* and *in vitro* (Boris, Suarez, Vazquez, & Barbes, 1998; Reid, Beuerman, Heinemann, & Bruce, 2001; Zarate & Nader-Macias, 2006), outcompete pathogenic microbes for colonization (Zarate & Nader-Macias, 2006), produce of bacteriocins (Aroutcheva et al., 2001), and lower the pH of the microbiota through the production of lactic acid and hydrogen peroxide to make an inhospitable environment for pathogens (Atassi & Servin, 2010).

To date, two of the most well documented probiotic strains related to women's reproductive health are GR-1 (*L. rhamnosus*) and RC-14 (*L. reuteri).* Both strains were isolated from the FGT of healthy women in the

1980's and were initially selected for their ability to compete with pathogenic bacteria and yeast in the FGT, and reduce the risk of bladder infections (Kohler, Assefa, & Reid, 2012; Reid, 2008).

1.6.2 Probiotic strains *L. rhamnosus* (GR-1) and *L. reuteri* (RC-14)

More than 20 million doses of GR-1 and RC-14 have been administered worldwide, and therefore the safety, efficacy, and characterization of these strains has been extensively documented (Reid, 2008). *In vitro,* these strains have been shown to adhere to vaginal epithelial cells, and to have anti-virucidal effects, however RC-14 produces higher levels of hydrogen peroxide compared to GR-1 (Kohler, et al., 2012; Reid, 2008; Reid, Cook, & Bruce, 1987; Wagner & Johnson, 2012). Both GR-1 and RC-14 have been extensively used in clinical studies to show the their beneficial effects in the treatment and prevention of vulvo vaginal candidiasis, urinary tract infections, and bacterial vaginosis (Anukam et al., 2006; Bruce, Reid, McGroarty, Taylor, & Preston, 1992; Martinez et al., 2009; Reid, et al., 2001; Reid, Bruce, & Taylor, 1995; Reid et al., 2003). Furthermore, BV has been associated with many secondary adverse heath outcomes such as increased risk of pre-term labor and low birth weight, pelvic inflammatory diseases, and STIs, including HIV, HSV-2, *Chlamydia trachomadis*, *Neisseira gonorrhoeae*, *Trichomonas vaginalis*, and cytomegalovirus (Bautista et al., 2016; Brotman et al., 2007; Cherpes, Meyn, Krohn, & Hillier, 2003; Gallo et al., 2008; Gravett, Hummel, Eschenbach, & Holmes, 1986; Jacobsson,

Pernevi, Chidekel, & Jorgen Platz-Christensen, 2002; Joesoef et al., 1996; Lata, Pradeep, Sujata, & Jain, 2010; Nilsson, Hellberg, Shoubnikova, Nilsson, & Mardh, 1997; Ross et al., 2005; Sewankambo et al., 1997; Sharami, Afrakhteh, & Shakiba, 2007). Thus by preventing or reducing BV, probiotics may also show beneficial effects against BV associated adverse health outcomes.

The alimentary canal and FGT are not internally connected. Therefore, initial probiotic therapy for the FGT was administered locally through intravaginal capsules of GR-1 and RC-14, to ensure that the treatment was present in the target area (Bruce, et al., 1992; Reid, et al., 1995). The current thought regarding bacterial colonization of the FGT is that orally ingested bacteria travel through the alimentary canal, from the intestine to the rectum and eventually pass the perineum to the FGT (Cribby, Taylor, & Reid, 2008; El Aila et al., 2009; Reid, 2008). Therefore, more recently, the concept of delivering probiotic therapy orally to repopulate the FGT microbiota has been suggested (Cribby, et al., 2008; Reid, et al., 2001; Reid & Bruce, 2003).

In a cohort of healthy women, Reid *et al.,* assessed the effect of oral probiotic administration on vaginal health and the potential side effects (Reid, et al., 2001). Results showed that daily oral ingestion of GR-1/RC-14 capsules significantly improved the vaginal flora in terms of *Lactobacillus* species present compared to baseline and the placebo control group. Additionally, yeast and coliform bacteria were significantly depleted in the

women receiving probiotic treatment. Furthermore, daily oral administration did not induce any side effects (Reid, et al., 2003). The use of GR-1/RC-14 oral capsules has also shown to be efficacious in the treatments of BV. In a study conducted using forty-two healthy women with or without asymptomatic BV, results showed that 82% of patients diagnosed with an intermediate nugent score or asymptomatic BV at day zero converted to a healthy nugent score when administered the GR-1/RC-14 oral probiotic at dosages of over 10^8 viable organisms (Reid, et al., 2001). The use of GR-1/RC-14 for treatment of BV is further exemplified when used in combination with a single dose of antibiotic treatment. A randomized double blind control trial of sixty-four BV positive Brazilian women showed that antibiotic treatment with oral capsules of GR-1 and RC-14 resulted in 87.5% relief in BV associated symptoms compared to a 50% in the antibiotic alone treatment (Martinez, et al., 2009). Notably, this study only assessed BV status after treatment at day 28. Given that continual recurrence of BV is an issue, further follow up assessments at prolonged time points should be evaluated. These initial studies serve as a basis to support the use of the GR-1/RC-14 combination as a therapeutic probiotic to increase FGT microbial health.

1.6.3 Use of Probiotics to Prevent STIs

An increasing amount of evidence is suggesting that STI acquisition is facilitated by a BV associated FGT microbiota (Bautista, et al., 2016). By classifying women into clusters based on microbial profile of their FGT,

Borgdoff *et al.,* showed women in the cluster with the least microbial diversity and a dominance of lactobacilli were significantly less likely to have viral infections and no bacterial STIs (Borgdorff et al., 2014). Interestingly, a positive correlation was observed between increasing microbial diversity, decreasing lactobacilli and prevalence of STIs, suggesting that the microbial composition of the FGT facilitates the transmission of STIs. Furthermore, increasing microbial diversity and decreased lactobacilli was associated with increased amounts of shed HIV virus within the FGT, showing a potential for increased transmission of virus to a sexual partner (Borgdorff, et al., 2014). Consequently, lactobacilli colonization exhibits an important role regarding STIs infection risk in both male and female counterparts.

Exogenously supplied lactobacilli probiotics have been used to alter a woman's genital microbiome from a BV-associated state to a 'healthy' *Lactobacillus* dominated environment (Martinez, et al., 2009; Reid, 2008). Therefore, the use of probiotics to not only alter the microbiota to a 'healthy' state but also as a prophylactic strategy to reduce the acquisition of STIs has been postulated. *In vitro*, studies have given further insight to the role of lactobacilli in prevention of STIs. A major mechanism by which lactobacilli are able to exert their anti-viral and anti-bacterial effects is through the production of lactic acid, hydrogen peroxide and the overall ability to create acidic environment (Atassi & Servin, 2010). For example, a study by St. Amant *et al.,* demonstrated that lactobacilli inhibited *Neisseria gonorrhoeae* in both

acidic and neutral pH conditions, however, the inhibition effect was not observed when hydrogen peroxide was neutralized (St Amant, Valentin-Bon, & Jerse, 2002). The opposite effect was observed when lactobacilli were incubated with *Chlamydia trachomatis*. The production of lactic acid by lactobacilli was sufficient to inactivate *Chlamydia trachomatis* however production of hydrogen peroxide was not capable of inactivation (Gong, Luna, Yu, & Fan, 2014). An additional study suggested that lactobacilli were able to inhibit Chlamydia elementary bodies (EB), in a concentration dependent manner through the production of metabolites such as organic acids, which inversely correlated with pH (Nardini et al., 2016). Furthermore, the amount of organic acid production by *Lactobacillus* species. was dependent on strain suggesting that specific strains of lactobacilli exert better protection in the FGT (Nardini, et al., 2016). Lactobacilli have also been shown to reduce viral replication of HIV-1 and HSV-2 *in vitro* (Zabihollahi et al., 2012). Interestingly, the observed anti-viral effect was suggested to be due to production of bacteriocins or molecules with viral neutralization activity, not pH or hydrogen peroxide. Together, these studies suggest a role for the therapeutic potential of lactobacilli probiotics for the reduction of STIs, including HIV in *vivo*.

1.6.4 Role of Probiotics in Reducing Inflammation and Strengthening the Epithelial Barrier

Both epithelial barrier breakage and inflammation are associated with increased acquisition of STIs in the FGT. The use of lactobacilli in the gut has

been extensively studied, as an anti-inflammatory and to increase epithelial barrier function in inflammatory bowel disease (IBD) (Bai & Ouyang, 2006). An *in vitro* study by Anderson *et al.,* showed a significant increase in the transepithelial electrical resistance (TER), a measure of barrier integrity in an intestinal epithelial cell (IEC) line when treated with lactobacilli (Anderson et al., 2010). Using microarray data, 19 tight-junction related genes were up regulated in the presence of lactobacilli, further verifying the increase in barrier integrity. In addition, tight junction staining was performed on the IEC monolayers and showed an increase in fluorescence intensity for cells treated with lactobacilli (Anderson, et al., 2010). An additional study using the same IEC cell line showed that the up-regulation of tight junction proteins by lactobacilli occurred through the stimulation of TLR-2 (Karczewski et al., 2010). Lactobacilli treatment was also shown to protect the IEC barrier in the presence of substances known to induce barrier damage such as TNF-α (Eun, et al., 2011). The protection observed by lactobacilli in the presence of TNF-α was diminished when cells were treated with a mitogen activated protein kinase (MAPK) pathway inhibitor, suggesting that the protection of the IEC barrier in the presence of lactobacilli is dependent on the MAPK pathway (Eun, et al., 2011). In addition to the beneficial effects on the IEC barrier, lactobacilli have been shown to modulate inflammatory factors. This has been well demonstrated using enterotoxigenic *Escherichia coli* (ETEC) K88 infection to induce damage in a porcine epithelial cell model (Roselli et al.,

2007). The treatment of infected cells with lactobacilli, reduced ETEC adhesion, prevented membrane damage by inhibiting disruption of tight junction protein ZO-1, up-regulated IL-10 expression and inhibited ETEC induced IL-8 (Roselli, et al., 2007). It has been suggested that lactobacilli inhibit the NF-κB activation through both the myeloid differentiation primary response gene 88 (MyD88)-dependent and independent pathways in murine intestinal epithelial cells, thus providing a mechanism for the decrease in inflammation (Petrof et al., 2009). Majority of research has shown that direct bacterium-cell contact is necessary for the beneficial effects associated with probiotics. However, recent research has shown that lactobacilli derived soluble proteins are responsible for some the beneficial effects observed by lactobacilli. For example, *L. rhamnosus*-GG derived soluble proteins p75 and p40 have been shown in both human and mouse colonic epithelial cells to induce protein kinase B (Akt), inhibit apoptosis induced by TNF-α, and promote cell growth (Yan et al., 2007). Furthermore, when p75 and p40 were immunodepleted from *L. rhamnosus*-GG conditioned media the reduction in TNF-α induced epithelial cell damage was reversed (Yan, et al., 2007). These findings suggest a role for probiotic bacteria in the prevention of epithelial barrier damage and adverse health outcomes mediated by inflammatory cytokine. Additionally, this supports the movement to assess these barrier and anti-inflammatory effects of probiotic lactobacilli in the FGT.

1.7 Regulation of the Female Genital Tract Microbiome by Sex Hormones and Hormonal Contraceptives

The menstrual cycle is able to alter the FGT microbiota (Wira, et al., 2015). Cyclic changes observed with the differing phases of the menstrual cycle involving factors such as hormones, pH, and glycogen content, affect bacterial adherence, their ability to colonize epithelial cells, and composition of microbial species (Eschenbach et al., 2000; Gajer et al., 2012). High concentration of estrogen increases glycogen deposition in epithelial cells, which is released into the lumen and serves as an important carbohydrate source for lactobacilli (Boskey, et al., 2001; Cruickshank, 1934; Mirmonsef et al., 2014; Paavonen, 1983; Wilson et al., 2010). In addition, high concentration of estrogen is associated with increased adherence of lactobacilli to epithelial cells (Silva, Rey, & Elena Nader-Macias, 2004). The decrease in estrogen during menopause results in lower amounts of *Lactobacillus* species in the FGT and this is associated with increased susceptibility to genital tract infections (Kumar et al., 2011). Thereby, these studies give evidence that the FGT microbiota is regulated by female sex hormones (Brotman, et al., 2014), and that FGT colonization by *Lactobacillus* species demonstrated protective mechanisms against pathogens (Reid, 2008). Together, studies in the literature suggest that increased estrogen acts on the microenvironment to increase the abundance of lactobacilli, which demonstrate immunomodulatory effects such as decreased pro-inflammatory

cytokines and HIV target cells, and reduced the risk of epithelial barrier disruption. This could lead to decreased susceptibility to HIV in the FGT. In comparison, the decreased glycogen deposition during the secretory phase of the menstrual cycle is correlated with a decreased abundance of Lactobacilli (Keane, Ison, & Taylor-Robinson, 1997). Given that lactobacilli have been shown to produce lactic acid as a protective mechanism in the FGT (Aldunate, et al., 2013), the association between increased susceptibility to HIV and decreased levels of lactobacilli may be related to decreased levels of lactic acid. Additionally, decreased lactobacilli would suggest that there is reduced competition for colonization on the surface of epithelial cells. This would allow for increased available surface area for pathogens to bind and induce a pro-inflammatory environment. Furthermore, clinical evidence from cross-sectional studies have linked contraceptive use with changes in vaginal microbiota (Achilles & Hillier, 2013). Miller et al., have shown that the use of DMPA significantly decreased colonization by hydrogen peroxide producing lactobacilli, while other strains of lactobacilli remained unchanged (C. M. Mitchell, et al., 2014). The production of hydrogen peroxide in the FGT is a non-specific host defense against a range of pathogens (Razzak, Al-Charrakh, & Al-Greitty, 2011). Therefore, the associated link between lack of hydrogen peroxide producing *Lactobacillus* species and DMPA use, may help explain the increased susceptibility to STIs in HC users. Given the benefits associated with increased *Lactobacillus* species in the FGT, the ability to

regulate lactobacilli colonization may be important when considering preventative strategies to inhibit HIV acquisition. Thus, the use of probiotic strains of lactobacilli that can modulate beneficial properties in the FGT should be considered.

1.8   Innate Immunity and the Microbiome

It has been suggested that the mucosal microbiota can modulate immune responses (Brotman, et al., 2014; McDermott & Huffnagle, 2014), however the specific mechanisms involved remain unknown. To date, majority of work done in this area has been conducted in the gastrointestinal tract where a clear relationship between the colonization of bacteria and the immune system has been observed (Arranz, Pena, & Bernardo, 2013; Jacobs & Braun, 2014; Round & Mazmanian, 2009; Sengupta et al., 2013). Similarly, it is thought that the microbiota within the FGT is able to influence innate mucosal immunity (Doerflinger, et al., 2014; Mirmonsef et al., 2011; C. Mitchell & Marrazzo, 2014; Witkin, Linhares, & Giraldo, 2007). This has been observed in studies which examined the outcome of incubating vaginal epithelial cells with strains of bacteria associated with either healthy or BV associated microbiota (Doerflinger, et al., 2014; Rose et al., 2012). For example, an experimental study reported that vaginal epithelial cells produced pro-inflammatory cytokines when incubated with *Atopobium vaginae* (BV-associated bacteria), however incubation with *L. cristpatus* (healthy FGT

associated bacteria) did not show a pro-inflammatory effect (Doerflinger, et al., 2014). Furthermore, Anahtar *et al.,* showed that increasing microbial diversity is positively correlated with inflammation in the FGT (Anahtar et al., 2015). This correlation was observed through both cross sectional and longitudinal analysis of the vaginal microbiome of healthy women. By further performing transcriptional profiling it was suggested that genital inflammation is a result of NF-kB activation by TLR4 on APCs that sense gram-negative bacteria (Anahtar, et al., 2015).

These studies show that microbial strains associated with a less healthy microbiota mediate a more pro-inflammatory environment in the FGT (Schwebke & Weiss, 2002). Studies from our lab and others have shown that a pro-inflammatory environment can lead to mucosal barrier disruption (Ferreira, et al., 2011; Nazli, et al., 2010). Therefore, it is possible that one of the reasons for increased susceptibility of STIs observed in women with BV may be attributed to decreased epithelial barrier functions as a result of the inflammatory environment (Ferreira, et al., 2011).

CHAPTER 2: RATIONALE, HYPOTHESIS AND AIMS

2.1   Rationale

Globally, over half of HIV infected individuals are women, with approximately 40% of transmission events occurring in the female genital tract (FGT). Epithelial cells lining the FGT comprise the first barrier to entry to HIV, and normally exist in the genital microenvironment in the presence of female sex hormones and the mucosal microbiota. Previous *in vivo* and *in vitro* studies have suggested that certain female sex hormones or a dysbiosis of the FGT microbiota may lead to inflammation and enhanced HIV acquisition. Additionally, epidemiological studies show conflicting evidence regarding the role of hormonal contraceptives, specifically the synthetic progestin Depo medroxyprogesterone acetate (DMPA), on HIV transmission in women. Although a *Lactobacillus*-dominant microenvironment in the FGT has been shown to have protective functions against viral pathogens, the impact of sex hormones and lactobacilli on epithelial barrier function and innate inflammatory factors remains to be determined.

Our lab has previously established that in a primary GEC culture system, HIV induces an inflammatory response, which causes epithelial barrier breakage and subsequent translocation of the virus. Both female sex hormones/hormonal contraceptives and the bacterial microbiota have been shown to affect HIV susceptibility in the FGT. Given that the epithelial barrier in the FGT is under constant regulation by female sex hormones/hormonal

contraceptive and the bacterial microenvironment we wanted to further study the role of hormones and protective bacteria from the microbiota (*Lactobacillus* species) on inflammation and epithelial cell barrier function within the primary GEC culture system.

2.2   Hypothesis

We hypothesize that lactobacilli strains (GR-1, RC-14 and L. crispatus) or E2 added to primary GEC cultures will increase barrier functions and reduce pro-inflammatory cytokine production. Conversely, the presence of P4 and MPA will not have a positive effect on these epithelial functions. In the presence of HIV, lactobacilli strains and E2 will play a protective role against HIV mediated barrier disruption and inflammation in GECs

2.3   Aims

To address this hypothesis, we proposed the following aims:

2.3.1   Aim 1) Examine female genital tract epithelial cell barrier function and innate immune responses in the presence of lactobacilli

2.3.2   Aim 2) Examine how the presence of female sex hormones influences barrier function and innate immune responses in genital epithelial cell cultures

2.3.3   Aim 3) Examine how interactions between hormones and lactobacilli modulate female genital tract epithelial cell barrier function and innate immune responses

2.3.4   Aim 4) Examine how interactions between hormones and lactobacilli modulate barrier function and innate immune responses of female genital tract epithelial cells in the presence of HIV

CHAPTER 3: RESULTS

3.1   Aim 1

Examine female genital tract epithelial cell barrier function and innate immune responses in the presence of lactobacilli

Rationale: A FGT microbiota dominated by *Lactobacillus* species has been shown to modulate immune responses and confer protection against pathogens (Reid, 2008), with *L. crispatus* being the most common species of *Lactobacillus* associated with a "healthy" vaginal microbiota. The probiotic strains *L. rhamnosus* and *L. reuteri,* also called GR-1 and RC-14 respectively, have been well characterized and extensively used for treatment of urogenital infections in women (Petrova, et al., 2015; Petrova, et al., 2013; Reid, 2008). The beneficial effects of *Lactobacillus* species have been observed in both *in vivo* and *in vitro* studies, many of which associate the presence of *Lactobacillus* in gut epithelial cells with increased epithelial barrier functions (Garcia-Lafuente, et al., 2001). However, the relationship between epithelial cells and lactobacilli on barrier function in the FGT has not been well characterized. We first adapted our epithelial cell culture system to standardize conditions for exposure to lactobacilli in order to then examine the interactions of lactobacilli with FGT epithelial cells.

Results: To optimize dose and exposure time, and measure GEC viability in the presence of lactobacilli, we exposed primary FGT epithelial

cells to either 10 or 100 CFU/epithelial cell concentrations of GR-1 and RC-14 for 1, 3, 6, 12 and 24 hours. An initial experiment was performed in anaerobic conditions given that *Lactobacillus* species are facultative anaerobes and are able to grow in either the presence or absence of oxygen. Results showed that the viability of epithelial cells was consistent between groups for the 1, 3, 6 and 12 hour time points, while a reduction in viability was observed at the 24 hour time point (Figure 1A). No differences in viability were observed between the different probiotic *Lactobacillus* strains.

We also looked at transepithelial electrical resistance (TER), which is a measure of cell monolayer integrity and is commonly used as a proxy indicator to monitor the growth and condition of polarized monolayers (Benson, Cramer, & Galla, 2013). With the exception of RC-14 (100 CFU/cell), all treatments and controls decreased TER measurements in GECs at the end of the 1, 3, 6 and 12-hour time points, compared to initial TER measurements at 0 hours (Figure 1B). At the 24-hour time point, all treatments and controls, with the exception of RC-14 (10 CFU/cell), decreased TER measurements. The TER decrease in the majority of treatments, including controls, suggested that the GECs had impaired cell function that affected membrane integrity within the anaerobic environment. Lastly, we examined the adherence of the *Lactobacillus* strains and found that adherence of both strains increased over time for the 1, 3, and 6 hour time points (Figure 1C). There was no significant difference in adherence of

lactobacilli between the 6 and 12 hour time points, however GR-1 showed increased adherence between the 12 and 24 hour time points, while RC-14 did not. There was a dose-dependent effect, with increased concentration of lactobacilli (100 CFU/cell) resulting in increased adherence when compared to the lower concentration (10 CFU/cell).

Initial experimental data gave insight as to how to better design future experiments. As previously mentioned, TER measurements decreased in all treatments, including control. We initially chose to perform experiments in anaerobic conditions as *Lactobacillus* species are facultative anaerobes and grow well in an anaerobic environment. However, FGT epithelial cells exist in the presence of oxygen and therefore the lack of oxygen may have contributed to the decrease in TER observed. The decreased viability of epithelial cells from 12 to 24 hours in the presence of probiotic *Lactobacillus* species compared to the no bacteria control suggested that the cells were not sustainable at the 24 hour time point in the presence of lactobacilli. Thus, we repeated these experiments using similar experimental treatment and design in aerobic conditions and using time points of 12 hours or less.

Results showed that the epithelial cells were >90% viable in the presence of probiotic strains of *Lactobacillus* for all time points (Figure 2A). Furthermore, when the experiment was performed in aerobic conditions, the presence of probiotic lactobacilli increased TER measurements beyond baseline for each treatment at all time points (Figure 2B). RC-14 significantly

increased TER at the 6 and 12-hour time points. This data indicates that 12 hours is an optimal exposure time of lactobacilli to primary GECs given that viability was >90% and TER measurements increased indicating an improvement of barrier integrity. Additionally, we looked at the time kinetics of bacterial adherence to GECs and measured bacteria in the cell culture supernatant as a proxy for bacterial replication. Both probiotic strains of *Lactobacillus* increased in adherence and enumeration over time (Figure 2C-D), while no significant differences were observed between the adherence or enumeration of GR-1 and RC-14. Monolayer permeability is an additional proxy measure of GEC barrier function. Given the previously observed increase in TER of GECs in the presence of probiotic lactobacilli, permeability was also measured to assess the 'leakiness' of the GEC monolayer. GEC permeability was assessed using a fluorescein isothiocyanate (FITC)–labeled dextran dye assay. By adding the FITC-labeled dextran dye to the apical compartment of the GEC monolayer and measuring the amount of dye in the basolateral compartment 24-48 hours later, we were able to measure the movement of the dye from the apical to the basolateral compartments of the transwell system. The presence of probiotic lactobacilli in GEC cultures significantly decreased the permeability of the monolayer compared to the control suggesting that in the presence of lactobacilli, GECs have less permeability or 'leakage' (Figure 3). A decrease in permeability suggests a more intact monolayer, further supporting that probiotic strains of lactobacilli

are able to increase GEC barrier functions. Furthermore, tight junctions between adjacent columnar GECs regulate TER and permeability of GEC monolayers. Therefore, fluorescent staining for the tight junction protein ZO-1 was performed, along with visualization by confocal microscopy. Results showed no significance difference in the fluorescence intensity of cell monolayers treated with lactobacilli compared to controls (Figure 4). Within columnar epithelial cells, multiple proteins regulate the function of tight junctions. Although the increase in TER and decrease in permeability was not attributed to ZO-1 expression, it may be a result of other tight junction proteins such as occludin or claudin 1-4. Lastly, to assess the effect of lactobacilli on innate inflammatory factors, we used the production of the pro-inflammatory cytokine TNF-α as an initial representative read-out for inflammation. The presence of GR-1 and RC-14 did not induce a significant TNF-α response beyond that seen in the no treatment control, indicating that these strains of lactobacilli do not induce a pro-inflammatory response (Figure 5).

After observing the increase in barrier integrity seen by the probiotic strains of *Lactobacillus*, we wanted to use a non-probiotic species of *Lactobacillus* as a comparison. For this, we chose *L. crispatus*, which is the most common species of lactobacilli found in the FGT of women considered to have a 'healthy' microbiota. We obtained *L. crispatus* (ATCC 33820) from collaborators and performed an initial standardization experiment with *L.*

crispatus using the same experimental design as described in Figures 2-5. Results showed that similar to the probiotic strains of *Lactobacillus,* viability was not altered over the 3, 6, or 12-hour time points in the presence of *L. crispatus*, and that TER measurements were increased in comparison to control treatments, although this was not statistically significant (Figure 6A-B). The adherence and enumeration of *L. crispatus* within the GEC culture system (Figure 6C-D) was lower when compared to the previous experiments using GR-1 and RC-14 (Figure 2C-D). Similar to results observed with probiotic lactobacilli (Figure 3), *L. crispatus* also demonstrated a significant decrease in the amount of dextran dye 'leakage' from the apical to basolateral compartment of the transwell compared to control (Figure 7). ZO-1 fluorescence was observed, however results revealed no significant increase in fluorescence by GECs treated with *L. crispatus* compared to controls (Figure 8). Additionally, GECs treated with *L. crispatus* induced a significant increase in TNF-α production (Figure 9) unlike GR-1 and RC-14 (Figure 5) suggesting that the response of GECs to probiotic strains of *Lactobacillus* may be different compared to non-probiotic strains. Every primary GEC culture is from a different donor and may produce donor specific responses to treatments. Therefore, we next conducted an experiment to compare all three strains of *Lactobacillus* within the same GEC culture to confirm if the results were reproducible. As previously observed, TER measurements were

increased in the presence of all three strains of lactobacilli (GR-1, RC-14, and *L. crispatus*), however only RC-14 significantly increased compared to untreated controls (Figure 10A). No significant difference in bacterial adherence or enumeration was observed for GECs treated with stains of probiotic *Lactobacillus* (Figure 10B-C). However, *L. crispatus* showed a decrease in adherence and enumeration (Figure 10B-C) compared to GR-1 and RC-14, further supporting the results previously described (Figure 2C-D and 6C-D). Increased TNF-α production by GECs in the presence of *L. crispatus* (Figure 11) was consistent with previous experiments (Figure 9). Furthermore, in the presence of *L. crispatus*, GECs also showed an increase in other pro-inflammatory cytokines such as IL-1β, IL-1α, and GM-CSF, some of which were significantly increased (Figure 11). These results suggest that initial experiments (Figure 2-9) are indeed reproducible and that regardless of the source of donor from which GEC cultures are grown, GECs exhibit a differential response to probiotic strains of *Lactobacillus* compared to *L. crispatus*. Therefore, additional experiments were performed on other tissues, comparing all three strains of *Lactobacillus* species and data was combined for overall analysis of four separate experiments (Figure 12-16).

Cumulatively, the data indicated that *L. crispatus* and probiotic strains of *Lactobacillus* significantly increased TER in comparison to untreated controls (Figure 12A). Bacterial adherence and enumeration of *L. crispatus* was significantly lower compared to both probiotic strains of *Lactobacillus*

(Figure 12C-D). The permeability of the GEC monolayer showed a decrease in cells treated with all strains of lactobacilli, however the decrease was only significant in the presence of probiotic lactobacilli (Figure 13). There was no significant increase in ZO-1 fluorescence intensity in GECs treated with any of the *Lactobacillus* species (Figure 14). The production of TNF-α was significantly increased in GECs in the presence of *L. crispatus* (Figure 15). *L. crispatus* is commonly the most abundant species in the lower FGT, and is associated with a 'healthy' microbiota. However, the microbiota of the upper FGT is not as well characterized, since it was previously thought to be sterile. Recently, it has been hypothesized that the most common *Lactobacillus* species in the upper FGT is *L. iners or* other bacterial species such as *Bacteroides xylanisolvens* (Aldunate, et al., 2013). Therefore, the decreased adherence and enumeration and increased TNF-α response of *L. crispatus* within the GEC culture system may be a result of its naturally low presence in the UGT. Although it is the primary *Lactobacillus* species associated with a health microbiota in the LGT, it may not be the case in the UGT.

Since bacterial adherence and enumeration were significantly lower for *L. crispatus* compared to GR-1 or RC-14, we wanted to determine if the GEC culture conditions were having an effect. Bacterial growth curves show changes in bacterial population size over time. To study the growth kinetics of the *Lactobacillus* species, we performed bacterial growth curves. Growth curves were initially performed in MRS broth, to provide the bacteria optimal

50

growth conditions. In order to determine if culture conditions might have an effect, we also assessed lactobacilli growth within the GEC culture medium. Optical density (OD_{600}) is a proxy measure of bacterial growth. To quantify the bacterial growth at each optical density reading, a sample was serially diluted and plated on MRS agar. After incubation the number of colonies that grew indicated the actual number of viable bacteria. By reading optical density values and plating samples to count colonies of viable bacteria every 30 minutes, we found that the three strains of lactobacilli (GR-1, RC-14 and *L. crispatus*) performed similarly when grown in MRS broth (Figure 16A-B). Despite differences in adherence and enumeration of *L. crispatus* compared to the probiotic strains of *Lactobacillus* when incubated with GECs, there were no differences in growth curves when the strains were grown in MRS broth. Conversely, when the lactobacilli were incubated in the GEC culture medium, no distinct phases of growth were observed, as there was no significant variation in optical density values or colony forming units within each individual strain of lactobacilli over time, suggesting that the bacterial strains were bacteriostatic in GEC culture medium. However, *L. crispatus* showed decreased colony forming units and optical density values compared to GR-1 and RC-14 (Figure 16C-D). The decreased colony forming units of *L. crispatus* when incubated in GEC culture medium suggests that *L. crispatus* is less tolerant in media compared to GR-1 and RC-14. Although GEC culture

51

medium is not ideal for bacterial growth, this data suggests that lactobacilli in GEC culture medium were viable and sustained over time.

Additionally, most *Lactobacillus* species are able to confer inhibitory effects against pathogens in the FGT (Petrova, et al., 2015), therefore, we also wanted to investigate at the differences in anti-microbial characteristics between *Lactobacillus* strains. Hydrogen peroxide is produced by some *Lactobacillus* species to prevent the overgrowth of pathogenic bacteria, fungi, and to protect against viruses (Boris & Barbes, 2000; Klebanoff, Hillier, Eschenbach, & Waltersdorph, 1991). Studies indicate that *in vitro* hydrogen peroxide produced by lactobacilli is able to inactivate HIV (Klebanoff & Coombs, 1991). Therefore, we wanted to determine if the *Lactobacillus* species in our model were producing hydrogen peroxide. RC-14 is known to produce high levels of hydrogen peroxide, while GR-1 produces low levels of hydrogen peroxide (Reid & Bruce, 2001). Our measurements of hydrogen peroxide production by the *Lactobacillus* strains were in agreement with previous literature, as RC-14 and *L. crispatus* produced significantly higher levels of hydrogen peroxide compared to GR-1 and the media control (Figure 17). These results suggest that GR-1 has less anti-pathogenic activity, with respect to hydrogen peroxide production, in comparison to RC-14 and *L. crispatus* in our model (Figure 17).

In conclusion, these results indicate that *Lactobacillus* species play a role in the increase of barrier integrity as observed by the significant increase

in TER measurements compared to control. Interestingly, there were clear differences in adherence, enumeration, and inflammatory cytokine production between the probiotic strains of *Lactobacillus* and *L. crispatus.* The probiotic *Lactobacillus* species showed increased adherence and enumeration compared to *L. crispatus.* Furthermore, *L. crispatus* conferred a significant increase in inflammatory cytokine production (TNF-α, MIP-1α, GM-CSF and IL-1β) compared to no bacteria control. Based on the results of the above-mentioned experiments, we have established an optimal time point and concentration to study the effects of *Lactobacillus* on GEC barrier function and innate immune responses. Given that GEC viability decreased at 24 hours (Figure 1A), and that viability was >80% up to 12 hours (Figure 2A and 6A), future experiments were performed using 12-hours or less of bacterial exposure.
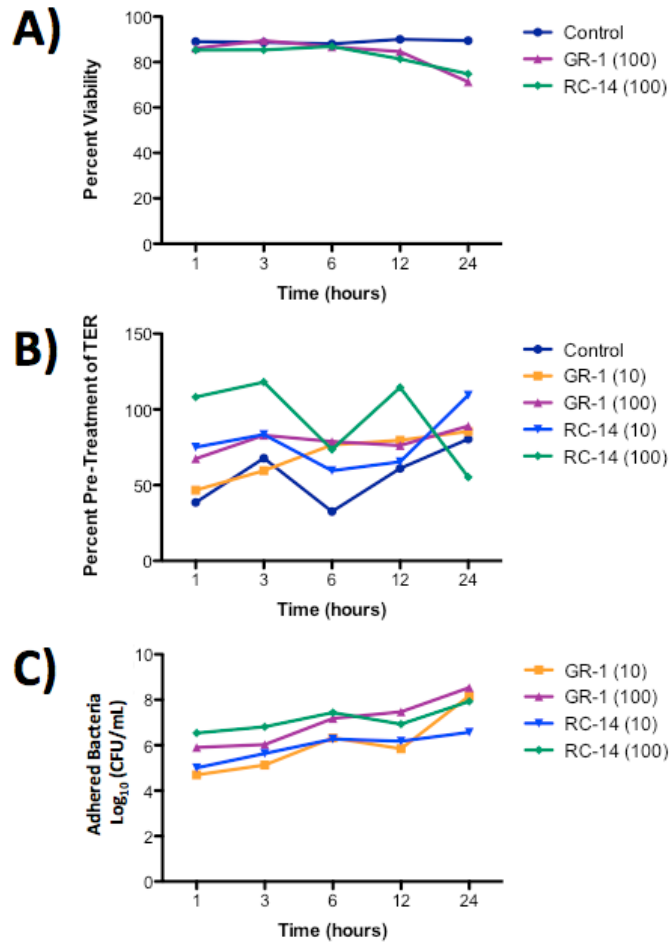
Figure 1. Time kinetics and parameters of genital epithelial cells exposed to probiotic strains of _Lactobacillus_ (GR-1 and RC-14) in an anaerobic environment. Primary genital epithelial cells exposed to probiotic strains of Lactobacilli (GR-1; _L. rhamnosus_ and RC-14; _L. reuteri_) and control (no bacteria), at 10 CFU/cell and 100 CFU/cell for 1, 3, 6, 12, and 24 hours. **A)** Cell viability was measured using a trypan blue exclusion assay to count total live and dead cells at the end of each time point. Data is shown as the percent of live cells. One replicate per experimental condition was included. **B)** TER values were observed at 0 hours and at the end of each time point and are expressed as percent pre-treatment at each time point. Two replicates per experimental condition were included. **C)** The number of viable bacteria adhering to the cell surface monolayer at the end of each time point were determined by collecting the adhered bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as Log10 CFU/mL. All data represents one biological sample with two technical replicates per time point.
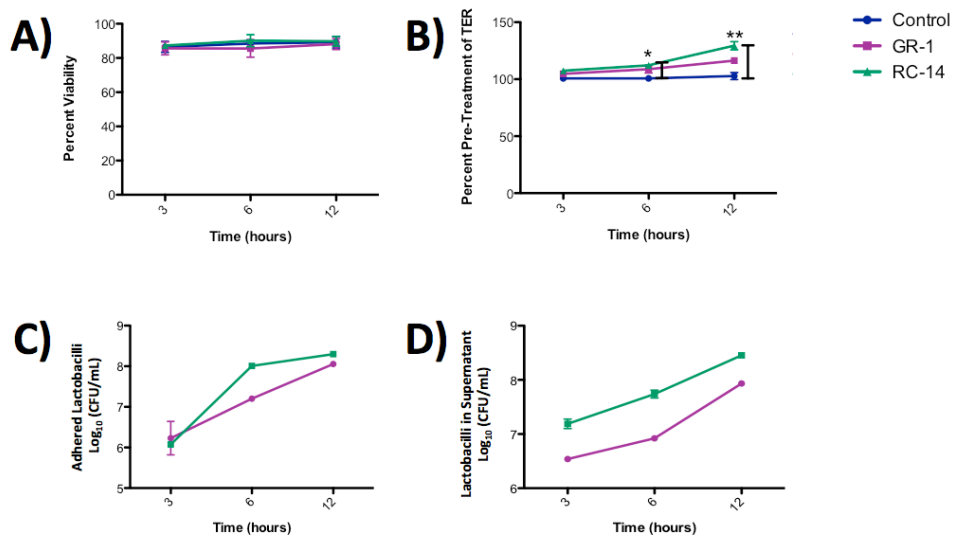
Figure 2. Time kinetics and parameters of genital epithelial cells exposed to probiotic strains of *Lactobacillus* (GR-1 and RC-14) in an aerobic environment. Primary genital epithelial cells exposed to probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*) and control (no bacteria), at 100 CFU/cell for 3, 6, and 12 hours. **A)** Cell viability was measured using a trypan blue exclusion assay to count total live and dead cells at the end of each time point. Data is shown as the percent of live cells. Two technical replicates per experimental condition were included. **B)** TER values were observed at 0 hours and at the end of each time point and are expressed as percent pre-treatment of each time point. Seven technical replicates per experimental condition were included. **C)** The number of viable bacteria adhering to the cell surface monolayer at the end of each time point were determined by collecting the adhered bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as Log10 CFU/mL. Three technical replicates per experimental condition were included. **D)** The number of viable bacteria in the cell culture supernatant observed at the end of each time point by collecting the bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as Log10 CFU/mL. Three technical replicates per experimental condition were included. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *$p<0.05$, **$p<0.01$, ***$p<0.001$. All data represents one biological sample.

Figure 3. The percent of FITC-labeled dextran dye leakage though polarized GECs is significantly decreased in the presence of probiotic lactobacilli. Primary genital epithelial cells pre-treated with probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*) and control (no bacteria), at 100 CFU/cell were incubated with an apical treatment 2.3mg/mL of 4 kDa FITC-labeled dextran dye for 24 hours. At 24 hours 50uL of apical and basolateral supernatant were collected in duplicate and placed into a 96 well plate. Fluorescence of FITC-labeled dextran dye was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices; Sunnyvale, CA) at an excitiation of 490nm and emission of 520nm. The dextran leakage in the basolateral compartment is expressed as a percentage of dextran added to the apical compartment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. Data represents one biological sample with two technical replicates per time point.
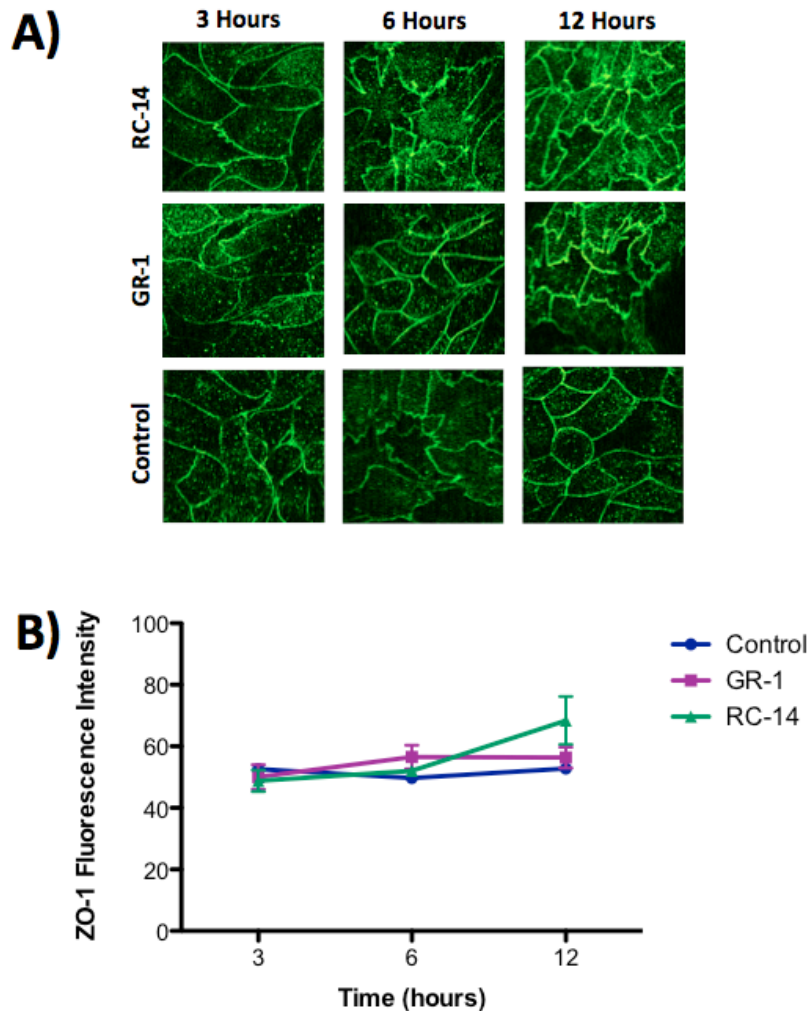
Figure 4. Time kinetics of ZO-1 staining and quantification of genital epithelial cells exposed to probiotic strains of *Lactobacillus* (GR-1 and RC-14) in an aerobic environment. Primary genital epithelial cells exposed to probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*) and control (no bacteria), at 100 CFU/cell for 3, 6, and 12 hours. **A)** Cell monolayers were fixed at each time point and were stained for ZO-1 tight junction protein. Images were captured using a laser scanning confocal microscope. One technical replicate per experimental condition was included. **B)** Quantification of ZO-1 staining was determined by measuring fluorescence intensity of ZO-1 using Image J Software. One technical replicate per experimental condition with three separate images per condition were included. All data represents one biological sample.
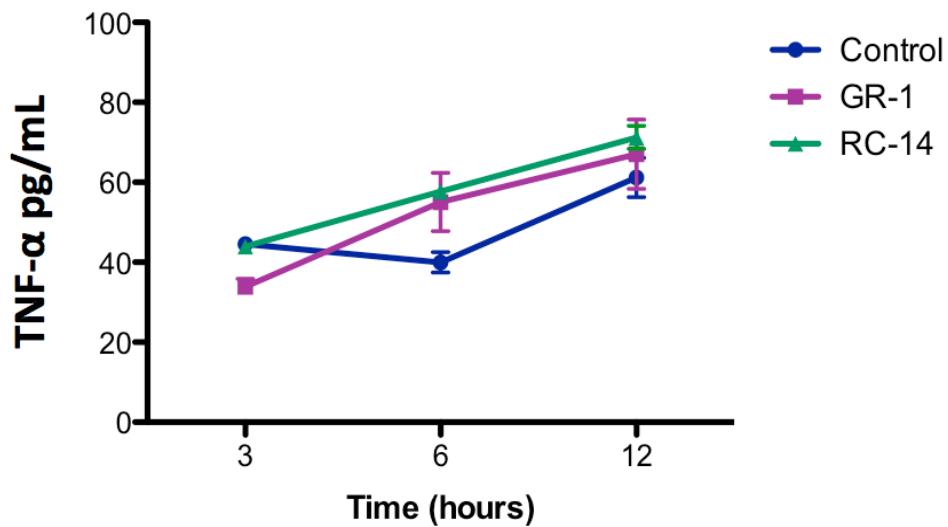
Figure 5. Time kinetic of TNF-α production by genital epithelial cells exposed to probiotic strains of *Lactobacillus* (GR-1 and RC-14) in an aerobic environment. Primary genital epithelial cells exposed to probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*) and control (no bacteria), at 100 CFU/cell for 3, 6, and 12 hours. Apical supernatants were collected at each time point and production of TNF-α was measured using a TNF-α ELISA. Data is expressed as pg/mL. All data represents one biological sample with two technical replicates per time point and treatment.
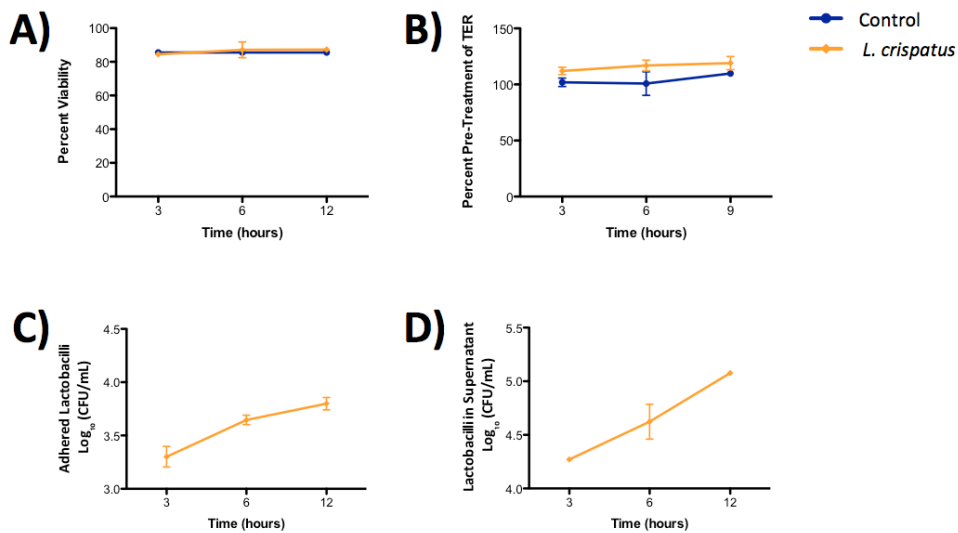
Figure 6. Time kinetics and parameters of genital epithelial cells exposed to *L. crispatus* in an aerobic environment. Primary genital epithelial cells exposed to *L. crispatus* and control (no bacteria), at 100 CFU/cell for 3, 6, and 12 hours. **A)** Cell viability was measured using a trypan blue exclusion assay to count total live and dead cells at the end of each time point. Data is shown as the percent of live cells. Two technical replicates per experimental condition were included. **B)** TER values were observed at 0 hours and at the end of each time point and are expressed as percent pre-treatment of each time point. Seven technical replicates per experimental condition were included. **C)** The number of viable bacteria adhering to the cell surface monolayer at the end of each time point were determined by collecting the adhered bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as Log10 CFU/mL. Three technical replicates per experimental condition were included. **D)** The number of viable bacteria in the cell culture supernatant observed at the end of each time point by collecting the bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as Log10 CFU/mL. Three technical replicates per experimental condition were included. All data represents one biological sample.

Figure 7. The percent of FITC-labeled dextran dye leakage though polarized GECs is significantly decreased in the presence *L. crispatus*. Primary genital epithelial cells pre-treated *L. crispatus* or no bacteria control, at 100 CFU/cell were incubated with an apical treatment of 2.3mg/mL of 4 kDa FITC-labeled dextran dye for 24 hours. At 24 hours 50uL of apical and basolateral supernatant were collected in duplicate and placed into a 96 well plate. Fluorescence of FITC- labeled dextran dye was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices; Sunnyvale, CA) at an excitation of 490nm and emission of 520nm. The dextran leakage in the basolateral compartment is expressed as a percentage of dextran added to the apical compartment. Statistical analysis was performed using an unpaired t-test to compare each treatment to control. All data represents one biological sample with two technical replicates per treatment.
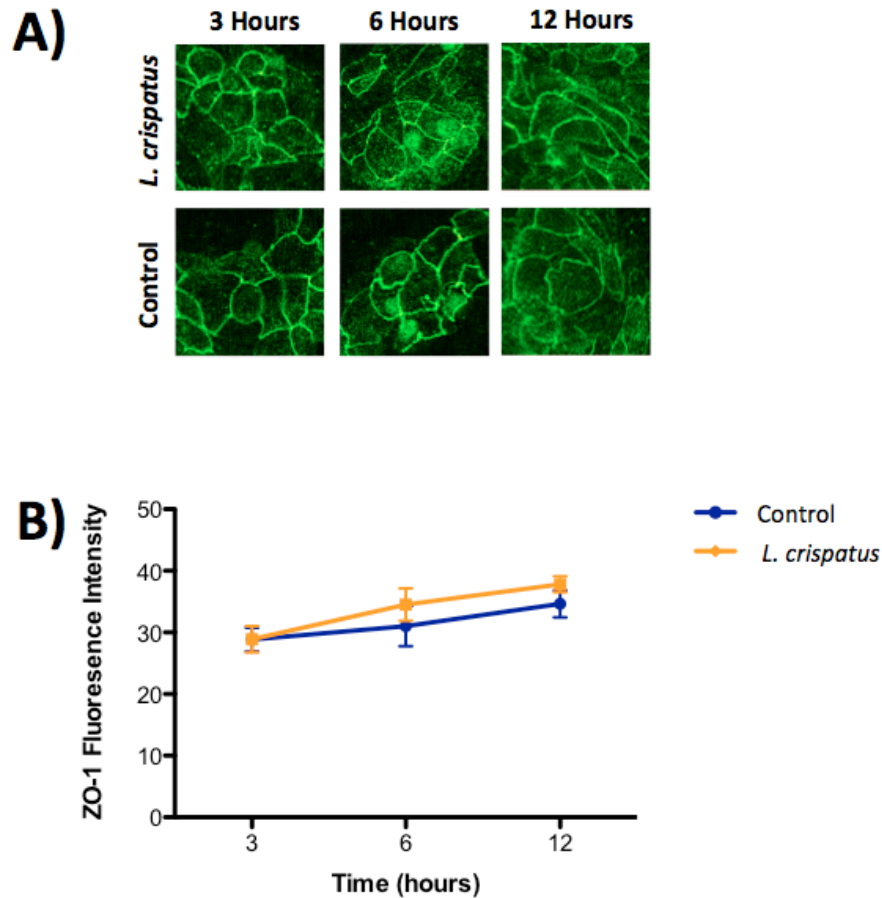
Figure 8. Time kinetics of ZO-1 staining and quantification of genital epithelial cells exposed to *L. crispatus* in an aerobic environment. Primary genital epithelial cells exposed to probiotic strains of *L. crispatus* and control (no bacteria), at 100 CFU/cell for 3, 6, and 12 hours. **A)** Cell monolayers were fixed at each time point and were stained for ZO-1 tight junction protein. Images were captured using a laser scanning confocal microscope. One technical replicate per experimental condition was included. **B)** Quantification of ZO-1 staining was determined by measuring fluorescence intensity of ZO-1 using Image J Software. One technical replicate per experimental condition with three separate images per condition were included. All data represents one biological sample.
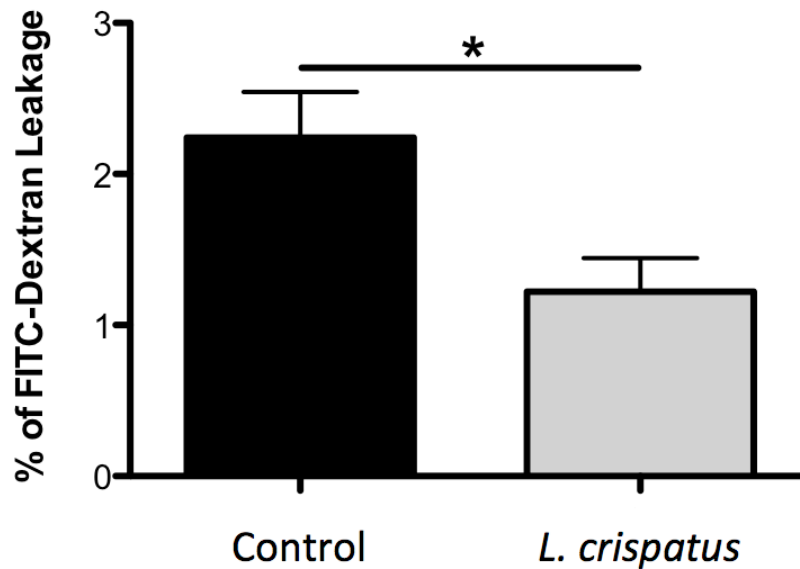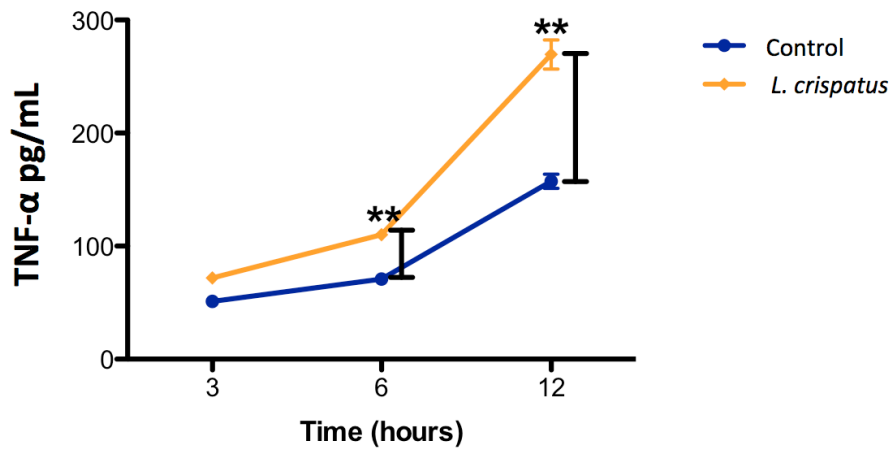
Figure 9. Time kinetic of TNF-α production by genital epithelial cells to *L. crispatus* in an aerobic environment. Primary genital epithelial cells exposed to *L. crispatus* and control (no bacteria), at 100 CFU/cell for 3, 6, and 12 hours. **A)** Apical supernatants were collected at each time point and production of TNF-α was measured using a TNF-α ELISA. Data is expressed as pg/mL. Data was analyzed using a two-tailed unpaired t-test, **p<0.005. Two technical replicates per experimental condition were included. All data represents one biological sample.
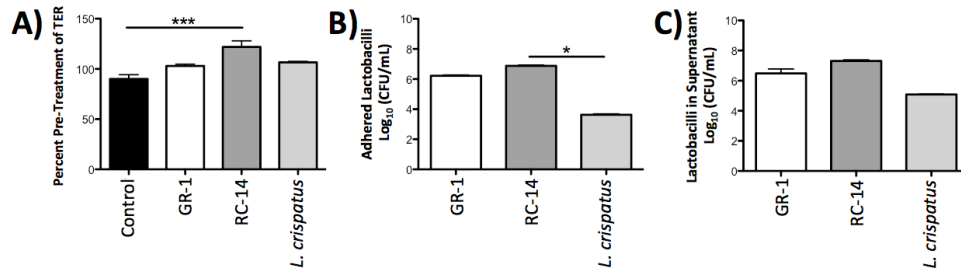
Figure 10. Comparison of parameters of genital epithelial cells exposed to *Lactobacillus* species (GR-1, RC-14 and *L. crispatus*). Primary genital epithelial cells were exposed to the most common *Lactobacillus* strain associated with a 'healthy' vaginal microbiota (*L. crispatus)*, probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*), and control (no bacteria), at 100 CFU/cell for 12 hours. **A)** TER values were observed at 0 hours and 12 hours and are expressed as percent pre-treatment of each time point. Five technical replicates per experimental condition were included. **B**) The number of viable bacteria adhering to the cell surface monolayer at the end 12 hours were determined by collecting the adhered bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as CFU/cm^2. Three technical replicates per experimental condition were included. **C)** The number of viable bacteria in the cell culture supernatant observed at 12 hours were by collecting the bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as Log10 CFU/mL. Three technical replicates per experimental condition were included. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All data represents one biological sample.
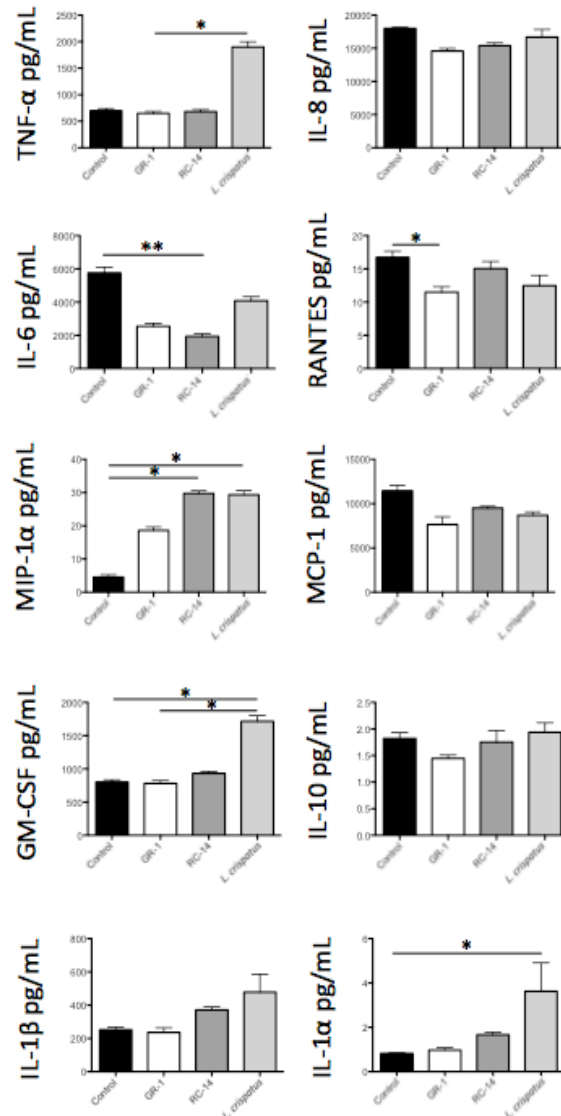
Figure 11. Comparison of cytokine/chemokine responses from genital epithelial cells exposed to *Lactobacillus* species (GR-1, RC-14 and *L. crispatus*). Primary genital epithelial cells were exposed to the most common *Lactobacillus* strain associated with a 'healthy' vaginal microbiota (*L. crispatus)*, probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*), and control (no bacteria), at 100 CFU/cell for 12 hours. Apical supernatants were collected at 12 hours. A Millipore multi-analyte assay was used to measure the production of cytokines and chemokines: IL-1α, IL-1β, IL-6, IL-8, IL-10, GM-CSF, MCP-1, MIP-1α, RANTES, and TNF-α, and are expressed as pg/mL of cytokine/chemokine production. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. Two technical replicates per experimental condition were included. All data represents one biological sample.
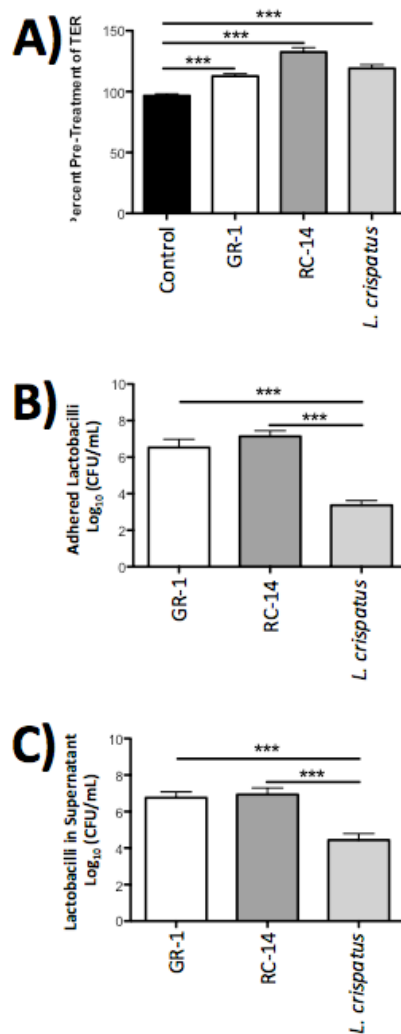
Figure 12. Cumulative comparison of *Lactobacillus species* (GR-1, RC-14 and *L. crispatus*) on genital epithelial cells. Primary genital epithelial cells were exposed to the most common *Lactobacillus* strain associated with a 'healthy' vaginal microbiota (*L. crispatus)*, probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*), and control (no bacteria), at 100 CFU/cell for 12 hours. **A)** TER values were observed at 0 hours and 12 hours and are expressed as percent pre-treatment of each time point. A minimum of 27 technical replicates per experimental condition were included. **B)** The number of viable bacteria adhering to the cell surface monolayer at the end 12 hours were determined by collecting the adhered bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as Log 10 CFU/mL. A minimum of 14 technical replicates per experimental condition were included. **C)** The number of viable bacteria in the cell culture supernatant observed at 12 hours were determined by collecting the bacteria and plating on MRS agar in serial dilutions. Bacteria were quantified by counting colonies grown on plates and are expressed as Log10 CFU/mL. A minimum of 14 technical replicates per experimental condition were included. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All data represents a combination of four biological samples.

Figure 13. Cumulative comparison of FITC-labeled dextran dye leakage though polarized GECs is significantly decreased in the presence of *Lactobacillus species* (GR-1, RC-14 and *L. crispatus*). Primary genital epithelial cells pre-treated with GR-1, RC-14, *L. crispatus* or no bacteria control, at 100 CFU/cell were incubated with an apical treatment of 2.3mg/mL of 4 kDa FITC-labeled dextran dye for 24 hours. At 24 hours 50uL of apical and basolateral supernatant were collected in duplicate and placed into a 96 well plate. Fluorescence of FITC- labeled dextran dye was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices; Sunnyvale, CA) at an excitation of 490nm and emission of 520nm. The dextran leakage in the basolateral compartment is expressed as a percentage of dextran added to the apical compartment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. All data represents a combination of two biological samples with two technical replicates per experimental condition.
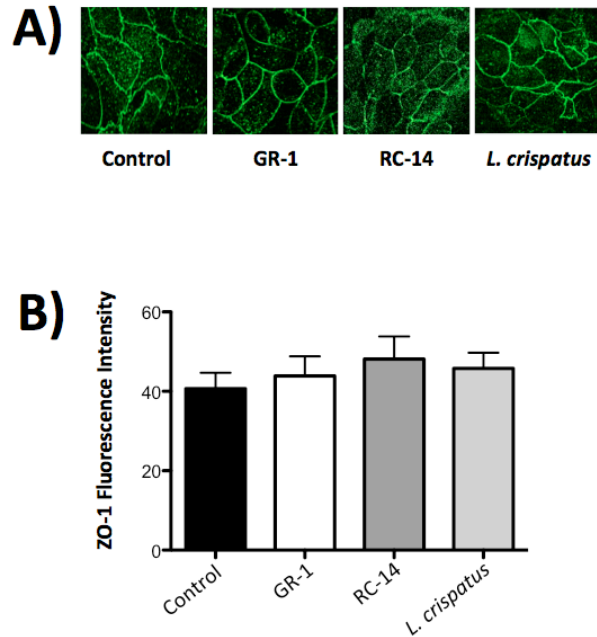
Figure 14. Cumulative comparison of ZO-1 staining and quantification of *Lactobacillus species* (GR-1, RC-14 and *L. crispatus)* on genital epithelial cells. Primary genital epithelial cells were exposed to the most common *Lactobacillus* strain associated with a 'healthy' vaginal microbiota (*L. crispatus)*, probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*), and control (no bacteria), at 100 CFU/cell for 12 hours. **A)** Cell monolayers were fixed at each time point and were stained for ZO-1 tight junction protein. Images were captured using a laser scanning confocal microscope. Images are representative of three replicates per experimental condition. **B)** Quantification of ZO-1 staining was determined by measuring fluorescence intensity of ZO-1 using Image J Software. Three technical replicates per experimental condition with three separate images per condition were included. All data represents a combination of two biological samples.
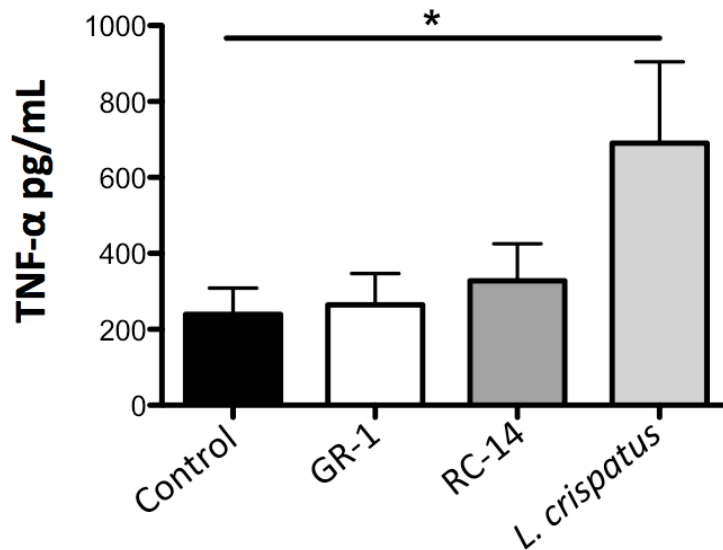
Figure 15. Cumulative comparison of TNF-a production by genital epithelial cells exposed to *Lactobacillus species* (GR-1, RC-14 and *L. crispatus)*. Primary genital epithelial cells were exposed to the most common *Lactobacillus* strain associated with a 'healthy' vaginal microbiota (*L. crispatus)*, probiotic strains of Lactobacilli (GR-1; *L. rhamnosus* and RC-14; *L. reuteri*), and control (no bacteria), at 100 CFU/cell for 12 hours. Apical supernatants were collected at 12 hours and TNF-α production was measured by ELISA and is expressed as pg/mL on the y-axis. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001). A minimum of 5 technical replicates per experimental condition were included. All data represents a combination of four biological samples.
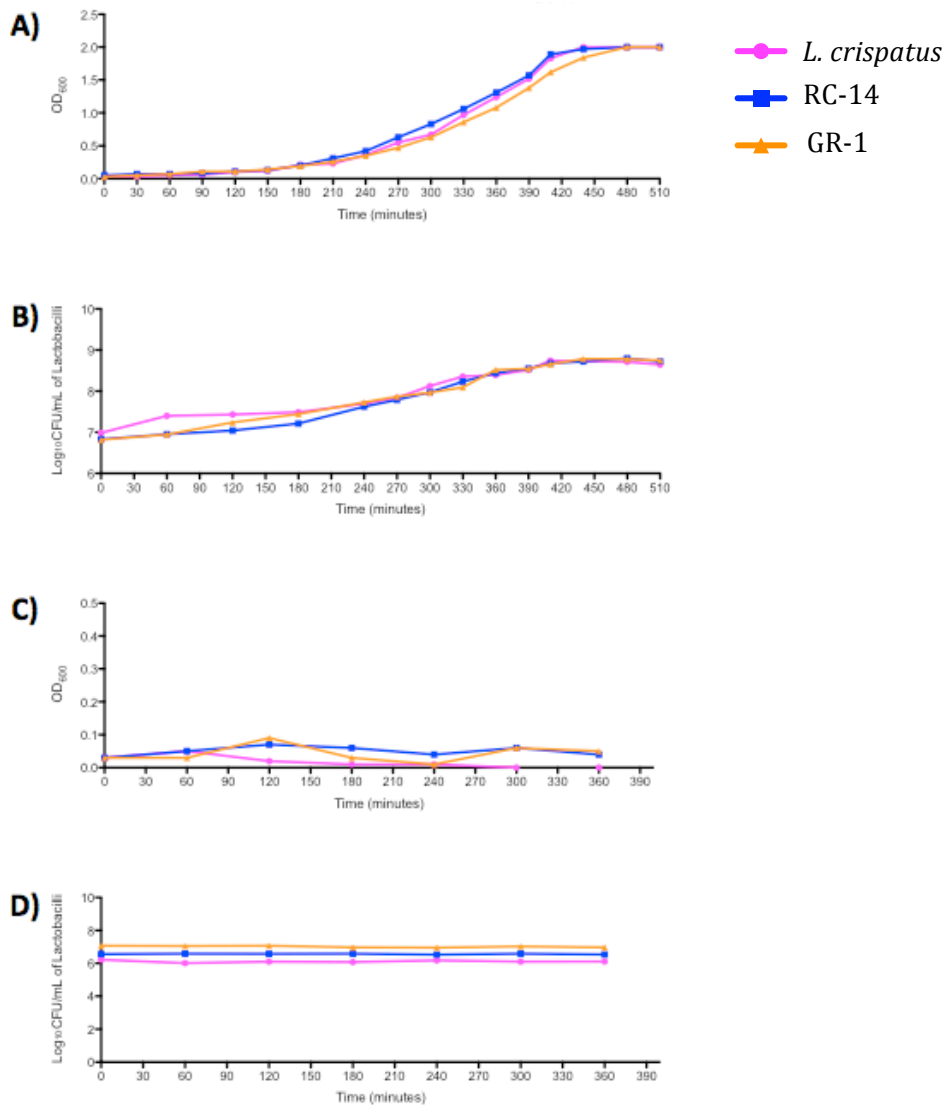
Figure 16. Bacterial growth of GR-1, RC-14 and *L. crispatus* is inhibited when incubated in GEC culture media. Bacterial growth was assessed for GR-1, RC-14 and *L. crispatus* in MRS agar and GEC culture media (DMEM/F12; phenol red free). Optical density at 600nm (OD_{600}) was measured every 30 minutes and 100uL was taken at each time point to plate serial dilutions on MRS agar. Agar plates were incubated for 24 hours and counted. Viable bacteria are expressed as Log10 CFU/mL. **A)** OD_{600} measurements of GR-1, RC-14 and *L. crispatus* in MRS broth. **B)** GR-1, RC-14 and *L. crispatus* CFU/mL sampled from growth curve stock solution in MRS broth. **C)** OD_{600} measurements of GR-1, RC-14 and *L. crispatus* in GEC cell culture media. **D)** GR-1, RC-14 and *L. crispatus* CFU/mL sampled from growth curve stock solution in GEC cell culture media. All data in A-D is representative of two separate experiments.
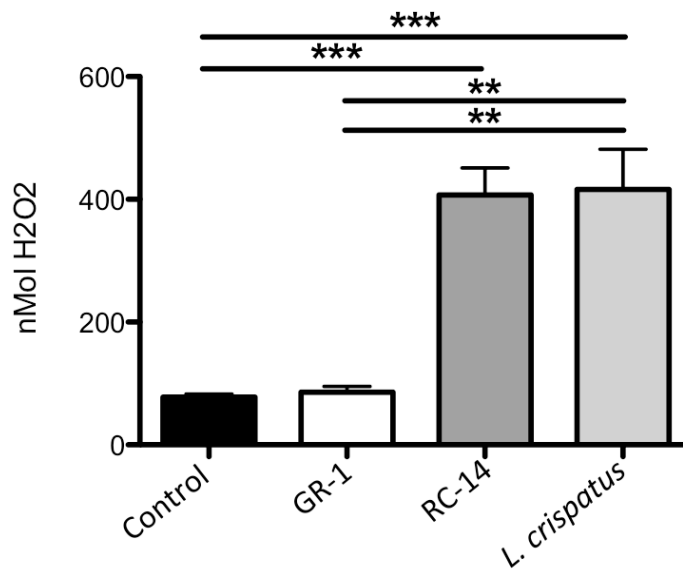
Figure 17. Cumulative comparison of hydrogen peroxide production by _Lactobacillus species_ (GR-1, RC-14 and _L. crispatus)_ in genital epithelial culture conditions. Primary genital epithelial cells were exposed to the most common _Lactobacillus_ strain associated with a 'healthy' vaginal microbiota (_L. crispatus)_, probiotic strains of Lactobacilli (GR-1; _L. rhamnosus_ and RC-14; _L. reuteri_), and control (no bacteria), at 100 CFU/cell for 12 hours. Apical supernatants were collected and assayed for hydrogen peroxide, represented as nMol H_2O_2 on the y-axis, using a commercial detection kit. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Two technical replicates per experimental condition were included. All data represents a combination of two biological samples.

3.2   Aim 2

Examine how the presence of female sex hormones influences barrier function and innate immune responses in genital epithelial cell cultures

Rationale: Many studies have demonstrated that female sex hormones regulate immune responses in the genital tract of women (Brotman, et al., 2014), however epithelial cell responses to these hormones remain unclear. More specifically, the effect of sex hormones on barrier function has not been established. Epithelial cells in the FGT are under constant regulation by fluctuations in sex hormones such as E2 and P4. Several studies using animal models or *ex vivo* cervical explants have shown that E2 plays a protective role in HIV infection while P4 increases susceptibility to HIV (Saba, et al., 2013; Smith, et al., 2000).

Globally, over 150 million women use hormonal contraceptives (HCs), with 50 million of them using injectable contraceptives such as depot-medroxyprogesterone acetate (DMPA) (Bureau, 2002). Medroxyprogesterone acetate (MPA), the primary constituent in DMPA, has been reported in literature to increase susceptibility to sexually transmitted infections including HIV (Baeten, et al., 2007; Crook, et al., 2014; Marx, et al., 1996; Mascola, et al., 2000; Polis & Curtis, 2013; Trunova, et al., 2006). The effect of MPA on the genital epithelial cell barrier as a mechanism of increased susceptibility requires further investigation. To better understand the role of sex hormones

in the FGT, we examined how E2, P4, and MPA influence the FGT epithelial barrier functions and the modulation epithelial immune responses.

Results: To study the effect of female sex hormones on the epithelial barrier, primary FGT epithelial cells were grown in the presence or absence of physiological concentrations of E2 (10^{-9}), P4 (10^{-7}), MPA (10^{-9}), or a no hormone negative control for a minimum of 7 days. These experiments examined the barrier function of GECs cultured in the presence of female sex hormones, or the hormonal contraceptive MPA, over time. No differences in TER measurements of cells grown in the presence of E2, P4, or MPA were observed over time (Figure 18A-B). To further elucidate the effect of hormones on GEC barrier function a FITC-labeled dextran dye was used to assess the permeability of the GEC monolayer. GEC permeability was determined by measuring the movement of FITC-dextran dye through the GEC monolayer from the apical to the basolateral compartment of the transwell system. Permeability was assessed at 24 (Figure 19A) and 48-hour (Figure 19B) time points. GECs grown in the presence of hormones did not significantly influence permeability at the 24 or 48-hour time point. Staining for ZO-1 was also visualized and quantified. Similar to TER, the presence of hormones did not alter ZO-1 staining or quantification (Figure 20A-B). Together, these data indicate that hormones alone do not alter the barrier function of primary GECs in culture (Figure 18-20).

To assess the inflammatory environment of GECs grown in the presence of female sex hormones, supernatants were collected to measure a panel of cytokines/chemokines (IL-1α, IL-1β, IL-6, IL-8, IL-10, GM-CSF, MCP-1, MIP-1α, RANTES, and TNF-α) using a Millipore multiplex assay. GECs grown in the presence of E2 showed a significant decrease in TNF-α, IL-1α, IL-1β, and IL-8 compared to no hormone treated cells suggesting that E2 was conferring an anti-inflammatory environment (Figure 21). A significant decrease in MIP-1α was observed for cells grown in the presence of MPA compared to no hormone control (Figure 21). Additional inflammatory cytokines and chemokines were also measured, such as IL-6, IL-10, GM-CSF, MCP-1 and RANTES, however no significant differences were observed (Figure 21). To verify that E2 was inducing an anti-inflammatory environment in our GEC system, we measured TNF-α by ELISA as a proxy measure of inflammation. Combined data from eleven GEC cultures showed that E2 and MPA conferred a significant decrease in TNF-α production compared to the no hormone control (Figure 22).

A number of studies have provided evidence for a direct relationship between estrogen and glycogen in the FGT of humans and animal models (Ayehunie et al., 2015; Ayre, 1951; Gregoire & Parakkal, 1972; Gregoire, Ramsey, & Adams, 1967; Gregoire & Richardson, 1970; Wrenn, Bitman, & Wood, 1968). During a woman's reproductive years, a mid-cycle peak in estrogen correlates with GEC proliferation and intracellular glycogen

deposition (Farage & Maibach, 2006). Women using DMPA have suppressed endogenous estrogen levels similar to post-menopausal women and subsequently, have decreased genital tract glycogen levels (Jeppsson, Gershagen, Johansson, & Rannevik, 1982; Miller et al., 2000; Mirmonsef et al., 2015; C. M. Mitchell, et al., 2014). Glycogen within the FGT is metabolized by *Lactobacillus* species to produce a product with known anti-pathogenic activity, lactic acid (Conti, Malacrino, & Mastromarino, 2009; Graver & Wade, 2011; Juarez Tomas, Ocana, Wiese, & Nader-Macias, 2003; Lai, et al., 2009; O'Hanlon, Moench, & Cone, 2013). Therefore, we measured glycogen levels in genital epithelial cell supernatants and cell lysates in the presence or absence of hormones. Although the results were not statistically significant, apparent trends suggested that E2- and no hormone-treated epithelial cells have similar levels of glycogen production within the cells (Figure 23). MPA treated cells showed no glycogen deposition within the GEC cultures (Figure 23).

These experiments indicate that hormones do not alter the barrier function of GECs grown in hormones, as evidenced by a lack of difference in ZO-1 staining, TER measurements, and FITC-dextran dye permeability between cells grown in the presence or absence of hormones. However, while E2, P4 and MPA alone do not affect barrier functions in GECs, E2 and MPA do influence innate immune responses in the FGT epithelial cells, as

74

evidenced by decreased production of pro-inflammatory cytokines in their

presence.
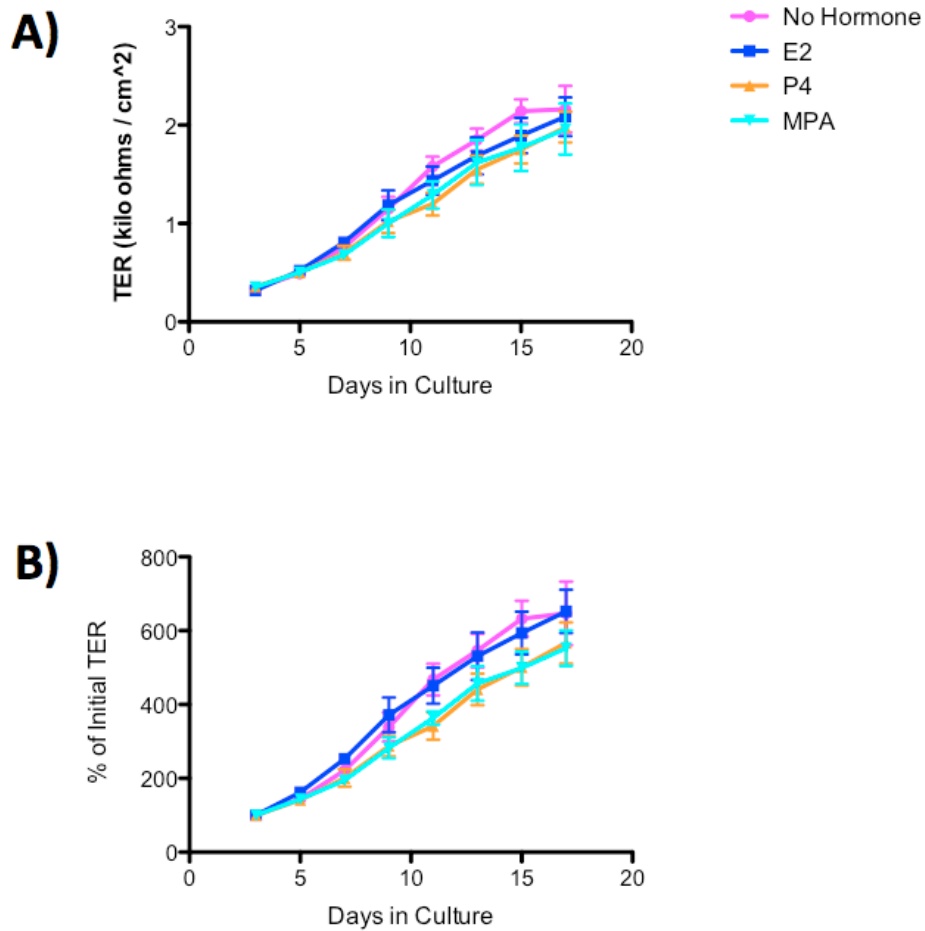
Figure 18. Cumulative comparison of the effect hormones on genital epithelial cells TER over time. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) for 7-17 days. **A)** TER measurements were observed every second day starting at day 3. **B)** TER measurements expressed as percent of initial (day 3 TER). All data represents a combination of four biological samples with a minimum of five technical replicates per experimental condition.

A)

B)
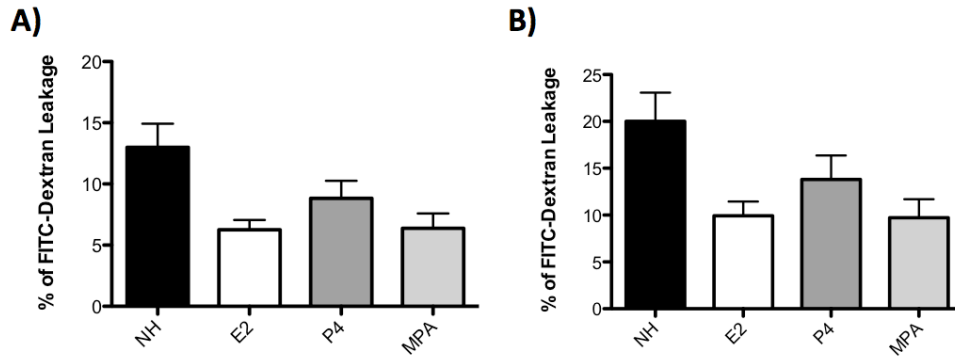
Figure 19. Cumulative comparison of the effect hormones on genital epithelial cell barrier permeability. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) for 7-17 days. Barrier permeability was measured by adding FITC-labelled dextran dye to the apical side of the epithelial cell monolayer. At 24 **(A)** and 48 **(B)** hours 50uL of basolateral supernatant was collected and fluorescence (excitation 490; emmision 520) was measured. The leakage is expressed as the percentage of dye in the basolateral compartment compared to the apical compartment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons (*p<0.05, **p<0.01, ***p<0.001). All data represents a combination of four biological samples with two technical replicates per experimental condition.

A)

NH E2 P4 MPA

B)

ZO-1 Fluorescence Intensity
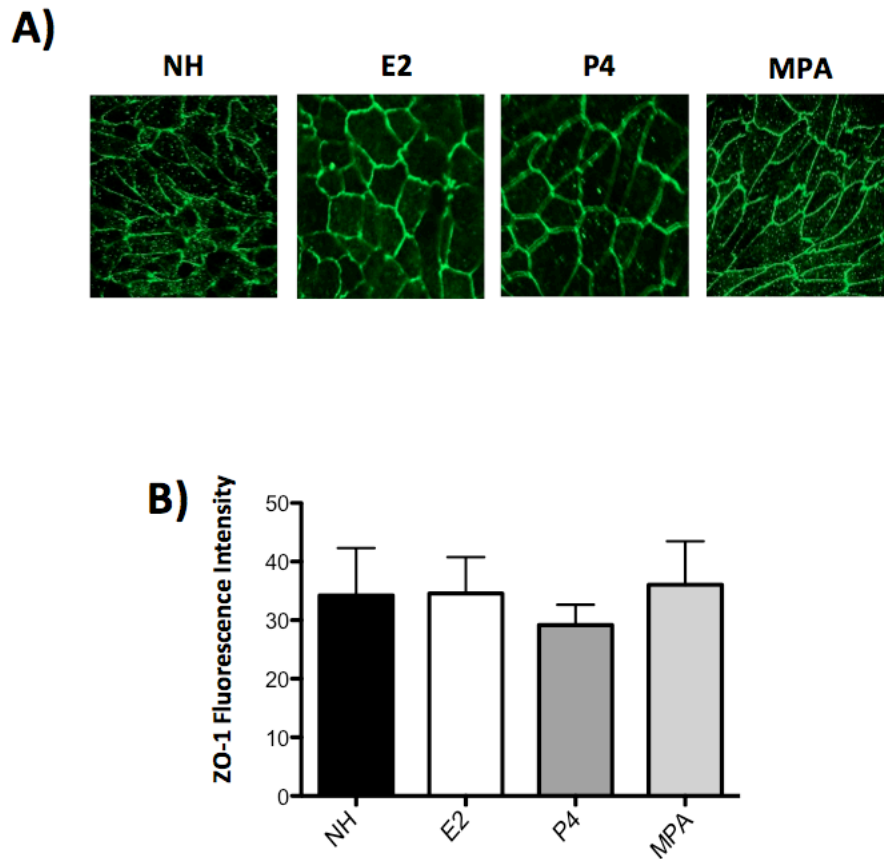
Figure 20. Cumulative comparison of the effect hormones on genital epithelial cells ZO-1 staining and quantification. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) for 7-17 days. **A)** Cell monolayers were fixed at day 10 and were stained for ZO-1 tight junction protein. Images were captured using a laser scanning confocal microscope. Images are representative of three replicates per experimental condition. **B)** Quantification of ZO-1 staining was determined by measuring fluorescence intensity of ZO-1 using Image J Software. Two technical replicates per experimental condition with three separate images per condition were included. All data represents a combination of two biological samples.
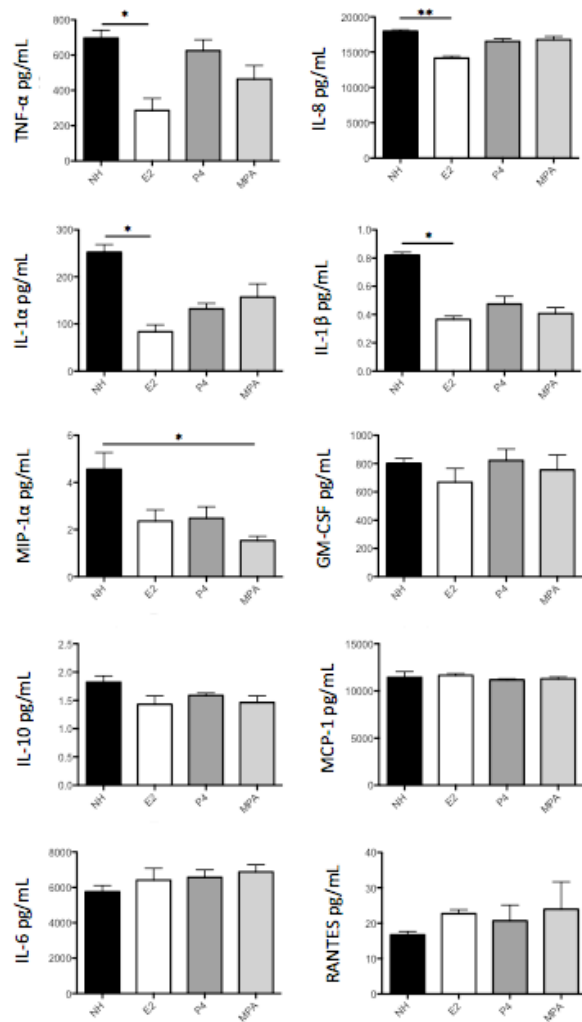
Figure 21. Effect of female sex hormones on genital epithelial cell cytokine production. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) for 10 days. Apical supernatants were collected and assayed by Millipore multi-analyte kit for the following cytokines and chemokines: IL-1α, IL-1β, IL-6, IL-8, IL-10, GM-CSF, MCP-1, MIP-1α, RANTES, and TNF-α, and are expressed as pg/mL of cytokine/chemokine production. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. Two technical replicates per experimental condition were included. All data represents one biological sample.
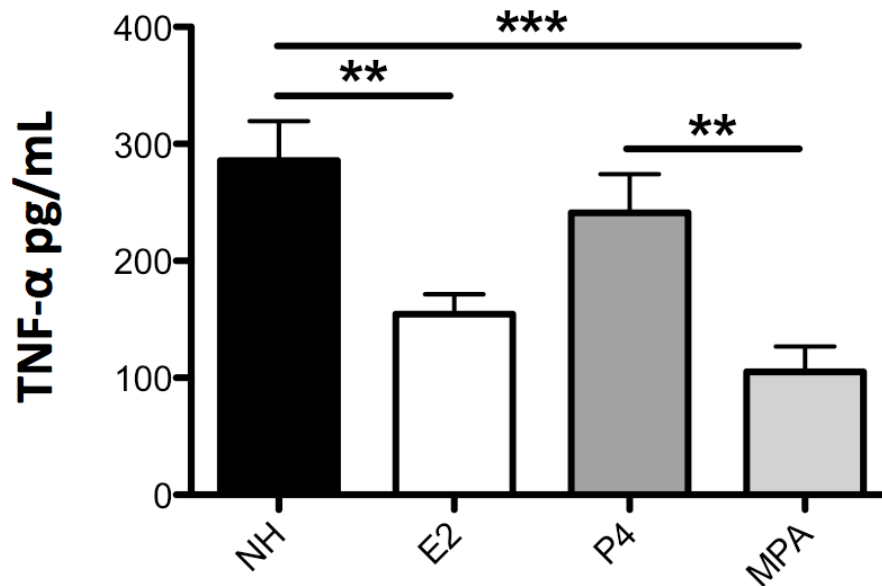
Figure 22. Cumulative comparison of the effect hormones on genital epithelial cell TNF-α production. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) for 7-17 days. Apical supernatants were collected and TNF-α production was measured by ELISA and is expressed as pg/mL. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons (*$p<0.05$, **$p<0.01$, ***$p<0.001$). All data represents a combination of 11 biological samples with a minimum of 2 technical replicates per experimental condition were included.
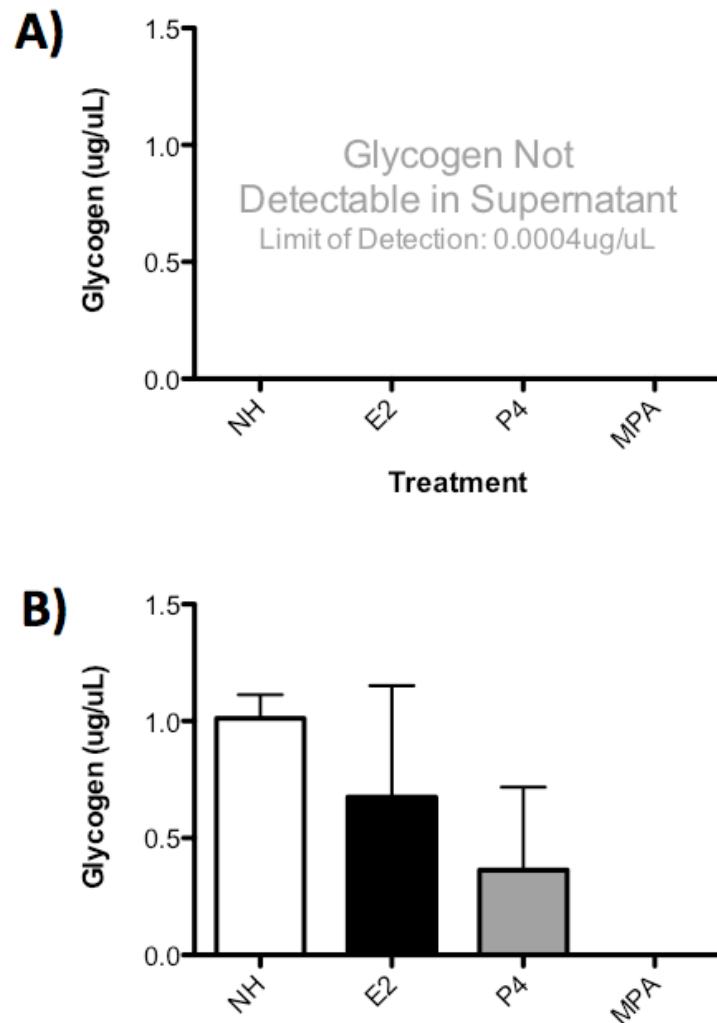
Figure 23. Glycogen deposition in primary genital epithelial cells exposed to female sex hormones. Primary genital epithelial cells were grown in the presence or absence of the female sex hormones E2 (10^{-9}), P4 (10^{-7}) or the hormonal contraceptive MPA (10^{-9}) for 7-10 days. Apical supernatants and cell lysates were collected and assayed for glycogen production represented as ug/ul of glycogen on the y-axis, using a commercial glycogen detection kit. A minimum of ten replicates per experimental condition were pooled for collection of cell lysates and two replicates per experimental condition were collected for assessment of apical supernatants. **A)** Glycogen detection in apical supernatants. **B)** Glycogen detection in cell lysates of cells treated with E2 and no hormone (control). All data represents a combination of three biological samples with a minimum of 1 technical replicates per experimental condition.

3.3   Aim 3

Examine how interactions between hormones and lactobacilli modulate female genital tract epithelial cell barrier function and innate immune responses

**Rationale:** Female sex hormones have been shown to alter the composition of the microbiota in the FGT (Brotman, et al., 2014). Studies have reported that the proliferative phase (E2 dominant) of the menstrual cycle is associated with increased colonization of *Lactobacillus* species in comparison to the secretory phase (P4 dominant) (Eschenbach, et al., 2000). However, the interaction of female sex hormones in combination with *Lactobacillus* in the context of epithelial cell barrier function and innate responses has not been determined.

Results: To study the interactions between a combination of hormones and lactobacilli on the GEC barrier, primary GECs were grown in the presence or absence of hormones (E2, P4, or MPA) for 7-10 days, followed by exposure to lactobacilli. When comparing different *Lactobacillus* strains in combination with hormones, experiments demonstrated that epithelial barrier integrity, as measured primarily by TER, was significantly increased in the presence of GR-1, RC-14, and *L. crispatus* for epithelial monolayers grown in the presence of No Hormone, E2, and MPA. GECs grown in the presence of P4 only had a significant increase in TER when exposed to RC-14 (Figure 24A). When comparing hormone treatments by bacterial strain, cells grown in

the presence of E2 or P4 had significantly higher TER compared to those grown in the presence of MPA, when exposed to RC-14. Additionally, E2 treatment also resulted in significantly higher TER compared to MPA treatment when exposed to *L. crispatus* (Figure 24B). Primary GECs grown in the presence of no hormone, E2, and P4 significantly decreased barrier permeability in the presence of probiotic lactobacilli (Figure 25A). The addition of *L. crispatus* to GEC monolayers significantly decreased permeability when cells were grown in the presence of E2, although a decrease was observed in all hormonal conditions (Figure 25A). GECs grown in the presence of MPA showed a similar trend to other hormonal treatments in the presence of *Lactobacillus* species, however results did not reach significance (Figure 25A). Furthermore, when comparing hormone treatment by bacterial strain, both GR-1 and *L. crispatus* showed a significant decrease in E2, P4, and MPA treatments compared to the no hormone control, whereas RC-14 did not (Figure 25B).

Based on clinical research, which previously reported a decrease in hydrogen peroxide-producing lactobacilli in the FGT of women using DMPA, we predicted that the presence of MPA would result in decreased adherence and enumeration of the hydrogen peroxide-producing RC-14 *Lactobacillus* strain. In contrast, we expected that cells grown in the presence of E2 would have increased adherence and enumeration of lactobacilli. In our model, no differences were observed in bacterial adherence or enumeration of GECs

grown in the presence or absence of hormones. However, *L. crispatus* showed consistently less adherence and enumeration compared to GR-1 and RC-14 in all hormone groups (Figure 26 and 27).

Given that our previous results showed an increase in the production of pro-inflammatory cytokines by GECs in response to *L. crispatus,* and a decrease in the production of pro-inflammatory cytokines by GECs in the presence of E2, we next aimed to characterize the inflammatory response to *L. crispatus* treatment in the presence of E2. As expected, combined data from three separate experiments showed that the induction of certain pro-inflammatory cytokines (IL-1α and TNF-α) by the addition of *L. crispatus* to the GEC monolayers was prevented when cells were grown in the presence of E2 (Figure 28 and 29). This further suggests that GECs grown in the presence of E2 may have anti-inflammatory effects and that E2 may confer protection against inflammation related outcomes, such as HIV acquisition through the FGT. Interestingly, TNF-α responses induced by *L. crispatus* were also significantly dampened by MPA, while P4 induced an increase in TNF-α responses.

Previous literature has suggested that women using the hormonal contraceptive DMPA have decreased hydrogen peroxide-producing lactobacilli (Miller, et al., 2000). Given that there were no major changes observed in the enumeration or adherence of lactobacilli between hormone-treated cells, the amount of hydrogen peroxide being produced from

lactobacilli in different hormonal conditions was measured. It was consistently observed that RC-14 and *L. crispatus* produced increased amounts of hydrogen peroxide compared to GR-1. No significant differences were observed in the amount of hydrogen peroxide production between hormone conditions, suggesting that hormones do not alter the production of hydrogen peroxide by lactobacilli in our model (Figure 30).

In summary, these experiments suggest that in the presence of lactobacilli, female sex hormones or hormonal contraceptives can alter barrier functions and innate immune responses within the GEC culture system. Specifically, E2 was anti-inflammatory and all *Lactobacillus* species enhanced barrier integrity regardless of hormone treatment. However, female sex hormones and hormonal contraceptives did not alter the amount of hydrogen peroxide produced by lactobacilli, nor the enumeration or adherence of GR-1, RC-14 or *L. crispatus*.
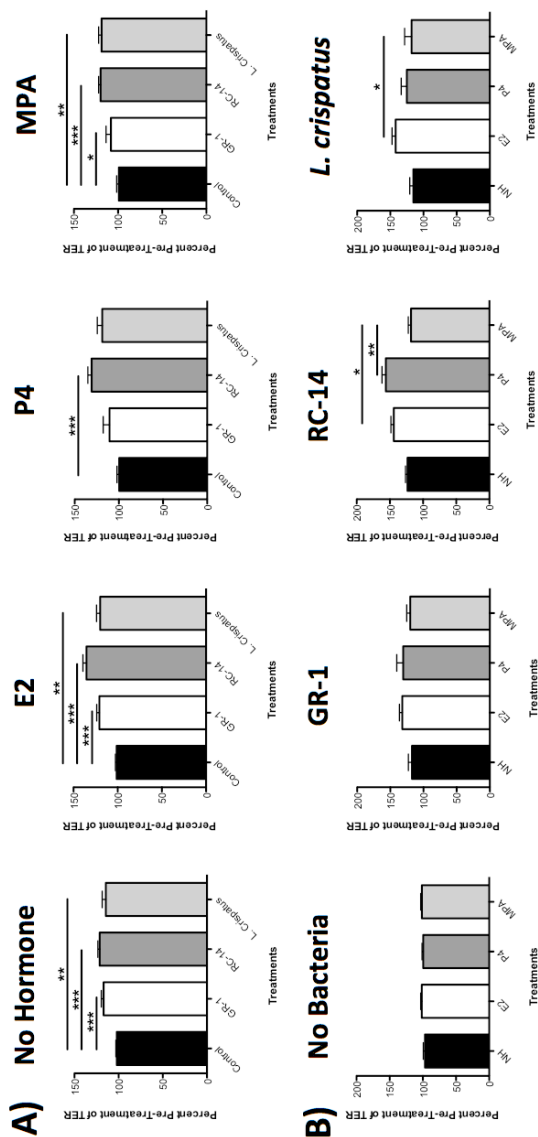
Figure 24. Effect of *Lactobacillus* strains GR-1, RC-14 and *L. crispatus* and female sex hormones on genital epithelial cell TER measurements. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) and exposed to *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus*) at a concentration of 100 CFU/cell for 12 hours. TER measurements were observed at time 0 and 12 hours post bacterial exposure. TERs are expressed as percent pre-treatment of initial TER at 0 hours on the y-axis. A minimum of five replicates per experimental condition were included. Panel A and B consist of the same data. **A)** Data is presented to compare hormone treatments within each strain of *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus).* **B)** Data is presented to compare the three strains of *Lactobacillus spp.* within each hormone treatment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons $*p<0.05$, $**p<0.01$, $***p<0.001$. All data is representative of a combination of three biological samples with 2 technical replicates per experimental condition.
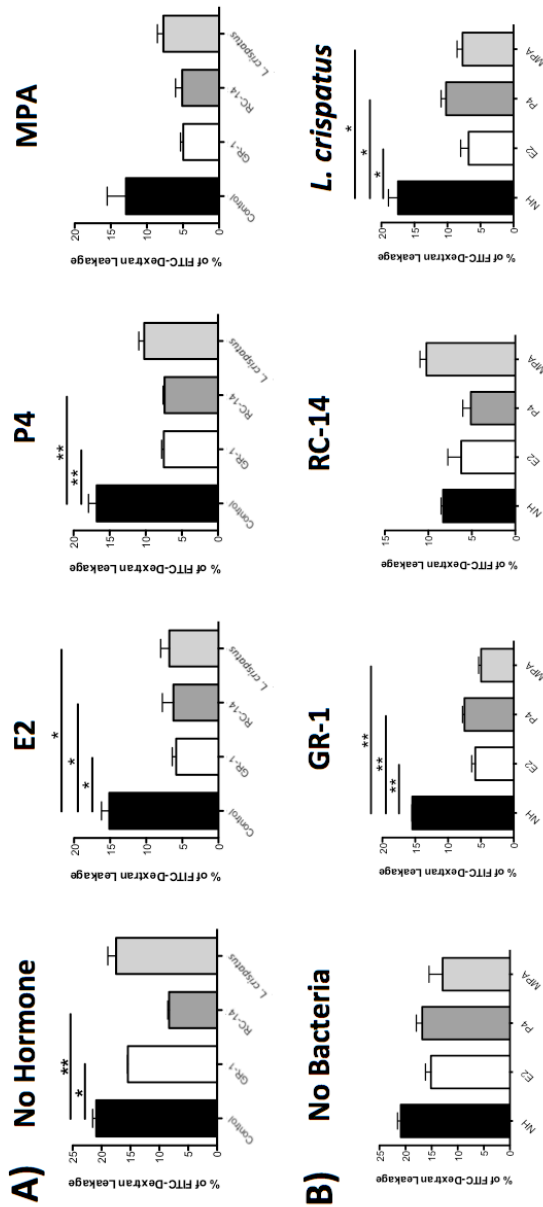
Figure 25. Effect of *Lactobacillus* strains GR-1, RC-14 and *L. crispatus* and female sex hormones on genital epithelial cell barrier permeability. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) were pre-treated with *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus)* at a concentration of 100 CFU/cell. Barrier permeability was measured by adding FITC-labeled dextran dye to the apical side of the epithelial cell monolayer. At 24 hours 50uL of basolateral supernatant was collected and fluorescence (excitation 490; emission 520) was measured. The leakage is expressed as the percentage of dye in the basolateral compartment compared to the apical compartment. **A)** Data is presented to compare hormone treatments within each strain of *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus).* **B)** Data is presented to compare the three strains of *Lactobacillus spp.* within each hormone treatment. Data was analyzed by a one way analysis of variance using the Bonferonni post-test. Data is representative of two biological replicates with two technical replicates per experimental condition.
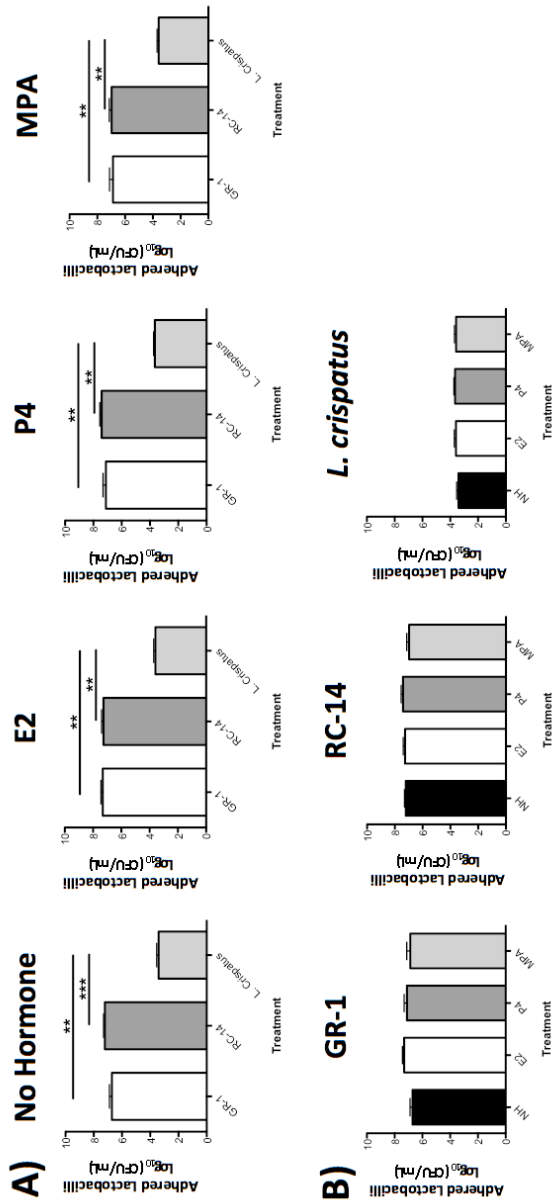
Figure 26. Effect of genital epithelial cells and female sex hormones on bacterial adherence of *Lactobacillus* strains GR-1, RC-14 and *L. crispatus*. The number of viable bacteria adhering to primary genital epithelial cells grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) and exposed to *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus)* at a concentration of 100 CFU/cell for 12 hours. Apical monolayers were washed twice with PBS to remove non-adherent bacteria. Adhered bacteria were collected, serially diluted in MRS broth and plated on MRS agar for 24 hours at 37 degrees Celsius. The colony forming units were counted and are expressed on the y-axis as Log10 CFU/mL. Panel A and B consist of the same data. **A)** Data is presented to compare hormone treatments within each strain of *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus).* **B)** Data is presented to compare the three strains of *Lactobacillus spp.* within each hormone treatment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. All data is representative of a combination of three biological replicates with a minimum of two technical replicates per experimental treatment.

Figure 27. Effect of genital epithelial cells and female sex hormones on bacterial enumeration of *Lactobacillus* strains GR-1, RC-14 and *L. crispatus*. The number of viable bacteria enumerating in primary genital epithelial cell cultures grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) and exposed to *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus)* at a concentration of 100 CFU/cell for 12 hours were quantified. Apical supernatants were collected. Appical monolayers were washed twice with PBS and added to the supernatant. The supernatant with added washes were centrifuged at 10,000rpm for 5 minutes. Collected bacteria was serially diluted in MRS broth and plated on MRS agar for 24 hours at 37°C. The colony forming units were counted and are expressed on the y-axis as Log10 CFU/mL. Panel A and B consist of the same data. **A)** Data is presented to compare hormone treatments within each strain of *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus).* **B)** Data is presented to compare the three strains of *Lactobacillus spp.* within each hormone treatment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons $*p<0.05$, $**p<0.01$, $***p<0.001$. All data is representative of a combination of three biological samples with a minimum of two technical replicates per experimental condition.
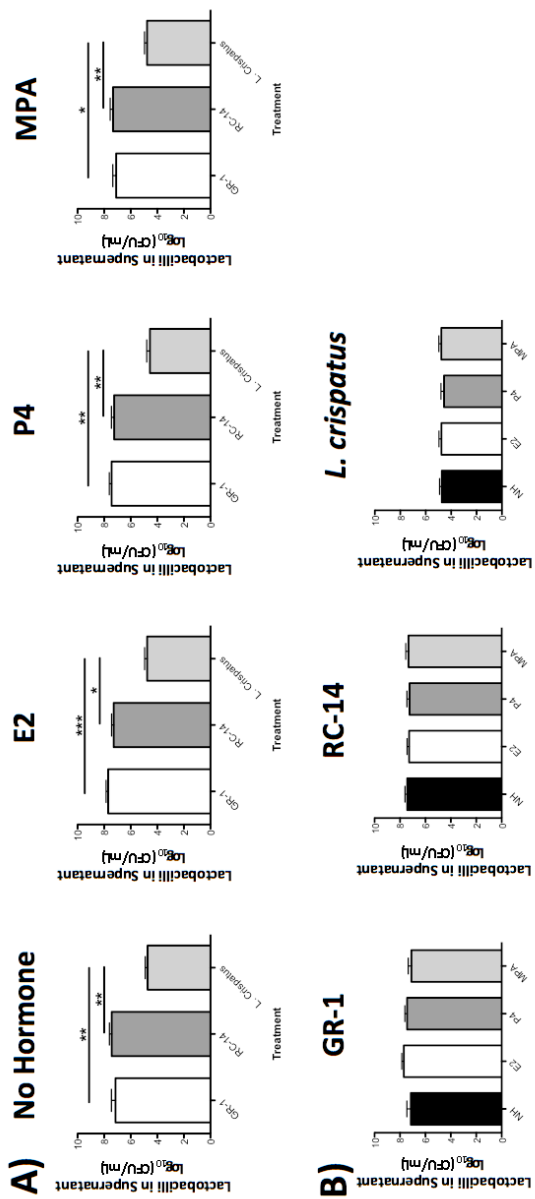
Figure 28. Effect of *Lactobacillus* strains GR-1, RC-14 and *L. crispatus* and female sex hormones on genital epithelial cell production of TNF-α. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) and exposed to *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus*) at a concentration of 100 CFU/cell for 12 hours. Apical supernatants were collected and assayed by Millipore multi-analyte kit or ELISA to measure TNF-a, and is expressed as pg/mL of cytokine production. Panel A and B consist of the same data. **A)** TNF-a production is presented to compare hormone treatments within each strain of *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus*). **B)** TNF-a production is presented to compare the three strains of *Lactobacillus spp.* within each hormone treatment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. All data is representative of a combination of three biological samples with two technical replicates per experiment condition.
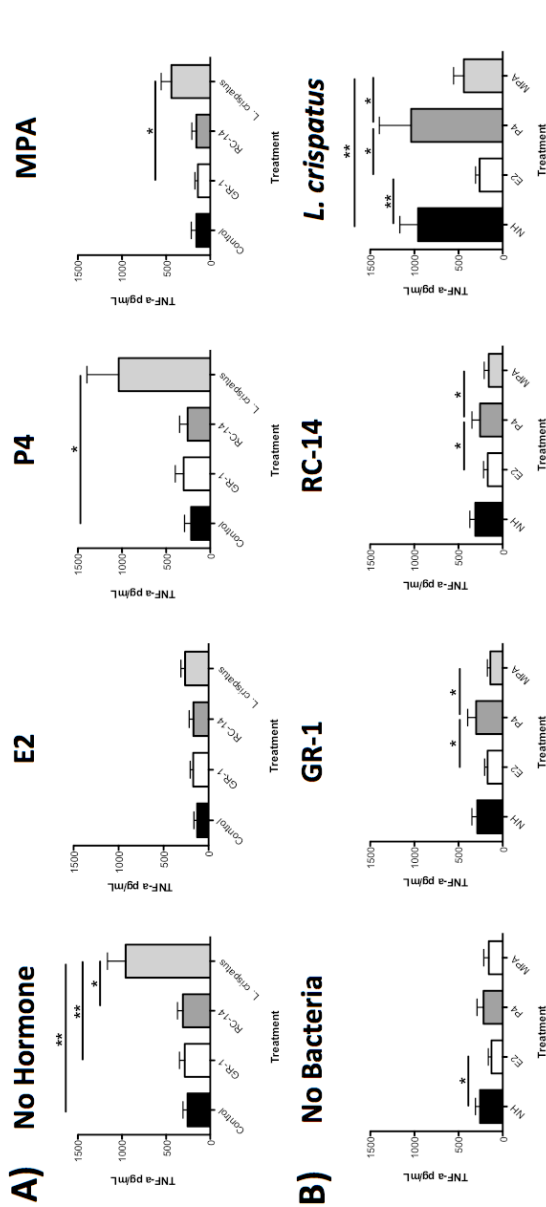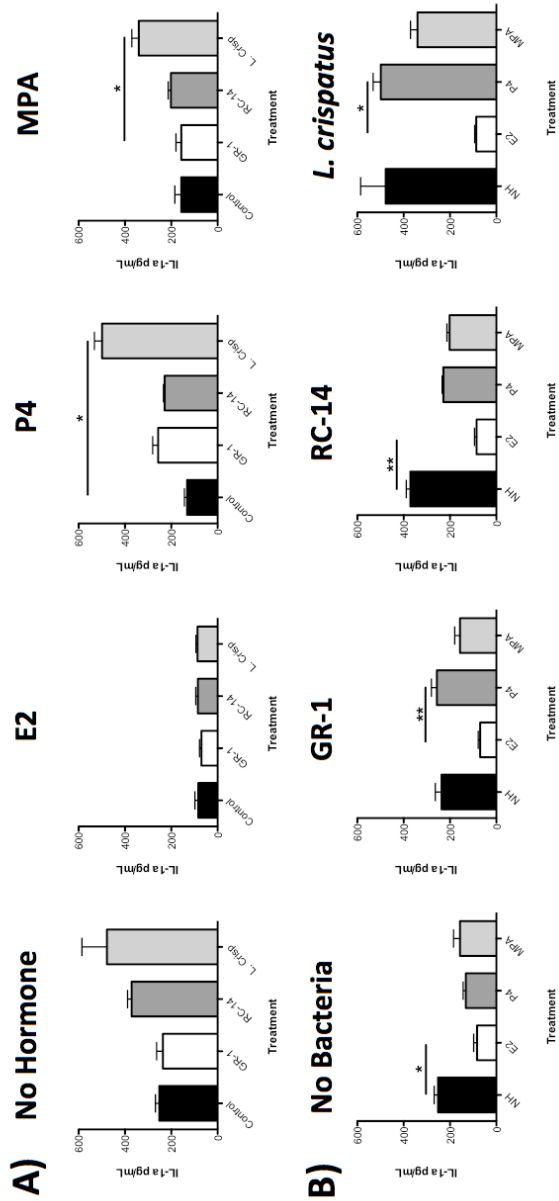
Figure 29. Effect of *Lactobacillus* strains GR-1, RC-14 and *L. crispatus* and female sex hormones on genital epithelial cell production of IL-1α. Primary genital epithelial cells were grown in the presence or absence of physiological concentrations of female sex hormones, E2 (10^{-9}) and P4 (10^{-7}) or hormonal contraceptive, MPA (10^{-9}) and exposed to *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus*) at a concentration of 100 CFU/cell for 12 hours. Apical supernatants were collected and assayed by Millipore multi-analyte kit to measure IL-1a, and is expressed as pg/mL of cytokine production. Panel A and B consist of the same data. **A)** IL-1a production is presented to compare hormone treatments within each strain of *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus).* **B)** IL-1a production is presented to compare the three strains of *Lactobacillus spp.* within each hormone treatment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. All data is representative of one biological sample with two technical replicates per experimental condition.

91
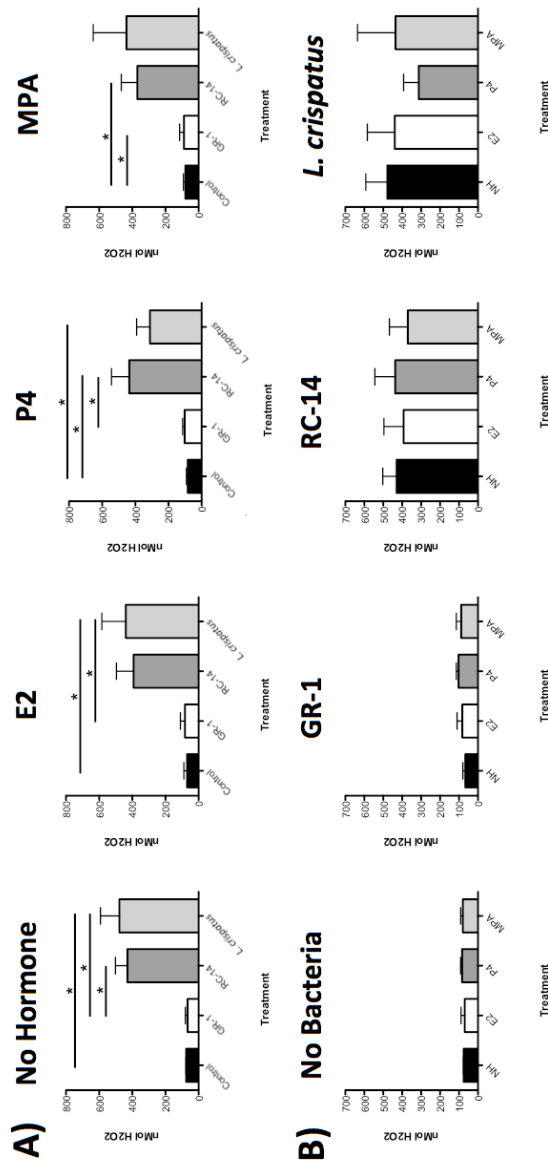
Figure 30. Hydrogen peroxide production by lactobacilli added to genital epithelial cells exposed to female sex hormones. Primary genital epithelial cells were grown in the presence or absence of the female sex hormones E2 (10^{-9}), P4 (10^{-7}) or the hormonal contraceptive MPA (10^{-9}) followed by a 12 hour exposure to 100 CFU/cell of GR-1, RC-14 or *L. crispatus*. Apical supernatants were collected and assayed for hydrogen peroxide, represented as nMol H_2O_2 on the y-axis, using a commercial detection kit. **A)** Data is presented to compare hormone treatments within each strain of *Lactobacillus spp.* (GR-1, RC-14 or *L. crispatus*). **B)** Data is presented to compare the three strains of *Lactobacillus spp.* within each hormone treatment. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *p<0.05, **p<0.01, ***p<0.001. All data is representative of a combination of two biological samples with two technical replicates per experimental condition.

3.4 Aim 4

Examine how interactions between hormones and lactobacilli modulate barrier function and innate immune responses of female genital tract epithelial cells in the presence of HIV

Rationale: Epithelial cells lining the FGT comprise the first barrier of entry to HIV, and normally exist in the genital microenvironment in the presence of female sex hormones and the mucosal microbiota. Previous *in vivo* and *in vitro* studies have suggested that female sex hormones or a dysbiosis of the FGT microbiota may lead to inflammation and enhanced HIV acquisition (Ferreira, Dizzell, et al., 2014; Hedges, Barrientes, Desmond, & Schwebke, 2006; C. Mitchell & Marrazzo, 2014; Onderdonk, Delaney, & Fichorova, 2016; Passmore, et al., 2016; Smith, et al., 2000; Thurman et al., 2015; Trunova, et al., 2006). Recent studies performed on genital tract tissue explants indicate that HIV susceptibility may be altered depending on the phase of the menstrual cycle (Wira, et al., 2015). Since hormone levels fluctuate throughout the menstrual cycle, it is possible that the underlying reason for changes in susceptibility may be due to the presence of female sex hormones. Additionally, epidemiological studies have shown conflicting evidence regarding the role of hormonal contraceptives, specifically the synthetic progestin Depot-medroxyprogesterone acetate (DMPA), on HIV-1 transmission in women (Huijbregts, et al., 2013). Studies have suggested that female sex hormones regulate microbial populations within the FGT

93

(Eschenbach, et al., 2000). Interestingly, HIV susceptibility has also been correlated with certain microbial compositions (Myer, Kuhn, Stein, Wright, & Denny, 2005). Although a *Lactobacillus*-dominant microenvironment in the FGT has been shown to have protective functions against viral pathogens, the impact of sex hormones and *Lactobacillus* on epithelial barrier function and innate inflammatory factors remains to be determined. Previous studies in our lab have shown that HIV directly disrupts GEC barrier function and increases permeability (Nazli, et al., 2010).

Thus far, our results have shown that lactobacilli (GR-1, RC-14, and *L. crispatus*) enhanced barrier functions independent from hormone treatment and that E2 and MPA appear to be exerting anti-inflammatory effects in our GEC culture system. Therefore, we next examined how the increased barrier function by lactobacilli and the anti-inflammatory response of E2 and MPA are affected by the presence of HIV in our GEC culture system.

Results: To study the interaction of hormones, lactobacilli, and HIV, GECs were grown to confluence in the presence or absence of E2, P4, and MPA, then pre-treated with lactobacilli, and later exposed to HIV. We first assessed barrier function using TER as a proxy measure of barrier integrity. Similar to previous experiments, exposure to GR-1 and RC-14 in all hormonal conditions significantly increased TERs (Figure 31A-D). Compared to HIV exposure alone, significantly increased TERs were observed in HIV-exposed GECs pre-treated with GR-1 in the presence of E2 or no hormone (Figure

31A-B). Increased TERs were also observed in HIV-exposed GECs pre-treated with RC-14 in the presence of E2 or P4, compared to HIV exposure alone (Figure 31B-C). In contrast, pre-treatment with GR-1 or RC-14 did not significantly improve TERs of HIV-exposed GECs grown in the presence of MPA (Figure 31D). Given that all hormonal conditions were showing similar trends we combined the data for all hormonal groups to increase the replicate number and assess significance. When considering the combined data for GECs grown in all hormonal conditions, TER measurements were significantly increased in GR-1 and RC-14 pre-treated cells in the presence of HIV compared to HIV alone (Figure 31E). These data show that cells treated with GR-1 and RC-14 are able to maintain barrier integrity in the presence of HIV.

The pro-inflammatory cytokines TNF-α, IL-1α, and IL-1β are associated with an inflammatory environment in the FGT and have been shown to increase epithelial barrier permeability (Al-Sadi & Ma, 2007; Masson et al., 2016; Miloral, Miller, Sanmiguel, & Jansen, 2014; Nazli et al., 2010). Therefore, we wanted to examine the effect of hormones and lactobacilli on pro-inflammatory cytokine production by HIV exposed GECs.

Firstly, we observed the effect of HIV treatment on TNF-α production in the presence of hormones alone. All hormonal treatments resulted in an increase in TNF-α production when exposed to HIV (Figure 32). However, only no hormone, P4, and MPA resulted in a significant increase when

95

exposed to HIV and compared to a media control (Figure 32). Given that GECs grown in the presence of E2 did not produce a significant TNF-α response when exposed to HIV, it is suggested that E2 is able to confer a less inflammatory environment in the presence of HIV compared to no hormone, P4 and MPA. We next assessed the effect of HIV exposure on GECs pretreated with lactobacilli and grown in the presence of hormones. Pre-treatment with GR-1 and RC-14 significantly reduced HIV mediated induction in TNF-α in the presence of no hormone, P4 and MPA (Figure 33A and C-D). GECs grown in the presence of E2 and pre-treated with GR-1 or RC-14 did not show a significant decrease in TNF-α compared to HIV (Figure 33B). However, it should be noted that in the presence of E2, HIV did not induce a significant TNF-α response, unlike no hormone, P4, and MPA (Figure 32). This suggests that GECs exposed to HIV have a less inflammatory TNF-α response when grown in the presence of E2 compared to other hormones and that probiotic lactobacilli are able to reduce HIV mediated increases in TNF-α.

We next assessed the effect of HIV treatment on IL-1α production in the presence of hormones. In the presence of HIV, all hormonal conditions increased IL-1α production by GECs, however MPA was the only hormonal condition to significantly increase production of IL-1α (Figure 34). GECs grown in the presence of no hormone and P4 showed approximately a two-fold increase in IL-1α when exposed to HIV compared to their respective

media controls, while E2 showed levels of IL-1α similar to media control (Figure 34). Trends suggest that similar to results observed measuring TNF-α production, GECs grown in the presence of E2 and exposed to HIV confer a less inflammatory IL-1α response compared to other hormones. We next compared the IL-1α response by GECs in the presence of hormones and lactobacilli when exposed to HIV. Results showed that GECs grown in the presence of MPA significantly decreased IL-1α production when pre-treated with GR-1 or RC-14 and exposed to HIV in comparison to HIV treatment alone (Figure 35D). Comparable to results observed with GECs grown in the presence of MPA, the presence of no hormone, E2, and P4 showed a similar reduction in IL-1α production when cells were pre-treated with GR-1 and RC-14, however these results were not significant (Figure 34A-C). We further explored the effect of HIV treatment on pro-inflammatory cytokine production in the presence of hormones and lactobacilli by measuring IL-1β secretion. When comparing HIV treatment in hormonal conditions we observed similar results to TNF-α and IL-1α. Although no results were significant, trends suggested that E2 and MPA showed a moderate increase in the production of IL-1β when GECs were exposed to HIV while P4 and MPA resulted in a much greater increase (Figure 36). Furthermore, the production of IL-1β corroborated with TNF-α and IL-1α results when GECs were grown in the presence of no hormone, E2 and P4 pretreated with lactobacilli and exposed to HIV. There was a non-significant decrease in IL-1β for cells pretreated with

97

GR-1 or RC-14 and exposed to HIV compared to HIV alone treated cells (Figure 37A-C). In the presence of MPA, pretreatment with GR-1 and RC-14 resulted in an increase in the production of IL-1β, however these results were not significant (Figure 37D).

The production of pro-inflammatory cytokines, TNF-α, IL-1α, and IL-1β, by GECs exposed to HIV were decreased in the presence of E2 and MPA compared to no hormone and P4 (Figure 32, Figure 34 and Figure 36). Furthermore, trends suggest that inflammation induced through cytokine production by GECs in response to HIV exposure may be dampened by the presence of lactobacilli (Figure 33, Figure 35 and Figure 37). Given that TNF-α, IL-1α, and IL-1β are associated with reduced epithelial barrier function, mitigating their production by GECs via hormones such as E2, and lactobacilli may have prophylactic potential to reduce microbial translocation in the presence of HIV.

IL-8 is a pro-inflammatory chemokine that has been shown to induce epithelial barrier damage, and is associated with MPA treatment *in vitro* (Eade et al., 2010; Irvin & Harold, 2015; Sorrentino et al., 2008). Furthermore, IL-8 has been correlated with a BV associated microenvironment is suggested to play a role in HIV acquisition. Therefore, we examined production of IL-8 by GECs grown in the presence of hormones and exposed to HIV. An increase in IL-8 production by GECs was observed in all hormonal conditions when cells were exposed to HIV, however MPA was the only hormonal condition that

showed a significant increase (Figure 38). We further explored the production of IL-8 by GECs in the presence of hormones, pre-treated with lactobacilli and exposed to HIV. We observed that in the presence of MPA there was a significant decrease in IL-8 production when cells were pretreated with GR-1 or RC-14 and exposed to HIV in comparison to HIV treatment alone (Figure 39D). Furthermore, the presence of no hormone, E2, and P4 showed a trend towards a reduction in IL-8 production when cells were pre-treated with GR-1 or RC-14 and exposed to HIV compared to HIV treatment alone, however these results were not significant (Figure 39A-C).

An additional inflammation associated chemokine, RANTES, was found to be higher in the genital secretions of women using DMPA compared to women using no hormonal contraception (C. Morrison, et al., 2014). Therefore, we also examined the secretion of RANTES by GECs in our model. In the presence of all hormonal conditions, HIV exposure to GECs resulted in a significant increase in the production of RANTES (Figure 40). Furthermore, in the presence of P4 and MPA, GECs pre-treated with GR-1 or RC-14 and exposed to HIV showed a significant decrease in RANTES production compared to the HIV treatment alone (Figure 41C-D). In the presence of no hormone, GECs pretreated the GR-1 but not RC-14 showed a significant decrease in RANTES production compared to HIV treatment alone, however pretreatment with RC-14 also showed a reduction in RANTES production when exposed to HIV (Figure 41A). GR-1 and RC-14 pretreatment

reduced RANTES production when GECs were grown in the presence E2 and exposed to HIV, however results were not significant (Figure 41B).

The production of pro-inflammatory chemokines, IL-8 and RANTES by GECs were increased in all hormonal conditions when exposed to HIV. While a significant increase in the production of RANTES was observed in the presence of all hormonal conditions when exposed to HIV, only GECs grown in the presence of MPA showed a significant increase IL-8 (Figure 38 and Figure 40). Interestingly, unlike the pro-inflammatory cytokine expression of TNF-α, IL-1α, and IL-1β that were dampened in the presence of MPA, pro-inflammatory chemokine production was not dampened by E2 nor MPA. Furthermore, GECs grown in the presence of MPA and P4 showed a significant decrease in RANTES production when pre-treated with lactobacilli and exposed to HIV compared to HIV alone treatment (Figure 41C-D). While MPA also significantly decreased IL-8 secretion by GECs pretreated with lactobacilli and exposed to HIV, in the presence of no hormone, E2 and P4 the similar trend observed was not significant (Figure 39A-C). This further suggests that MPA results in immunosuppressive effects via pro-inflammatory cytokines but not chemokines. Additionally, lactobacilli pretreatment was able to significantly reduce HIV-mediated increase in the inflammatory chemokines, IL-8 and RANTES in the presence of MPA (Figure 39D and Figure 41D). An effect also observed by secretion of RANTES for GECs grown in the presence of P4 (Figure 41C). No hormone, E2 and P4 treated

GECs show similar trends to MPA in the reduction of IL-8 secreted by GECs pretreated with lactobacilli and exposed to HIV, however results are not significant (Figure 39 A-C). Lastly, GECs grown in the presence of no hormone and E2, pretreated with lactobacilli and exposed to HIV showed a similar reduction in RANTES secretion compares to MPA and P4, however results were not significant (Figure 41A-B).

Irrespective of hormonal condition, lactobacilli enhanced GEC barrier functions in this system. Pretreatment of GECs with lactobacilli showed a decrease in pro-inflammatory cytokine and chemokine production when compared to HIV treatment alone, suggesting that lactobacilli are able to reduce HIV-mediated induction of pro-inflammatory cytokines and chemokines. Additonalliy, E2 and MPA treated GECs conferred a less inflammatory response to HIV exposure compared to no hormone and P4 when measureing the pro-inflammatory cytokines TNF-α, IL-1α and IL-1β. Interestingly, when measuing the pro-inflammatory chemokines IL-8 and RANTES, the attenuated effect of E2 and MPA were not observed. Therefore, this suggests that hormones differentially regulate cytokines and chemokines in the presence of HIV. Lastly, the increase in barrier function observed by lactobacilli treatment and the decrease of inflammation observed in the presence of E2 may work together to reduce HIV acquisition.
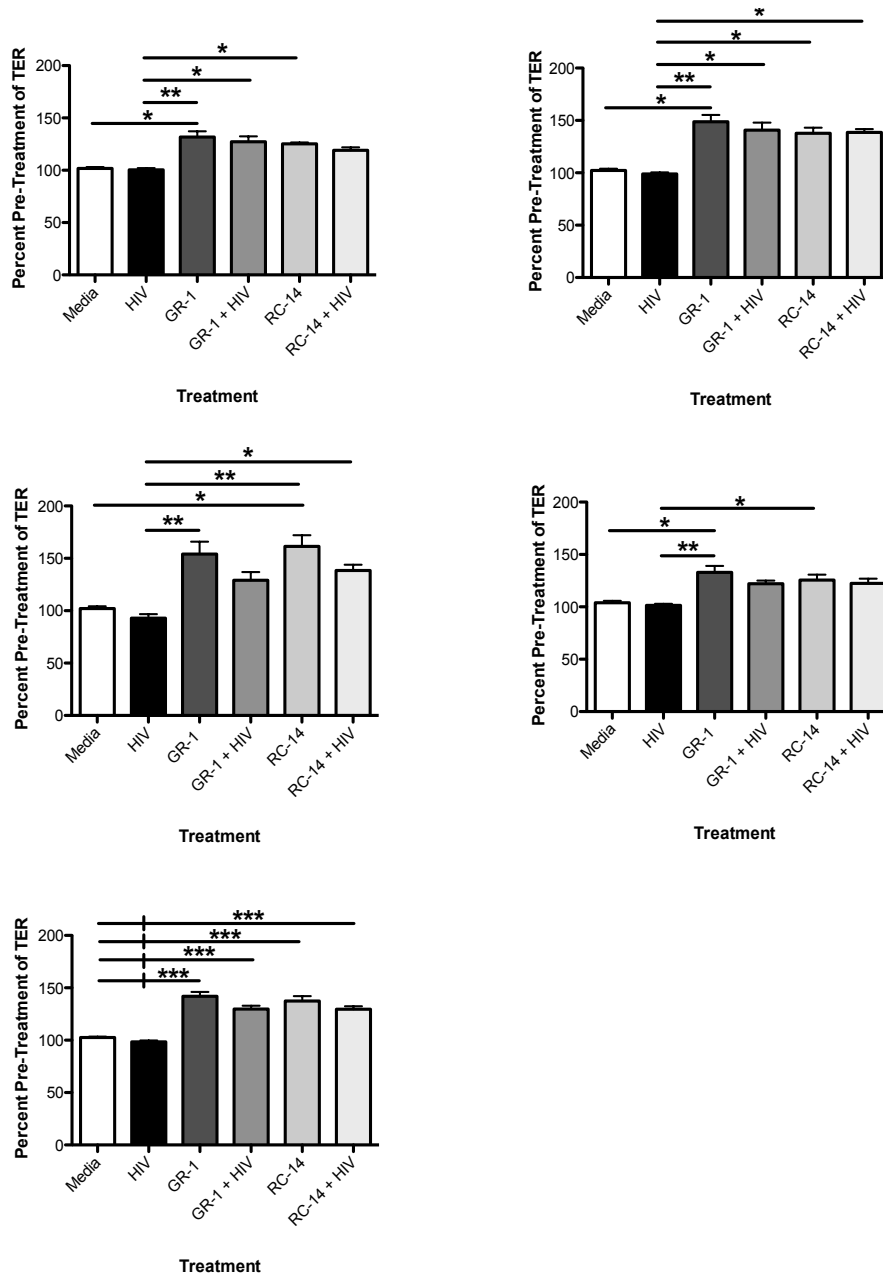
Figure 31. Primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14 and exposed to HIV-1 increase in TER measurements. TER of primary genital epithelial cells grown in hormones, pretreated with 100 CFU/cell of GR-1 or RC-14 for 2 hours followed by exposure to HIV (10^6 IU/mL) for 24 hours. TERs are expressed as percent pre-treatment of initial TER at time zero. **A)** Cells grown in No Hormone media. **B)** Cells grown in E2 media. **C)** Cells grown in P4 media. **D)** Cells grown in MPA media. (A-D) Data is combined from three separate experiments. A minimum of five technical replicates per experimental condition were included **E)** Combined data from all treatments in all hormones. A minimum of 15 technical replicates for each experimental condition. Data was analyzed by Kruskal-Wallis non-parametric analysis of variance with Dunn's test to correct for multiple comparisons *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All data is representative of a combination of three biological samples.
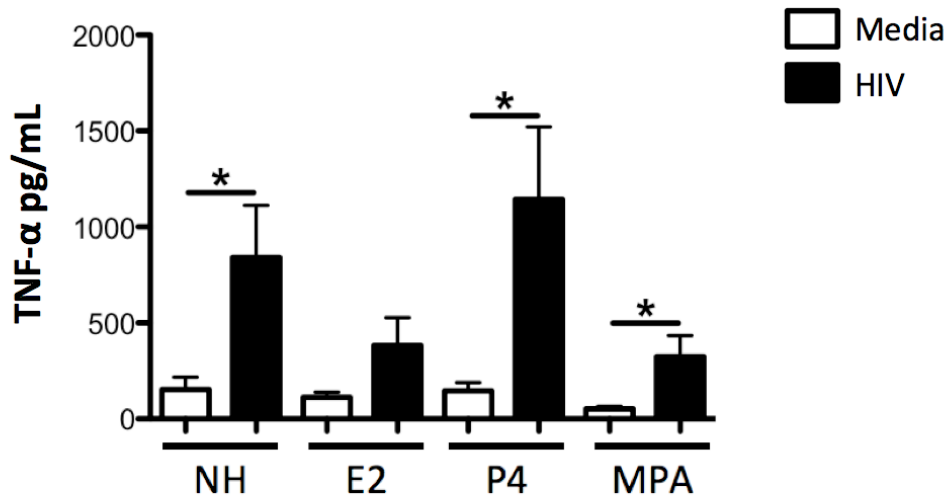
Figure 32. Production of TNF-α by primary genital epithelial cells grown in hormones and exposed to HIV. TNF-α production of primary genital epithelial cells grown in hormones, and exposed to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected TNF-α production was measured by Magpix assay and is expressed as pg/mL. Data was analyzed using an unpaired t-test to compare between media and HIV treatments for each hormone condition. All data is presented as a combination of three separate experiments. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.

Figure 33. Primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV decrease TNF-α production compared to HIV. TNF-α production of primary genital epithelial cells grown in hormones, pretreated with 100 CFU/cell of GR-1 or RC-14 for 2 hours followed by exposure to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected and TNF-α production was measured by Magpix assay and is expressed as pg/mL. **A)** Cells grown in No Hormone media. **B)** Cells grown in E2 media. **C)** Cells grown in P4 media. **D)** Cells grown in MPA media. Data was analyzed by a one way analysis of variance using the Bonferonni post-test. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.

Figure 34. Production of IL-α by primary genital epithelial cells grown in hormones and exposed to HIV. IL-1α production of primary genital epithelial cells grown in hormones, and exposed to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected IL-1α production was measured by Magpix assay and is expressed as pg/mL. Data was analyzed using an unpaired t-test to compare between media and HIV treatments for each hormone condition. All data is presented as a combination of three separate experiments. Five replicates per experimental condition are included.

Figure 35. Production of IL-1α by primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV. IL-1α production of primary genital epithelial cells grown in hormones, pretreated with 100 CFU/cell of GR-1 or RC-14 for 2 hours followed by exposure to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected and IL-1α production was measured by Magpix assay and is expressed as pg/mL. **A)** Cells grown in No Hormone media. **B)** Cells grown in E2 media. **C)** Cells grown in P4 media. **D)** Cells grown in MPA media. Data was analyzed by a one way analysis of variance using the Bonferonni post-test. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.

Figure 36. Production of IL-1β by primary genital epithelial cells grown in hormones and exposed to HIV. IL-1β production of primary genital epithelial cells grown in hormones, and exposed to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected IL-1β production was measured by Magpix assay and is expressed as pg/mL. Data was analyzed using an unpaired t-test to compare between media and HIV treatments for each hormone condition. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.

Figure 37. Production of IL-1β by primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV. IL-1β production of primary genital epithelial cells grown in hormones, pretreated with 100 CFU/cell of GR-1 or RC-14 for 2 hours followed by exposure to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected and IL-1β production was measured by Magpix assay and is expressed as pg/mL. **A)** Cells grown in No Hormone media. **B)** Cells grown in E2 media. **C)** Cells grown in P4 media. **D)** Cells grown in MPA media. Data was analyzed by a one way analysis of variance using the Bonferonni post-test. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.

Figure 38. Production of IL-8 by primary genital epithelial cells grown in hormones and exposed to HIV. IL-8 production of primary genital epithelial cells grown in hormones, and exposed to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected IL-8 production was measured by Magpix assay and is expressed as pg/mL. Data was analyzed using an unpaired t-test to compare between media and HIV treatments for each hormone condition. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.
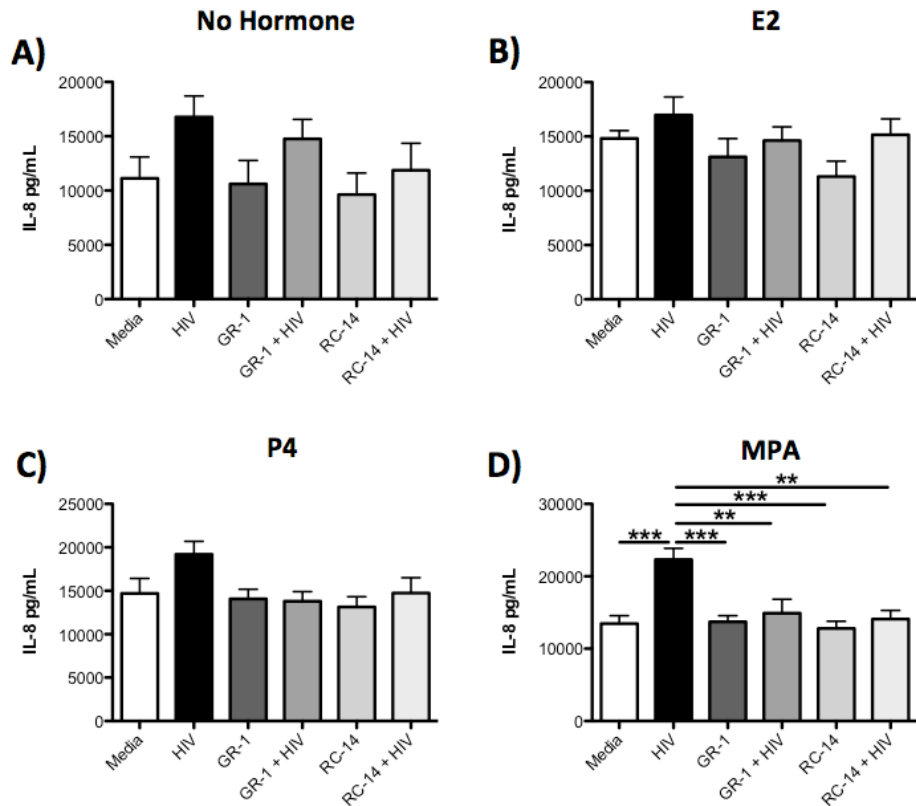
Figure 39. Production of IL-8 by primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV. IL-8 production of primary genital epithelial cells grown in hormones, pretreated with 100 CFU/cell of GR-1 or RC-14 for 2 hours followed by exposure to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected and IL-8 production was measured by Magpix assay and is expressed as pg/mL. **A)** Cells grown in No Hormone media. **B)** Cells grown in E2 media. **C)** Cells grown in P4 media. **D)** Cells grown in MPA media. Data was analyzed by a one way analysis of variance using the Bonferonni post-test. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.

Figure 40. Production of RANTES by primary genital epithelial cells grown in hormones and exposed to HIV. RANTES production of primary genital epithelial cells grown in hormones, and exposed to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected RANTES production was measured by Magpix assay and is expressed as pg/mL. Data was analyzed using an unpaired t-test to compare between media and HIV treatments for each hormone condition. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.
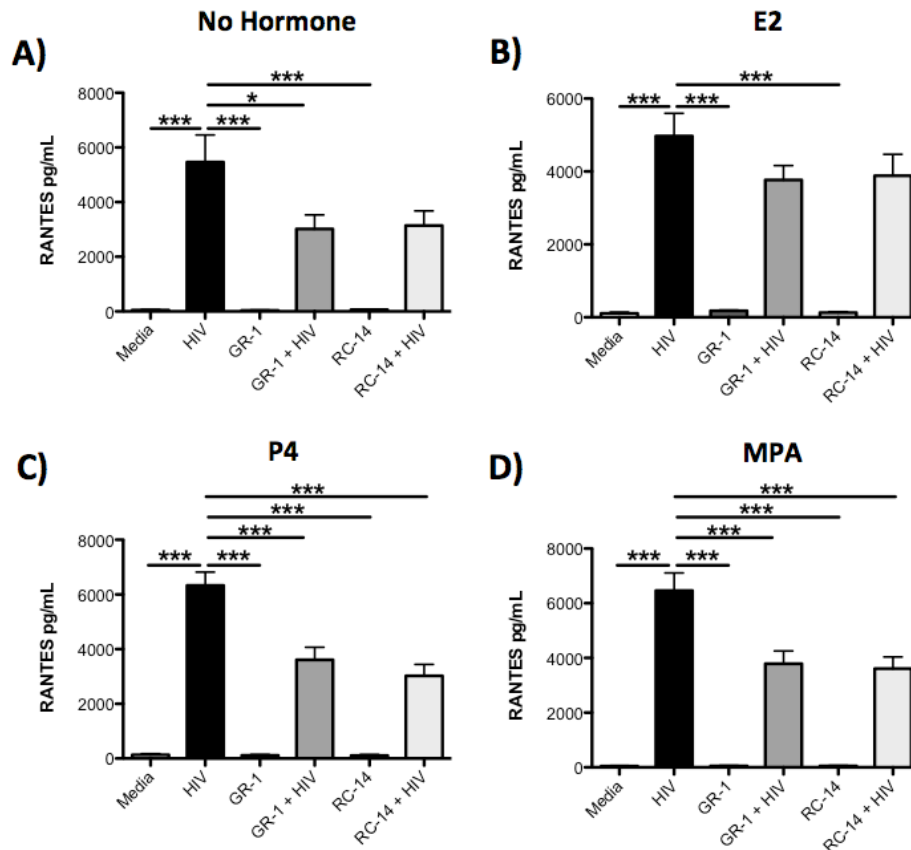
Figure 41. Production of RANTES by primary genital epithelial cells pre-treated with *Lactobacillus* strains GR-1 or RC-14, and exposed to HIV. RANTES production of primary genital epithelial cells grown in hormones, pretreated with 100 CFU/cell of GR-1 or RC-14 for 2 hours followed by exposure to HIV (10^6 IU/mL) for 24 hours. Apical supernatants were collected and RANTES production was measured by Magpix assay and is expressed as pg/mL. **A)** Cells grown in No Hormone media. **B)** Cells grown in E2 media. **C)** Cells grown in P4 media. **D)** Cells grown in MPA media. Data was analyzed by a one way analysis of variance using the Bonferonni post-test. All data is representative of a combination of three biological samples with five technical replicates per experimental condition.

CHAPTER 4: DISCUSSION
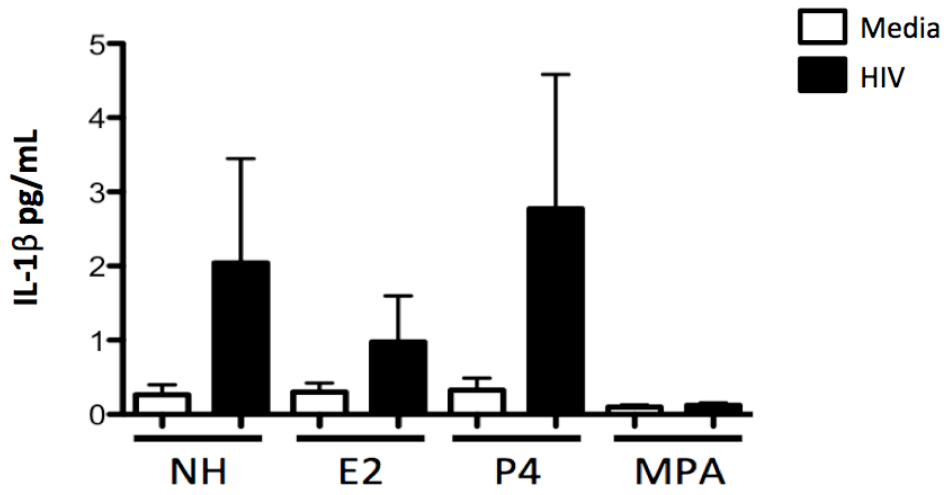
4.1   Summary

Within the FGT two critical components associated with HIV acquisition are epithelial barrier function and innate inflammatory responses. The epithelial cells of the FGT form a barrier against the entry of pathogens. In order for HIV to establish successful infection, the virus must cross this barrier in order to access target cells (Carias, et al., 2013; Haase, 2010; Kaushic, 2011; Shen, Richter, & Smith, 2014). GECs are under constant influence by female sex hormones, hormonal contraceptives, and the microbiota (Brotman, et al., 2014; Nguyen, et al., 2014; Petrova, et al., 2013; Wira, et al., 2010; Wira, et al., 2015). In the presence of HIV, the epithelial cells secrete a plethora of pro-inflammatory cytokines, including TNF-α which causes a decrease in the integrity of the epithelial cell barrier allowing the translocation of HIV across the epithelium as well as potential for other microbes (Nazli, et al., 2010). Previous *in vivo* and *in vitro* studies have suggested that female sex hormones alter a women's susceptibility to HIV (Kaushic, Roth, et al., 2011; Marx, et al., 1996; Saba, et al., 2013; Wira & Fahey, 2008). Estrogen is suggested to have protective effects against HIV acquisition while progesterone is suggested to increase HIV acquisition (Marx, et al., 1996; Saba, et al., 2013; Smith, et al., 2000). Additionally, epidemiological studies show conflicting evidence regarding the role of

hormonal contraceptives, specifically the synthetic progestin Depot-medroxyprogesterone acetate (DMPA) on HIV-1 transmission in women (C. S. Morrison, et al., 2015; Ralph, et al., 2015). Furthermore, dysbiosis of the FGT microbiota resulting in a more polymicrobial environment is associated with inflammation in the FGT (Anahtar, et al., 2015). Both inflammation and an increased polymicrobial environment are associated with increased acquisition of STIs (Borgdorff, et al., 2014; Masson et al., 2015; C. Mitchell & Marrazzo, 2014; Sewankambo, et al., 1997). Conversely, a FGT microbiota rich in *Lactobacillus* species has been associated with protective functions against viral pathogens and lowered acquisition of HIV (Borgdorff, et al., 2014; Sha et al., 2005). Although a *Lactobacillus*-dominant microenvironment in the FGT has been shown to have protective functions against viral pathogens, the impact of sex hormones and lactobacilli on epithelial barrier function and innate inflammatory factors has yet to be determined. Understanding the mechanism of barrier functions and inflammation in the FGT are critical to preventing HIV infection. These mechanisms are especially relevant in the context of columnar epithelial cells of the UGT, as clinical and experimental evidence indicates that the primary site of HIV acquisition is the endocervix, which consists of a single layer of columnar epithelial cells (Li, et al., 2009). Given the gap in literature, the goal of this work was to establish an *in vitro* model to examine the interactions of female sex hormones and lactobacilli on primary (GEC) barrier functions. The overall hypothesis of this

study was that, "*Lactobacillus* strains (GR-1, RC-14 and *L. crispatus*) or E2 added to primary GEC cultures would increase barrier function and reduce pro-inflammatory cytokine production. Conversely, the presence of P4 and MPA would not have a positive effect on these epithelial functions. In the presence of HIV, *Lactobacillus* strains and E2 would play a protective role against HIV mediated barrier disruption and inflammation in GECs". To address this hypothesis, we proposed the following aims:

Aim 1) Examine female genital tract epithelial cell barrier function and innate immune responses in the presence of lactobacilli.

Aim 2) Examine how the presence of female sex hormones influences barrier function and innate immune responses in genital epithelial cell cultures.

Aim 3) Examine how interactions between hormones and lactobacilli modulate female genital tract epithelial cell barrier function and innate immune responses.

Aim 4) Examine how interactions between hormones and lactobacilli modulate barrier function and innate immune responses of female genital tract epithelial cells in the presence of HIV.

The overarching themes discovered from the results of this study were: 1) All lactobacilli strains increase GEC barrier functions and probiotic strains confer increased barrier functions compared to *L. crispatus*. 2) Probiotic strains of lactobacilli do not induce pro-inflammatory cytokine production by

GECs, however *L. crispatus* exposure to GECs induces secretion of pro-inflammatory cytokines. 3) Female sex hormones and hormonal contraceptives do not alter GEC barrier functions. 4) E2 and MPA exert anti-inflammatory effect on GECs grown in their presence. 5) Hormones do not alter bacterial adherence, enumeration or production of hydrogen peroxide. 6) In the presence of HIV, probiotic lactobacilli are able to confer increased barrier functions. 7) E2 is able to decrease inflammation induced by HIV. Each aim will be further discussed in detail below.

4.2 Effect of *Lactobacillus* species on genital epithelial cell barrier function and innate immune responses

Lactobacillus species are associated with a healthy microbiome in the FGT (Lamont et al., 2011; Petrova, et al., 2015; Petrova, et al., 2013; van de Wijgert et al., 2014). The most commonly found bacterial species in the FGT that is associated with a healthy state is *L. crispatus* (B. Ma, et al., 2012; van de Wijgert, et al., 2014). *L. rhamnosus* and *L. reuteri* are also found in FGT, but are far less abundant than *L. crispatus, L. iners, L. jensenii or L. gasseri* (Gajer et al., 2012; Lamont et al., 2011; Ma et al., 2012; Ravel et al., 2011; Wertz et al., 2008). GR-1 (*L. rhamnosus*) and RC-14 (*L. reuteri*) are two strains that have been isolated from the vaginal tract and shown to have probiotic properties (Ravel et al., 2011; Reid, et al., 1987). These strains were specifically selected for their ability to adhere to vaginal epithelial cells and

outcompete pathogenic bacteria to eliminate undesirable pathogens in the FGT and have demonstrated beneficial health outcomes in the urogenital tract of women (Reid, 2008; Reid & Bruce, 2001; Saunders, Bocking, Challis, & Reid, 2007; Wagner & Johnson, 2012). Additionally, GR-1 and RC-14 are the constituents of Fem-Dophilus®, a commercially available oral probiotic that is marketed for improving composition of the FGT flora (Jarrow Formulas®: Fem-Dophilus® for vaginal and urinary tract health, 2014).

The effects of the microbiota on epithelial barrier function and innate inflammatory factors have been well studied in the columnar epithelial cells of the intestinal tract (Anderson, et al., 2010; Eun, et al., 2011; Yu, Yuan, Deng, & Yang, 2015). Previous *in vitro* studies have shown that lactobacilli are able to increase barrier functions and abrogate cytokine-mediated disruption of the epithelial barrier in IECs (Anderson et al., 2010; Eun et al., 2011). Lactobacilli have also been shown to reduce pathogen-induced secretion of pro-inflammatory cytokines and chemokines in IECs (Roselli, et al., 2007; Yu, et al., 2015). Given that both inflammation and a decrease in barrier function are associated with increased HIV acquisition and disease progression (Herold et al., 2013; Hunt et al., 2014; Masson, et al., 2015; Nazli, et al., 2010) we sought to explore the role of barrier functions and inflammation in our GECs. Cumulative data using our GEC culture system showed that all three strains of lactobacilli significantly increased TER, a measure barrier integrity (Figure 12A). By measuring the amount of dextran dye leakage from the apical to

basolateral side of the transwell system the permeability of the barrier was assessed. GECs treated with GR-1 and RC-14 significantly decreased epithelial barrier permeability (Figure 13). *L. crispatus* also decreased epithelial barrier permeability, however the result did not reach statistical significance (Figure 13). An increase in barrier integrity and a decrease in permeability implied that an overall increase in barrier functions occurred in GECs in the presence of lactobacilli. Therefore, we concluded that all three strains of lactobacilli increase barrier functions in our GEC culture system however; GR-1 and RC-14 perform better compared to *L. crispatus*. An increase in barrier functions is protective in the FGT, as pathogens such as HIV must cross the epithelial barrier to establish infection in target cells (Dezzutti & Hladik, 2013; Haase, 2010; Hladik & McElrath, 2008). By increasing barrier functions pathogens are less able to translocate between adjacent epithelial cells (Ferreira, et al., 2015; Klatt, Funderburg, & Brenchley, 2013; Nazli, et al., 2010). Furthermore, increases in barrier functions are often a result of increased tight junction protein expression between adjacent columnar epithelial cells (Karczewski, et al., 2010; Sultana, McBain, & O'Neill, 2013). The addition of *Lactobacillus* species to IECs has previously been shown to increase the protein expression of ZO-1 and increase expression of tight junction genes such as occluden, claudin-1, ZO-1 and ZO-2 (Anderson et al., 2010; Eun et al., 2011). In our studies, GECs treated with lactobacilli and stained for tight junction protein ZO-1 showed no

significant differences in fluorescence intensity compared to control (Figure 14). This suggests that the increase in barrier integrity was not due to enhanced ZO-1 protein expression in our model. Notably, numerous tight junction proteins have been observed to regulate barrier function of GECs (Blaskewicz, Pudney, & Anderson, 2011). Therefore, the increase in barrier integrity may be a result of other tight junction proteins such as occludin and caludin1-4. Additionally, adherens and desmosomal junction proteins as well as extracellular matrix proteins may play a role in regulation of GEC barrier functions (Blaskewicz, et al., 2011). Alternatively, fluorescence-based microscopy may not be an adequately sensitive technique to measure small but meaningful changes in ZO-1 localization.

A previous study has shown differences in protective functions from *G. vaginalis* biofilms by GR-1, RC-14 and *L. crispatus* (Saunders, et al., 2007). The RC-14 strain of lactobacilli was able to significantly deplete viable *G. vaginalis* and reduce the depth of *G. vaginalis* biofilms whereas GR-1 was better able to displace *G. vaginalis* in biofilms compared to RC-14 and *L. crispatus* demonstrating that different strains of *Lactobacillus* exert different functional capacities for protection from pathogens (Saunders, et al., 2007). In our model, all three strains of bacteria generated similar responses by GECs in terms of barrier functions, however, *L. crispatus* showed significantly different results when pro-inflammatory cytokine/chemokine production by GECs, adherence of bacteria to GECs, and quantification of the amount of

bacteria in the GEC supernatant were assessed (Figure 11 and Figure 12B-C). Previous research has documented conflicting evidence regarding the use of *L. crispatus* as prophylactic to ameliorate pathogen induced inflammation at mucosal sites (Borruel et al., 2002; Doerflinger, et al., 2014; Fichorova, Yamamoto, Delaney, Onderdonk, & Doncel, 2011; Rizzo et al., 2015; F. X. Zhou et al., 2012). While some studies have shown *L. crispatus* can reduce inflammation (Doerflinger, et al., 2014), other studies have suggested that *L. crispatus* does not affect inflammation or plays a role in exacerbating disease status by increasing inflammation (Borruel, et al., 2002; Rizzo, et al., 2015; F. X. Zhou, et al., 2012). For example, *in vivo* studies have shown that *L. crispatus* treatment worsened inflammatory bowel disease by increasing inflammation (Cui et al., 2016; F. X. Zhou, et al., 2012). A comprehensive study by Zhou *et al.,* used a murine dextran sodium sulfate (DSS)-induced colitis model to show that *L. crispatus* treatment induced markedly worse disease scores compared to the saline control treatment (F. X. Zhou, et al., 2012). By measuring mRNA and protein levels from the colonic tissue of DSS mice treated with *L. crispatus*, it was shown that there was a significant increase in IL-1β, IL-6, and TNF-α, while ZO-1 expression was significantly reduced. They further verified the adverse effects of *L. crispatus* by showing a significant up-regulation of NF-κB p65 in IECs treated with *L. crispatus*. In addition, *L. crispatus* was shown to induce NF-κB signaling and increase IL-8 in vaginal epithelial cells (Fichorova, et al., 2011). In our model GR-1 and

RC-14 did not influence the secretion of TNF-α by GECs, however *L. crispatus* induced a significant increase in TNF-α, IL-1β, GM-CSF, and MIP-1α production compared to media control (Figure 11 and Figure 15). Furthermore, *L. crispatus* showed significantly less adherence to GECs and decreased CFUs of *L. crispatus* were measured in the GEC culture supernatant compared to GR-1 and RC-14 (Figure 12B-C). This suggests that *L. crispatus* was less able to replicate *in vitro* compared to probiotic strains of lactobacilli. We explored the effect of the GEC culture medium on bacterial growth kinetics in order to elucidate the differential response of *L. crispatus* in comparison to GR-1 and RC-14 in our GEC culture system. *L. crispatus* showed a decrease in colony forming units and optical density values compared to GR-1 and RC-14 (Figure 16C-D) when grown in cell culture media. This difference was not observed when *L. crispatus* was grown in broth (Figure 16A-B), suggesting that *L. crispatus* is less tolerant of the epithelial cell media compared to GR-1 and RC-14. Although, *L. crispatus* showed decreased growth compared to GR-1 and RC-14, the bacterial numbers were maintained over time showing that while media conditions are not ideal for growth, it does not decrease viability of the bacteria. Given that the culture conditions were not decreasing viability of *L. crispatus,* other assumptions for the differences observed in inflammation, bacterial adherence, and amount of bacteria in supernatants may be attributed to strain isolation location (Edelman et al., 2002; Gardiner, Heinemann, Bruce,

Beuerman, & Reid, 2002; Reid & Bruce, 2001), genotypic and phenotypic differences by bacterial strains within a given species (Castagliuolo et al., 2005; Reid & Bruce, 2001) or differences in the upper and lower microbiota of the FGT (C. M. Mitchell, et al., 2015; Rampersaud, Randis, & Ratner, 2012; Verstraelen, et al., 2016).

The location of strain isolation may play a role in the probiotic potential of a *Lactobacillus* species. Experiments in our studies were performed using *L. crispatus* 33820 from the ATCC (American Type Culture Collection). This strain of *L. crispatus* was isolated from the human eye in 1953 (Brygoo & Aladame, 1953) while GR-1 and RC-14 were isolated from the FGT (Reid, et al., 1987). A study by Gardiner *et al.,* evaluated the probiotic potential of three commercially available probiotic *Lactobacillus* species, two of which were the same species but different strains isolated from distinct locations (Gardiner, Heinemann, Bruce, Beurman, & Reid, 2002). *L. reuteri* (RC-14) and *L. rhamnosus* (GR-1) were associated with beneficial urogenital health outcomes (Anukam, et al., 2006; Bruce, et al., 1992; Martinez, et al., 2009; Reid, et al., 2001; Reid, et al., 1995; Reid, et al., 2003), whereas *L. rhamnosus*-GG was isolated from the intestinal tract and has been well established to exhibit beneficial effects in the digestive tract (Alander et al., 1999; Johnson-Henry, Donato, Shen-Tu, Gordanpour, & Sherman, 2008; Madsen, 2001). All three strains were applied intravaginally in healthy human females and were evaluated at later time points by generating discrete DNA

fingerprints of selected bacteria and comparing them using randomly amplified polymorphic DNA (RAPD) analysis. It was concluded that *L. reuteri* RC-14, *L. rhamnosus* GR-1 were better able to adhere to vaginal epithelial cells and could be detected for longer periods of time in the FGT compared to *L. rhamnosus*-GG. This suggests the importance of the location of strain isolation and that each strain should be assessed for probiotic potential in the respective area where it is anticipated to be used. In addition to the differences observed between bacterial strains based on isolation location, bacterial strains isolated from the same location have also been shown to exert phenotypic differences. A study by Edelman *et al.,* isolated multiple *L. crispatus* strains, ST1, A33, 134mi from the crop of chickens and compared their *in vitro* adherence to tissue and cell types along the alimentary canal to a non-indigenous strain of *L. crispatus* (ATCC 33280) (Edelman et al., 2002). When comparing indigenous *L. crispatus* strains, the STI and A33 strains adhered well to multiple areas of the crop and colon, while ST1 was the only strain to adhere to cecum. Strain A33, adhered to the epithelium of the crop and the duodenal epithelium. This supports that within a specific species of bacteria, phenotypic differences can occur between strains. Additionally, the *L. crispatus* ATCC strain 33820 was observed to only have a very weak adherence in the colon, suggesting that indigenous strains have increased adherence to alimentary canal epithelial cells of the chicken compared to non-indigenous strains (Edelman et al., 2002). This further

supports the importance of isolation location when assessing bacterial strains for a targeted area. Furthermore, studies have demonstrated that certain isogenic variants of *L. crispatus* (M247 and MU5) show differential adherence capacities both *in vitro* and *in vivo* despite being genetically identical (Castagliuolo, et al., 2005; Cesena et al., 2001; Voltan et al., 2007). This difference is attributed to reduced hydrophobicity in the MU5 strain of lactobacilli, thus resulting in decreased adherence to hydrophilic cells (Castagliuolo, et al., 2005)*.* A comprehensive study by Douillard *et al.,* demonstrated functional differences in strain isolation location as well as genomic and phenotypic difference between strains of the same bacterial species (Douillard et al., 2013). The study assessed 100 different *L. rhamonusus* strains isolated from diverse sources and compared them to the *L. rhamonusus*-GG reference genome. By identifying 17 highly variable regions that encode functional characteristics such as carbohydrate transport and metabolism, production of mucus-binding pili, bile salt resistance, prophages and CRISPR adaptive immunity the bacterial strains were able to be grouped based on similarities of their phenotypes and genotypes. Interestingly, the grouped phenotypes showed an association between bacterial strains and their respective ecological niches suggesting that isolated bacterial species have adaptive traits that are specific to their isolation environment (Douillard, et al., 2013). This study provides additional support that the location of strain isolation

plays an important role probiotic potential and must be taken into consideration when choosing strains for targeted beneficial effects.

To date, human trials using probiotics have proven to be safe with no obvious side effects. However, there is conflicting evidence regarding the efficacy of probiotics as some of them, including *L. crispatus* CCTCC M206119 aggravate disease symptoms of ulcerative colitis in mice (F. X. Zhou, et al., 2012). This highlights the importance of selecting bacterial strains that have been well characterized and thoroughly investigated using relevant *in vivo* and *in vitro* models. Therefore, the differences between *L. crispatus* and the probiotic strains of lactobacilli in our GEC culture system may be attributed to the fact that *L. crispatus* (ATCC 33280) was isolated from the human eye in 1953 and that it was not specifically assessed for beneficial functions in the FGT. Furthermore, although *L. crispatus* is associated with a healthy vaginal microbiome, we are just beginning to elucidate bacterial species located in the endometrial cavity. The majority of our current knowledge of the FGT microbiota is derived from studies performed on vaginal and cervical samples as the upper genital tract was previously thought to be sterile (Rampersaud, Randis, & Ratner, 2012). More recently, studies have documented a microbiotia in the upper FGT, showing that the composition and amount of bacteria in the upper and lower FGT differ (C. M. Mitchell et al., 2015; Verstraelen et al., 2016). The vaginal microbiota has been well characterized and in a healthy state is dominated by *Lactobacillus* species, namely *L.*

crispatus, L. iners, L. jensenii and *L. gasseri* (Petrova et al., 2016). A study by Mitchell *et al.,* characterizing the microbiota of hysterectomy patients used taxon-directed 16S rRNA gene TaqMan format qPCR assays for specifically selected bacterial species (Mitchell et al., 2015). By sampling the endometrial cavity of the excised uterus, *L. iners* was the most common bacteria in the UGT and the amount of bacteria were 2-4 $\log_{10}$ lower in the UGT compared to the LGT. A more comprehensive study of the endometrial microbiota by Verstraelen *et al.,* used deep sequencing of the V1-2 hypervariable regions of the 16S rRNA gene, yielding an average of 41,194 reads per sample and pursued taxanomic annotation by comparing available sequences through Ribosomal Database Project and the NCBI database (Verstraelen et al., 2016). Unlike the vaginal microbiota, the most common bacterial genus in the UGT in this study was Bacteriodes with *B. xylanisolvens*, *B. thetaiotaomicron*, and *B. fragilis* being the most common species. The Pelomonas genus, a relatively undefined genus with only two known species was the second most abundant. The most common *Lactobacillus* species were *L. crispatus* and *L. iners*, however *Lactobacillus* species represented a much lower proportion. This data suggests that the UGT has a very distinct microbiota compared to the LGT (Verstraelen et al., 2016).

Lactobacilli exert their beneficial functions in the FGT through a variety of direct and indirect effects (Aroutcheva et al., 2001; Atassi & Servin, 2010; Boris, Suarez, Vazquez, & Barbes, 1998; Mirmonsef & Spear, 2014; Petrova

et al., 2015; Reid, Beuerman, Heinemann, & Bruce, 2001; Zarate & Nader-Macias, 2006). A mechanism by which *Lactobacillus* species confer inhibitory effects against pathogens in the FGT is the production on hydrogen peroxide (Petrova, et al., 2015). It has been shown that hydrogen peroxide production by *Lactobacillus* species contributes to lowering the pH in the FGT, thus preventing the overgrowth of pathogenic bacteria, and creating an inhospitable environment to pathogens (Atassi & Servin, 2010). Given that *in vitro* studies have demonstrated that hydrogen peroxide is able to inactivate HIV, it is an important factor for our model (Klebanoff & Coombs, 1991). Previous literature has shown that both RC-14 and *L. crispatus* are significant producers of hydrogen peroxide while GR-1 is a low producer of hydrogen peroxide (Jin et al., 2006; Reid & Bruce, 2001). In our model, both RC-14 and *L. crispatus* produced significantly higher amounts of hydrogen peroxide compared to GR-1 and control (Figure 17). These results are in accord with the current literature and suggest that *L. crispatus* and RC-14 are likely able to exert anti-pathogenic effects through the production of hydrogen peroxide. Although GR-1 is not known for its ability to produce hydrogen peroxide, unlike RC-14 and *L. crispatus,* GR-1 is resistant to high concentrations of spermicides (McGroarty et al., 1992). Therefor GR-1 would be able to better colonize in presence of local vaginal formulations such as microbicides, spermicides, gels, and douches.

In conclusion, these results indicate that GR-1, RC-14 *and L. crispatus* strains of lactobacilli play a role in the increase of barrier integrity as observed by the significant increase in TER measurements and the decreased barrier permeability compared to control. As previously mentioned differences in adherence, enumeration, and inflammatory cytokine production between the probiotic strains of *Lactobacillus* and *L. crispatus* may be attributed to the strain isolation location.

4.3 Effect of female sex hormones and hormonal contraceptives on genital epithelial barrier function and innate immune responses

The epithelial cells of the FGT are under constant regulation by endogenous female sex hormones, estrogen (E2) and progesterone (P4) (Brotman, et al., 2014; Nguyen, et al., 2014; Petrova, et al., 2013; Wira, et al., 2010; Wira, et al., 2015). Exogenous hormone therapy is used by millions of women globally for hormonal contraceptive purposes and postmenopausal hormone regulation (Trends in Contraceptive Use Worldwide, United Nations, 2015). Endogenous hormones have been shown to regulate cell proliferation, apoptosis, and have microbicidal effects through secretion of anti-microbial peptides (AMP) by cells of the FGT (Brotman, et al., 2014; Nguyen, et al., 2014; Petrova, et al., 2013; Wira, et al., 2010; Wira, et al., 2015). Additionally, endogenous hormones regulate innate immune responses through the secretion of cytokines and chemokines by cells of the FGT (Ochiel, Fahey,

Ghosh, Haddad & Wira, 2008; Wira et al., 2010). More recently the effects of exogenous hormones on FGT cellular functions has come into question as MPA, the primary constituent in the hormonal contraceptive Depo-Provera has been associated with an increase in HIV acquisition (Baeten, et al., 2007; Crook, et al., 2014; Marx, et al., 1996; Mascola, et al., 2000; Polis & Curtis, 2013; Trunova, et al., 2006). Although many studies have established that female sex hormones and hormonal contraceptives regulate immune responses in the FGT, the direct effect on epithelial cell barrier functions and innate inflammatory factors is still not well understood. Given that the epithelial cells in the FGT are the primary barrier of entry to pathogens, we assessed the effect of E2, P4, and MPA on GEC barrier function and innate immune responses.

In our model, hormones did not show a significant effect on barrier integrity, barrier permeability, nor the expression of tight junction protein ZO-1 (Figure 18, Figure 19, Figure 20). This data suggests that in the primary GEC culture system, hormones do not alter barrier functions.

Increased inflammatory factors in the FGT have been associated with a decrease in barrier function. Therefore, we assessed the secretion of innate inflammatory cytokines by GECs in the presence of hormones. Results showed that both E2 and MPA exerted a significant anti-inflammatory effect on GECs, as seen by a reduction in the secretion of TNF-α (Figure 22), a cytokine well known for its ability to induce barrier damage. Pro-inflammatory

cytokines and chemokines IL-1α and IL-1β and IL-8 were also significantly decreased in the presence of E2 while MPA significantly decreased MIP-1α (Figure 21). This data suggests that E2 and MPA decrease pro-inflammatory cytokine production by GECs. Given that inflammation is a known contributor to STI acquisition in the FGT, the presence of E2 may play a protective role against pathogens. Notably, MPA also reduced pro-inflammatory cytokine production. This is consistent with current literature, as MPA has been shown to suppress both innate and adaptive immune functions (Huijbregts et al., 2013; Trunova et al., 2006). A study by Huijbregts *et al.,* observed a significant decrease in pro-inflammatory cytokines and chemokines, including TNF-α and MIP-1α in peripheral blood mononuclear cells (PBMCs) and vaginal mucosal mononuclear cells (VMMCs) when treated with MPA (Huijbregts et al., 2013). The increase in HIV acquisition for women using MPA based hormonal contraceptives may be due to a variety of other mechanisms. For example, T cells exposed to MPA *in vitro* showed an increase in expression of HIV co-receptors CXCR4 and CCR5 (Huijbregts et al., 2013). Interestingly, postmenopausal women with suppressed estrogen levels, similar to women taking DMPA, were also shown to have elevated CCR5 expression on cervical CD4+ T cells, suggesting that a decrease in estrogen such as the hypoestrogenic state induced by DMPA may be attributed to the increased risk of HIV acquisition (Metitz et al., 2012).

Further mechanisms suggesting that DMPA use is associated with increased HIV acquisition comes from an *in vivo* study assessing cervicovaginal secretions. Using proteomics to assess CVL samples, results showed that women using DMPA contained significantly less signatures of epithelial wound healing and reduced proteins associated with epithelial repair compared to women using no hormonal contraceptives, suggesting that DMPA affects wound healing in the FGT (Birse et al., 2016). This concept was also tested *in vitro*, using a scratch test to evaluate wound healing in the presence of MPA. Results indicated that wound healing was impaired in the presence of MPA compared to control treatment (Wira et al., 2016, P18.09). Interestingly, an *in vitro* study using Ki67 stain cells to determine epithelial cell proliferation demonstrated that E2 increased proliferation while MPA decreased proliferation in GECs (Blauer et al., 2005). Lastly, DMPA use has been associated with increased target cells for HIV in the FGT. A prospective study by Chandra *et al.,* compared vaginal biopsies of women before and after DMPA administration and reported an increase in CD45, CD3, CD8, CD68 and CCR5$^+$ immune cell phenotypes in the epithelium and lamina propria in DMPA users at week 12 compared to baseline measurements at week zero (Chandra et al., 2013). Therefore, although MPA did not decrease barrier function or increase inflammatory factors in our GEC culture system, other mechanisms may contribute to the association between MPA and increased HIV acquisition. Additionally, in our model E2 significantly decreased multiple cytokines and

chemokines. Literature has shown that E2 is associated with protective effects from HIV/SIV acquisition in tissue explants models and non-human primate models (Marx, et al., 1996; Saba, et al., 2013; Smith, et al., 2000). Thus, the protective effective may be due to the E2 induced decrease in pro-inflammatory cytokines and chemokines by GECs, as a decrease in inflammation results in less recruitment and activation of immune cells that could become targets of HIV infection.

During the menstrual cycle, high levels of estrogen correspond with increased glycogen deposition in the genital epithelium (Farage & Maibach, 2006). Glycogen serves as source of energy for most strains of lactobacilli and is anaerobically metabolized to lactic acid (Conti, et al., 2009; Graver & Wade, 2011; Juarez Tomas, et al., 2003; Lai, et al., 2009; O'Hanlon, et al., 2013). The use of DMPA suppresses endogenous estrogen levels and decreases glycogen deposition in the epithelium of the FGT (Jeppsson, et al., 1982; Miller, et al., 2000; Mirmonsef, et al., 2015; C. M. Mitchell, et al., 2014). A study by Mirmonsef et al., demonstrated that high levels of free glycogen in vaginal fluid had a strong positive correlation with the amount of lactobacilli in the FGT, suggesting that glycogen levels may serve as an indicator of a healthy FGT (Mirmonsef et al., 2014). In our model we were able to measure glycogen within the epithelial cells but not free glycogen in the cell culture supernatant (Figure 23A-B). In accordance with current literature, estrogen treatment resulted in a non-significant increase of glycogen content in the

epithelial cells compared to P4 and MPA (Figure 23 B). Interestingly, MPA treatment showed no glycogen content in epithelial cells, an effect similar to what is observed *in vivo* (Miller et al., 2000; Mitchell et al., 2014). The observed differences in glycogen content by hormone treatment suggested that in our model, hormone treatments were able to alter physiological functions of epithelial cells. Additionally, previous *in vitro* studies have attributed glycogen content within vaginal epithelial cells to successful lactobacilli colonization (Rose II et al., 2012). Therefore, this data suggested that hormone treatment may alter lactobacilli adherence to epithelial cells in our system.

In conclusion, our results show that hormones do not alter barrier functions in GECs. However, hormones do influence innate inflammatory factors secreted by GECs. In the presence of E2 and MPA, the production of pro-inflammatory cytokines was significantly decreased. These studies suggest a role hormonal regulation of the innate immune responses by GECs.

4.4 Effect of interaction between *Lactobacillus* species and female sex hormones/hormonal contraceptives on genital epithelial cell barrier function and innate immune responses

The epithelial cells that line the FGT are simultaneously influenced by female sex hormones and the microbiota (Brotman, et al., 2014). Our

previous results concur with current literature showing that lactobacilli are able to increase barrier functions (Anderson et al., 2010) and that E2 and MPA had anti-inflammatory effects. Therefore, we next assessed barrier functions of GECs in the presence of hormones and lactobacilli. Similar to results seen in GECs treated with lactobacilli alone, barrier integrity was increased in the presence of all lactobacilli strains for all hormone treatments, however only cells grown in no hormone, E2 and MPA reached significance (Figure 24A). This correlated with barrier permeability, which was decreased in the presence of all lactobacilli strains for all hormone treatments. Only cells grown in E2 significantly decreased barrier permeability when exposed to all lactobacilli strains, while no hormone and P4 showed a significant decrease in permeability when exposed to probiotic strains of lactobacilli (Figure 25A). Together, these results further support a role for lactobacilli strains GR-1, RC-14 and *L. crispatus* in increasing barrier functions. Notably, GR-1 and RC-14 show are better able to decrease barrier permeability compared to *L. crispatus.* Interestingly, only cells grown in the presence of E2 showed a significant increase in barrier integrity and decrease barrier permeability for all lactobacilli strains, suggesting a protective role in barrier functions mediated by E2 (Figure 24A and Figure 25A).

The microbial composition in the FGT is influenced by sex hormones (Braundmeier et al., 2015; Brotman et al., 2014; Gajer et al., 2012; Galask, 1988; Petrova et al., 2015). Cross sectional studies have suggested estrogen

plays a role in increasing lactobacilli adherence to epithelial cells allowing for increased colonization in the FGT (Chan, Bruce, & Reid, 1984; Heinemenn & Reid, 2005; Yoshimura et al., 2001). The estrogen high phase of the menstrual cycle is associated with increased glycogen deposition in the FGT. Studies have demonstrated that increased glycogen within the lumen of the FGT is associated with increased lactobacilli, as glycogen is an energy source for lactobacilli and is anaerobically metabolized to lactic acid (Conti, Malacrino, & Mastromarino, 2009; Graver & Wade; 2011; Mirmonsef et al., 2014). Furthermore, DMPA suppresses estrogen levels creating a hypoestrogenic environment within the FGT and this decreases glycogen deposition within the epithelium (Miller et al., 2000; Mitchell et al., 2014). The use of DMPA has also been associated with an altered FGT microbiota and decreased amounts of hydrogen peroxide producing *Lactobacillus* species (Miller et al., 2000; Mitchell et al., 2014). Similar to DMPA users, postmenopausal women have low levels of estrogen and decreased lactobacilli colonization in their FGT (Heinemenn & Reid, 2005; Jeppsson, Gershagen, Johansson, & Rannevick, 1982). Interestingly, postmenopausal women have been associated with an increased susceptibility to STIs, suggesting that the loss of lactobacilli and/or decreased E2 levels in the FGT is associated with decreased protection (Drew & Sherrard, 2008). Furthermore, hormone replacement therapy has been shown to alter the FGT of postmenopausal women to a healthy *Lactobacillus* dominant state and is

associated with a lower occurrence of adverse urogenital health outcomes such as STIs or urinary tract infections (Heinemenn & Reid, 2005; Kirkengen et al., 1993; Perrotta et al., 2008; Raz & Stamm, 2003; Yoshimura et al., 2001). Given the effects of hormones on the FGT microbiome observed with *in vivo* studies, and our previous results showing differential glycogen content in epithelial cells in response to hormones, we tested bacterial adherence to GECs and enumeration, as measured by the amount of bacteria in the cell culture supernatant in the presence of hormones. Results showed no difference in bacterial adherence or enumeration of *L. crispatus*, GR-1 or RC-14 in the presence of hormones (Figure 26B and Figure 27B). However, as previously observed, the probiotic strains of lactobacilli had increased adherence and bacteria in the cell culture supernatant compared to *L. crispatus* (Figure 26A and Figure 27A). Previous studies that observed a positive correlation between increased glycogen and lactobacilli in vaginal fluid measured the cell-free glycogen (Mirmonsef et al., 2014). Given that we were not able to measure cell-free glycogen in the cell culture supernatant it is possible that the lactobacilli strains were not able to utilize the glycogen. Interestingly, a study by Spear *et al.,* demonstrated that many *Lactobacillus* species do not actually utilize glycogen directly, but instead metabolize products from the enzymatic digestion of glycogen such as maltose, maltotriose, and maltotetraose (Spear et al., 2014). Furthermore, longitudinal studies that assess the FGT microbiome over the menstrual cycle show

conflicting evidence regarding microbiome regulation by sex hormones. By observing the FGT microbiome over 16 weeks, Gajer *et al.,* reported that cyclic changes during the menstrual cycle altered the FGT microbial composition. The study concluded that during the estrogen dominant phase of the menstrual cycle the microbial community was the most stable and during the onset of menses, the microbial community was least stable, suggesting that switching of community state types primarily occurs mid-cycle (Gajer et al., 2012). Srinivasan *et al.,* also observed that fluctuations in the microbial composition of the FGT primarily occurred during menses, namely an increase in *G. vaginalis.* The increase in *G. vaginalis* was attributed to iron availability from menstrual blood as after the cessation of menses *G. vaginalis* decreased and the microbial community returned to its original state (Srinivasan et al., 2010). A similar observation was made by Santiago *et al.,* as they measured a 100-fold decrease in *L. crispatus* and an increase in *L. iners, G. vaginalis, A. vaginae*, and *P. bivia,* only during menstruation (Santiago et al., 2012). Conversely, a study of Canadian women showed no significant changes in the FGT microbiome associated with menstrual phases (Chaban et al., 2014).

Increased inflammatory cytokines in the FGT are associated with decreased barrier functions. Previously, we have shown that E2 and MPA are anti-inflammatory compared to no hormone, and that probiotic lactobacilli do not exert an inflammatory response, however, *L. crispatus* does induce

137

inflammation in GECs. Therefore, we assessed the production of inflammatory cytokines by GECs in response to both hormones and lactobacilli. Both TNF-α and IL-1α have been associated with BV and an inflammatory response in the FGT (Masson et al., 2016). Additionally, they have been shown to decrease barrier functions in the FGT (Milora, Miller, Sanmiguel, & Jensen, 2014; Nazli et al., 2010). Combined results indicated that induction of the pro-inflammatory cytokines IL-1α and TNF-α by the addition of *L. crispatus* to the GEC monolayers was prevented when cells were grown in the presence of E2 (Figure 28 and 29). This further suggests that E2 has anti-inflammatory effects on GECs grown in its presence and that E2 may confer protection against inflammation related outcomes, such as HIV acquisition through the FGT. Additionally, TNF-α responses induced by *L. crispatus* were also significantly dampened by MPA, while P4 induced an increase in TNF-α responses (Figure 28).

Given that previous results exposing lactobacilli to GECs grown in the presence of hormones showed no differences between bacterial adherence or enumeration we assessed the production of hydrogen peroxide, an anti-pathogenic compound secreted by lactobacilli in the presence of hormones. As mentioned, previous literature has shown that women using DMPA have less hydrogen peroxide producing lactobacilli in their FGT microbiota. Our results show that the presence of hormones does not alter hydrogen peroxide production by lactobacilli (Figure 30). As previously observed, both RC-14

and *L. crispatus,* the known producers of hydrogen peroxide significantly increased hydrogen peroxide production compared to GR-1 and control (Figure 30). The hydrogen peroxide results provide further support that in our model, RC-14 and *L. crispatus* retain their anti-pathogenic functions.

In conclusion, these results show that GR-1, RC-14 and *L. crispatus* increase barrier functions, and E2 is able to abrogate pro-inflammatory cytokine production mediated by bacterial stimulus. Additionally, female sex hormones and hormonal contraceptives do not alter bacterial adherence, enumeration, or hydrogen peroxide production in our primary GEC model. Given that HIV is regulated by inflammation, the ability of E2 to decrease inflammation induced by an environmental stimuli is an important function to mitigate HIV acquisition. The reduction of inflammation through treatment of E2 and the increased barrier function by exposure to GR-1 or RC-14 are important mechanisms to assist in protection of the FGT, especially in the context of HIV. Given that *L. crispatus* (ATCC strain 33820) caused an inflammatory response by GECs as well as decreased adherence and enumeration, other strains of *L. crispatus* should be assessed for the probiotic potential, as it is the dominant bacteria within a 'healthy' FGT.

4.5 Effect of interaction between *Lactobacillus* species and female sex hormones/hormonal contraceptives on genital epithelial cell barrier function and innate immune responses in the presence of HIV

The primary route of HIV infections is through heterosexual transmission in the FGT (Hladik & Hope, 2009). The epithelial cells that line the FGT are the first barrier to HIV. In order for HIV to establish a productive infection, the virus must move from the lumen to the lamina propria by crossing the epithelial cell barrier (Carias et al., 2013; Haase, 2010; Tugizov, 2016). Within the lamina propria, the virus has access to target cells. Early events in HIV transmission remain poorly defined. An inflammatory environment in the FGT is associated with a disruption in epithelial barrier function, which increases permeability between adjacent cells allowing for microbial translocation of the virus (Masson et al., 2015; Nazli et al., 2010). By targeting strategies that decrease inflammation and increase barrier function in the FGT, the ability of HIV to cross the epithelial barrier would be decreased and thus provide protection from HIV acquisition.

Probiotics have previously been used to increase barrier function in both intestinal and genital tract cell lines (Anderson et al., 2010). In our GEC model, lactobacilli were able to confer an increase in barrier integrity in the presence of HIV (Figure 31). Notably, E2 was the only hormone to significantly increase barrier integrity of both GR-1 and RC-14 in the presence

of HIV (Figure 31B). The increase in barrier integrity observed by exposing GECs to lactobacilli suggests that lactobacilli may be able to mediate protection by strengthening the barrier and reducing the ability of HIV to translocate.

HIV exposure to GECs has been shown to increase production of pro-inflammatory cytokines, namely TNF-α, which can cause barrier damage (Nazli, et al., 2010). We have previously observed that GECs grown in the presence of E2 show a decrease in pro-inflammatory cytokine production (Figure 21 and 22). Previous literature from intestinal epithelial cells has also shown that lactobacilli reduce pathogen-induced inflammation (Roselli, et al., 2007; Yan, et al., 2007). Therefore, we assessed the production of TNF-α in the presence of HIV. Exposure to HIV resulted in an increase in TNF-α production by GECs under all hormonal treatments (Figure 32). However, only no hormone, P4, and MPA resulted in a significant increase when exposed to HIV and compared to a media control (Figure 32). Since GECs grown in the presence of E2 did not produce a significant TNF-α response when exposed to HIV, this implies that E2 is able to confer a less inflammatory environment in the presence of HIV compared to no hormone, P4 and MPA.

A pro-inflammatory environment in the FGT, including increased IL-1α and IL-1β has been associated with BV and increased acquisition of STIs (Masson et al., 2016). Furthermore, IL-1β has been shown to induce tight

junction permeability in IECs and IL-1α released by keratinized epithelial cells in response to HSV-1 resulted in a loss of viability causing decreased membrane potential (Al-Sadi & Ma, 2007; Milora, Miller, Sanmiguel, & Jensen, 2014). Given that both IL-1α and IL-1β are associated with an inflammatory environment in the FGT and have also been linked to decreased barrier function and loss of viability we wanted to assess their function in our system. Similar to the trends observed for TNF-α, all hormonal conditions increased IL-1α productions by GECs. In the presence of HIV however, MPA was the only hormonal condition to significantly increase production of IL-1α (Figure 34). GECs grown in the presence of no hormone and P4 showed an approximately two-fold increase in IL-1α when exposed to HIV compared to their respective media controls, while E2 showed levels of IL-1α similar to media control (Figure 34). By assessing secretion of IL-1β by GECs we observed similar trends to TNF-α and IL-1α. Although results did not reach significance, trends suggested that E2 and MPA showed a moderate increase in the production of IL-1β when GECs were exposed to HIV while P4 and no hormone treatment resulted in a much greater increase (Figure 36). Overall, the cytokine secretion by GECs in the presence of HIV decreased in the presence of E2 and MPA compared to no hormone and P4 (Figure 32, Figure 34 and Figure 36).

Both IL-8 and RANTES are chemokines constitutively expressed by GECs. *In vitro* experiments have shown that levels of IL-8 and RANTES are

142

increased in vaginal, endocervical, and endometrial cell lines, in the presence of BV-associated bacteria (Eade et al., 2012). Additionally, collected samples of mucosal vaginal fluid showed a positive correlation between IL-8 and BV-associated bacteria while an inverse relationship between lactobacilli and IL-8 was observed (Spear, Zariffard, Cohen, & Sha, 2008). Furthermore, when IL-8 was added to columnar lung epithelial cells, a significant increase in barrier permeability was observed (Sorrentino et al., 2008). *In vitro* studies exposing vaginal epithelial cell lines to high doses of MPA also showed increases in chemokine production, namely IL-8 (Irvin & Herold, 2015). Given that IL-8 is able to induce epithelial barrier damage, is associated with MPA treatment *in vitro* and has been correlated with a BV associated microenvironment it may play a role in HIV acquisition. Therefore, we first assess production of IL-8 by GECs grown in the presence of hormones and exposed to HIV. An increase in IL-8 production by GECs was observed in all hormonal conditions when cells were exposed to HIV, however MPA was the only hormonal condition that showed a significant increase (Figure 38).

RANTES plays subjective role in HIV acquisition, as it has been associated with both increased and decreased HIV infection in literature. RANTES has been shown to suppress HIV replication *in vitro* (Cocchi, et al., 1995; Deng et al., 1996), by binding to the CCR5 receptor on cells and preventing access to this receptor by the HIV viral envelope protein (gp120) (Cocchi, et al., 1995; Deng, et al., 1996). It has also been suggested that

following the binding of RANTES to the CCR5 receptor, dimerization and internalization occurs, completely removing the receptor from the surface of cells and reducing the amount of co-receptors for gp120 to bind (Deng, et al., 1996; Vila-Coro et al., 2000). Conversely, RANTES recruits HIV target cells, such as to CD4+ T cells, monocytes, and dendritic cells to mucosal areas of injury or infection (Caux et al., 2000). Samples of cervical fluid comparing HIV seroconversion rates showed that levels of RANTES were higher in women who seroconverted to a HIV positive status (C. Morrison et al., 2014). Additionally, certain chemokines, such as RANTES were found to be higher in the genital secretions of women using DMPA compared to women using no hormonal contraception (C. Morrison, et al., 2014). Therefore, we measured the secretion of RANTES by GECs in our model. Initially we observed RANTES production by GECs grown in hormones and exposed to HIV. In the presence of all hormonal conditions, HIV exposure to GECs resulted in a robust significant increase in the production of RANTES (Figure 40). Interestingly, unlike the pro-inflammatory cytokine expression of TNF-α, IL-1α, and IL-1β that were decreased in the presence of E2 and MPA, pro-inflammatory chemokine production was neither dampened by E2 nor MPA.

Given the previously observed increase in barrier integrity in the presence of lactobacilli and HIV, we next assessed pro-inflammatory cytokine and chemokine effects of lactobacilli and hormones in the presence of HIV. Pre-treatment with GR-1 and RC-14 significantly reduced HIV mediated

induction in TNF-α in the presence of no hormone, P4 and MPA (Figure 33A and C-D) while GECs grown in the presence of E2 did not show a significant decrease in TNF-α compared to HIV (Figure 33B). Notably, in the presence of E2, HIV did not induce a significant TNF-α response, unlike no hormone, P4, and MPA, suggesting that GECs exposed to HIV have a less inflammatory TNF-α response when grown in the presence of E2 compared to other hormones (Figure 32). GECs grown in the presence no hormone, E2, and P4 showed a similar reduction in IL-1α production when cells were pre-treated with GR-1 and RC-14, however these results were not significant (Figure 34A-C). Comparable to results observed with GECs grown in the presence of no hormone, E2 and P4, the presence of MPA significantly decreased IL-1α production when pre-treated with GR-1 or RC-14 and exposed to HIV in comparison to HIV treatment alone (Figure 35D). Lastly, a non-significant decrease was observed in IL-1β production for cells pretreated with GR-1 or RC-14 and exposed to HIV compared to HIV alone treated cells (Figure 37A-C). In contrast to previously observed results, the presence of MPA, pretreatment with GR-1 and RC-14 resulted in an increase in the production of IL-1β, however these results were not significant (Figure 37D). The pro-inflammatory cytokines TNF-α, IL-1α, and IL-1β are associated with reduced epithelial barrier function. Therefore, by mitigating their production by GECs through hormones, namely E2, and lactobacilli treatment may have

prophylactic potential to reduce microbial translocation in the presence of HIV.

Pro-inflammatory chemokine production by GECs in the presence of hormones and lactobacilli in the presence of HIV was also measured. In the presence of MPA there was a significant decrease in IL-8 production when cells were pretreated with GR-1 or RC-14 and exposed to HIV in comparison to HIV treatment alone (Figure 39D). Additionally, the presence of no hormone, E2, and P4 showed a trend towards a reduction in IL-8 production when cells were pre-treated with GR-1 or RC-14 and exposed to HIV compared to HIV treatment alone, however these results were not significant (Figure 39A-C). Furthermore, in the presence of P4 and MPA, GECs pre-treated with GR-1 or RC-14 and exposed to HIV showed a significant decrease in RANTES production compared to the HIV treatment alone (Figure 41C-D). In the presence of no hormone, GECs pretreated with GR-1 but not RC-14 showed a significant decrease in RANTES production compared to HIV treatment alone, however pretreatment with RC-14 also showed a reduction in RANTES production when exposed to HIV (Figure 41A). GR-1 and RC-14 pretreatment reduced RANTES production when GECs were grown in the presence E2 and exposed to HIV, however results were not significant (Figure 41B).

Overall, in this system, lactobacilli enhanced GEC barrier functions irrespective of hormonal condition. GECs pretreated with lactobacilli showed

a decrease in pro-inflammatory cytokine and chemokine production when compared to HIV treatment alone, suggesting that lactobacilli are able to reduce HIV-mediated induction of pro-inflammatory cytokines and chemokines. Furthermore, GECs grown in the presence of E2 and MPA conferred a less inflammatory response to HIV treatment compared to no hormone and P4 when measuring the pro-inflammatory cytokines TNF-α, IL-1α and IL-1β. However, when measuing the pro-inflammatory chemokines IL-8 and RANTES, the attenuated effect of E2 and MPA were not observed. This suggests that hormones differentially regulate cytokines and chemokines in the presence of HIV.

Studies comparing the genital secretions of highly exposed seronegative (HESN) sex workers to HIV-positive and HIV-negative controls showed that levels of IL-1α, IL-1β, IL-8, and RANTES were lower in the HESN women (Lajoie et al., 2012; C. Morrison, et al., 2014; Yao et al., 2014). Enhanced barrier function and decreased inflammation correlate with decrease in HIV acquisition, replication and shedding (Mitchell et al., 2011; Masson et al., 2015; Nazli et al., 2010). Given the ability of probiotic lactobacilli GR-1 and RC-14 to increase barrier functions and reduce inflammatory markers such as TNF-α, IL-1α, IL-1β, IL-8, and RANTES in the presence of HIV, lactobacilli may play a role in the protection observed in HESN women. Furthermore, previous studies have shown that women using DMPA are at higher risk of HIV acqusitions compared to women not using

147

hormonal conraceptives. Therefore, it is possible that although MPA is having an anti-inflammatory cytokine effect it may be the increased chemokine levels that recruit target cells or increased expression of HIV co-receptors such as CCR5 and CXCR4 that are the basis for the increase in HIV susceptibility (Chandra, et al., 2013; Huijbregts, et al., 2013). These results provide insight into how factors in the genital microenvironment can affect HIV acquisition and will subsequently assist in the development of prophylactic strategies to reduce HIV transmission.

CHAPTER 5: FUTURE DIRECTIONS AND LIMITATIONS

This thesis establishes a novel *ex vivo* cell culture model to study the effects of hormones and lactobacilli on primary GECs from the FGT in the presence and absence of HIV. We have assessed barrier functions, production of pro-inflammatory cytokines and chemokines, bacterial measures, glycogen content in GECs, and hydrogen peroxide production, however additional measures remain to be assessed. Further measures which will provide insights to barrier functions and innate immune factors in the presence of sex hormones and lactobacilli include, the production of AMPs, secretion of bacteriocins, mucin expression, measurement of additional tight-junction proteins such as claudin and occludin. Additionally, several clinical trials have demonstrated the health benefits conferred by certain strains of lactobacilli. However, the mechanisms of the *in vivo* efficacy observed by specific strains of lactobacilli remains a major challenge to this field of research. Therefore, future experiments for this study should consider the molecular mechanisms of lactobacilli.

It is well established that HIV binds to TLR2 and -4 activates the MAPK, PI3K, and NF-κB pathways, which results in up-regulation of pro-inflammatory cytokines (Al-Sadi et al., 2013; Al-Sadi et al., 2012; Bai et al., 2008; Dayanithi et al., 1995; Saatian et al., 2013; Wu et al., 2013). Subsequently, up-regulation pro-inflammatory cytokines results in further activation of myosin light chain kinase (MLCK) and myosin light chain (MLC) phosphorylation and cytoskeleton

149

contraction, which induce delocalization of ZO-1 from tight junction specific areas and causes assembled junctional proteins such as occludin and claudins 1, 3 and 4 to internalize (Bruewer et al., 2003; Ma et al., 2005; Ye, Ma, & Ma, 2006; Marchiando et al., 2013). Thus leading to decreased barrier function and increased translocation of pathogens across the epithelium. Both *in vitro* and *in vivo* studies of the intestinal tract have observed increased tight-junction protein expression using Pam_3-Cys-SK4 (PSK3), a TLR-2 agonist (Cario, Gerken, & Podolsky, 2004; Cario, Gerken, & Podolsky, 2007). Interestingly, *in vitro* studies using IECs have suggested that the increased in barrier function observed in the presence of lactobacilli occurs via tight-junction protein expression mediated by TLR-2 pathway (Karczewski et al., 2010). Therefore, future directions of this project should include elucidating the mechanisms of lactobacilli to increase barrier function in GECs of the FGT and should include the exploration of the role of TLR-2 expression and signaling as it is involved in both HIV mediated barrier disruption and lactobacilli mediated increase in barrier integrity. Future studies should also assess the molecular mechanisms of hormone regulation on innate immune production of pro-inflammatory cytokines. Understanding how lactobacilli and hormones can alter intracellular pathways involved in innate immunity and barrier function is essential to generate prophylactic strategies that specifically target pathways that may assist in mitigating HIV acquisition.

The FGT hosts a wide variety of bacterial species at any given time. Therefore, a limitation to this study is the use of only a single bacterial species with GECs. Future studies should include the assessment of multiple bacterial species from the FGT in this model. Given that women with a BV-associated microbiota are at an increased risk of HIV acquisition, experiments in the future should assess the affects of bacteria prevalent in BV such as *Garderella* and *Prevotella*. Furthermore, GR-1 and RC-14 are used in combination as an oral probiotic. Thus, future experimental conditions may include treatment with these probiotic strains of lactobacilli in combination.

As previously described the upper FGT is composed of a different bacterial profile compared to the lower FGT (Miller et al., 2015; Verstraelen et al., 2016). A comprehensive study of the upper FGT microbiota suggested that the endometrial cavity is primarily composed of *Bacteroides* species. Consequently, an addiotnal limitation to the current study is that we did not assess the effect of *Bacteroides* species on barrier function and innate immune factors in our GEC culture system. Notably, few studies have assessed the microbiota of the upper FGT, and therefore more research needs to be performed in this area to support *Bacteroides* species as the dominant bacteria in the upper FGT. Furthermore, future studies to analyze the effect of *Bacteroides* species on GEC barrier function and innate immune factors would be beneficial.

Lastly, as in all *in vitro* and *ex vivo* culture models, this model is limited in its ability to mimic the *in vivo* microenvironment. Within the FGT, epithelial cells communicate with the underlying stromal cells and vice versa through soluble signals (Wira, Grant-Tschudy, & Crane-Godreau, 2005). Our system lacks cross talk between stromal cells and epithelial cells and thus is a limitation to these studies. Furthermore, the GECs in our system lack influence from other immune cells and components of the microenvironment that would be present *in vivo.*

Although several limitations of this study are noted, the GEC culture model developed persists as a relevant and novel system to specifically assess the effect of lactobacilli and sex hormones on the epithelial cell barrier and innate immune factors secreted by GECs.

CHAPTER 6: CONCLUSIONS

Genital epithelial cells are the primary barrier to HIV acquisition. Given the importance of this barrier, the goal of this work was to establish a system where GEC barrier functions and innate immune factors could be assessed when under the influence of sex hormones and lactobacilli, and in the presence or absence of HIV. The main findings of this thesis were that all lactobacilli strains (GR-1, RC-14 and *L. crispatus*) increased GEC barrier functions however, probiotic strains conferred increased barrier functions compared to *L. crispatus*. When measuring innate immune factors, probiotic strains of lactobacilli did not induce pro-inflammatory cytokine production by GECs, while *L. crispatus* exposure to GECs induced secretion of pro-inflammatory cytokines. Additionally, female sex hormones and hormonal contraceptives did not alter GEC barrier functions. Interestingly, E2 and MPA exerted anti-inflammatory effects on GECs grown in their presence. Furthermore, hormones did not alter bacterial adherence, enumeration or production of hydrogen peroxide. Lastly, in the presence of HIV, probiotic lactobacilli were able to confer increased barrier functions and E2 was able to decrease inflammation induced by HIV. These studies successfully established a model to assess the effect of hormones and lactobacilli on the GEC barrier function and innate immune responses in the presence of HIV. This lays the foundation to examine intracellular/molecular pathways involved in regulating innate responses and barrier functions

CHAPTER 7: MATERIALS AND METHODS

6.1 Source of Tissues and Primary Epithelial Cell Preparation

Informed written consent was obtained in accordance with the approval of the Hamilton Health Sciences Research Ethics Board. Viable tissues were received from women aged 30-59 years of age undergoing hysterectomies for benign gynecological reasons. The most common reasons for surgery were prolapse, fibroids, and menorrhagia (heavy bleeding).

6.2 Culture of Primary Genital Tract Epithelial Cells

A detailed protocol for isolation and culture of primary genital epithelial cells has previously been described by Kaushic *et al.,* (Kaushic, Nazli, Ferreira, & Kafka, 2011). Briefly, endometrial or endocervical tissues were minced into small pieces (1-2mm^2) and digested with an enzyme mixture (described below) for 10-20 minutes at 37 $^{\circ}$C on an orbital shaker. However, digestions may take 30-40 minutes, as it is tissue dependent. The enzyme mixture was then filtered using a mesh filter (250µm pore size) to separate genital epithelial cells from undigested tissue, mainly myometrial tissue. The filtrate was collected and further purified using an additional mesh filter (20µm pore size) to separate the epithelial cells from stromal cells, erythrocytes, mucus, cell debris and other cells that may have been present. Purified epithelial cells (typically sheets of cells) were then collected and placed in

primary cell media (detailed below). The culture system was prepared by coating 0.4-µm pore-sized polycarbonate membrane tissue culture inserts (BD Falcon) with a layer of Matrigel (BD) and placing the inserts in a 24 well culture plate (BD Falcon). Cells were then seeded on the apical compartment of the transwell system at 1×10^5 in 300µL of media, and 500µL of media was added to the basolateral chamber of the transwell system. Tissue culture plates were incubated at 37°C with 5% CO_2. Media was Every was changed every 48 hours by removing the apical and basolateral media and replacing it with fresh primary cell media. Cells were cultured until they formed polarized confluent monolayers, which is measured by TER (transepithelial electrical resistance) using a Voltmeter (EVOM). Monolayers showing TER values greater than 1 kΩ/cm will be considered confluent. Polarized confluent monolayers were usually formed within 5–10 days.

6.2.1 Digestive enzyme mixture

The digestive enzyme mixture consists of 1.5mg/ml collagenase D, 3.45mg/ml pancreatin, 0.1mg/ml hyaluronidase, and 0.2mg/ml D-glucose in Hanks buffered salt solution (HBSS) (McMaster Media Centre) and 100 U/mL with penicillin/streptomycin (Sigma–Aldrich).

6.2.2 Primary Cell Media

Primary GEC media consisted of phenol red free DMEM/F12 (Life Technologies) supplemented with 10µM HEPES, 2µM L-glutamine, 100 U/mL penicillin/streptomycin, 2.5% Nu Serum culture supplement (VWR), 2.5% Hyclone defined fetal bovine serum (FBS; Fischer Scientific) and 500µL of Fungizone (Invitrogen).

6.3 Preparation of E2, P4, and MPA Media

E2 (Sigma-Aldrich), P4 (Sigma-Aldrich) and MPA (Sigma-Aldrich) were prepared to 10^{-9} M, 10^{-7} M and 10^{-9} M standard concentrations, respectively, in primary cell media. Prepared hormone supplemented media was kept at 4°C wrapped in foil due to light sensitivity.

6.4 Hormone Incubation with Cells

Primary genital epithelial cells were grown either in the presence or absence of E2, P4 or MPA by adding 300 µL of prepared hormone media to the apical compartment and 500 µL to the basolateral compartment of the transwell culture system. The media was changed every 48-hours until the cells became confluent. Hormone media was added for at least 7 days prior to use for experiments.

6.5 Bacterial Propagation and Stock Preparation

Commercially available, probiotic *Lactobacillus* strains RC-14 (*Lactobacillus reuteri)* and GR-1 (*Lactobacillus rhamnosus*) were received in the form of stab-cultures from the laboratory of Dr. Gregor Reid (Western University, London ON, Canada). *L. crispatus* was also received in the form of stab-cultures from the Reid laboratory and was initially obtained from the ATCC (33820).

Bacteria were grown anaerobically in MRS broth (VWR) at 37°C on an orbital shaker for 24 hours. Bacteria were then centrifuged at 3000 rpm for 15 minutes, re-suspended in fresh MRS Broth, aliquotted into cryovials with glycerol (500uL of MRS Broth with Bacteria and 20% glycerol), and stored at -80 $^{\circ}$C for later use and experimentation.

From the initial re-suspension of bacteria and MRS Broth, a sample is used to make 10-fold dilutions in MRS Broth. The 10-fold dilutions are plated on MRS agar (VWR) and placed in chambers (Mitsubishi boxes, Fisher Scientific) with an anaerobic generating sachet (VWR) at 37°C for 24 hours. The bacteria were then counted to obtain the stock concentration.

6.6 Addition of Bacteria to Cells

Prior to the addition of bacteria to cells, the bacteria was suspended in PBS medium and centrifuged at 3000 rpm for 10 minutes to remove the MRS Broth. The bacteria were then re-suspended in cell culture medium free of antibiotics to be added to the cell cultures.

6.7   Measurement of Transepithelial Electrical Resistance (TER)

TER (transepithelial electrical resistance) was measured using a volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA; Model EVOM, Serial No. 56734 G07E) with the STX2 chopstick electrode (World Precision Instruments, Sarasota, FL, USA) at 0 hours and at specified time points post exposure to hormones (NH, E2, P4 or MPA) and/or *Lactobacillus* strains (GR-1, RC-14, or *L. crispatus)*, and/or HIV-1 IIIB, and/or polyinosinic-polycytidylic acid (poly IC) (Sigma Aldrich). TER measurements are expressed as percent pre-treatment of initial TEER at time 0.

Calculation:

(TER at specified time point/TER at 0 hours)*100 = Percent Pre-Treatment of TER

6.8   Bacterial Adherence to Cells

Supernatants were collected from the apical and basolateral compartments of the transwell system. The apical compartment was washed twice with 500uL of PBS to remove non-adherent bacteria. Triton X-100 (Sigma-Aldrich) was added to the apical compartment of the transwell system (100uL) for 10 minutes at 37°C. Supernatants were collected, serially (10-fold) diluted in MRS Broth and plated on MRS Agar. The plates were then incubates anaerobically at 37 ^{o}C for 24 hours at which time bacterial colonies

were counted.

6.9   Bacteria Enumeration

The supernatant was collected from the apical compartment of the transwell system. The apical compartment was washed twice with 500uL of PBS and washes were added to the collected supernatant. The supernatant and washes were centrifuged at 10,000 rpm for 5 minutes. The supernatant was decanted and cells were re-suspended in MRS Broth, serially (10-fold) diluted, and plated on MRS Agar. The plates were then incubates anaerobically at 37 ^{o}C for 24 hours at which time bacterial colonies were counted.

6.10   Cell Viability

Supernatants were collected from the apical and basolateral compartments of the transwell system. Trypsin (10X) was added to the apical compartment of the cell culture system and incubated for 5-15 minutes at 37°C. Supernatants containing detached cells were collected and media containing fetal bovine serum was added to inactivate the 10X Trypsin. Supernatants were centrifuged at 1500 rpm for 5 minutes. The supernatant was decanted and cells were re-suspended in primary cell media. A trypan blue exclusion assay was performed using a hemocytometer. Live cells and dead cells were counted. Data was presented as a percentage of live cells/total cells for prevent viability.

6.11   Immunofluorescent staining of epithelial cell cultures

After 24 hours of exposure of GECs to serum free media, gp120 (100ng/mL, Immunodiagnostics), or UV-inactivated HIV (10^5 IVU/mL) the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences). The cells were permeabilized and blocked using 5% bovine serum albumin (Sigma Aldrich), and 5% goat serum (Life Technologies) in 0.1% Triton X-100 (Sigma Aldrich). Blocking solution was added to each well for 30 minutes and was incubated at room temperature. Blocking solution was removed and primary rabbit anti-human zona occludens-1 (ZO-1) antibody (Life Technologies) was diluted in blocking solution to a concentration of 2μg/mL. 100μL of the primary antibody was added to the cells incubated for one hour at room temperature. The primary antibody was removed and the wells were washed. Secondary antibody goat anti-rabbit IgG, Alexa Flour 488-congugated (Life Technologies) was then diluted in blocking solution to a final concentration of 1.5μg/mL. 100μL was added to each well for one hour and incubated at room temperature in the absence of light. The secondary antibody was removed and the cells were again washed. Propidium iodide (Life Technologies), a nuclear counter stain, was diluted (1:3) in 0.1% triton X-100 and 100μL was added to each well and was at room temperature. Transwell membranes were removed using a scalpel and mounted on slides using VectaShield (Vector Laboratories Inc.) mounting fluid. An inverted confocal laser scanning LSM

510 microscope (Carl Zeiss Canada) was used to acquire images. Standard operating conditions were used and the confocal setting for image acquisition remained identical between all treatments.

6.12 Magpix Multi-Analyte Assay for measurement of cytokines and chemokines

Supernatants from GECs were collected at various time points post exposure to hormones and/or *Lactobacillus* strains GR-1, RC-14, or *L. crispatus*, and/or HIV-1 IIIB, and/or polyinosinic-polycytidylic acid (poly IC) (Sigma Aldrich). The Magpix multi-analyte magnet bead based assay (Catalogue #HCYTOMAG) was used to measure cytokine and chemokine concentrations in supernatants using the Magpix technology system (Millipore, Billerica, MA, USA), as per the manufacturer's instructions. The following cytokines and chemokines were measured: TNF-α, IL-1α, IL-1β, IL-6, IL-10, GM-CSF, MCP-1, MIP-1α, RANTES and IL-8.

6.13 Cytokine measurement by Enzyme Linked Immunosorbent Assay (ELISA)

Supernatants from GECs were collected at various time points post exposure to hormones (NH, E2, P4 or MPA) and/or *Lactobacillus* strains (GR-1, RC-14, or *L. crispatus)*, and/or HIV-1 IIIB, and/or polyinosinic-polycytidylic acid (poly IC) (Sigma Aldrich). The concentration of TNF-α in the collected

supernatants was measured using R&D Systems Duoset TNF-α ELISA kit (R&D systems, Catalogue #DY210) as per manufacturer's instructions.

6.14 Fluorescein Isothiocyanate (FITC)-Labeled Dextran Dye Assay

4kDa (Sigma; FD4-250MG) was dissolved in primary media to obtain a final concentration of 2.3mg/mL and added to the apical surface of GEC monolayers. At various time points post exposure to hormones (NH, E2, P4 or MPA) and/or *Lactobacillus* strains (GR-1, RC-14, or *L. crispatus)*, and/or HIV-1 IIIB, and/or poly IC, 50uL of basolateral medium was sampled and placed in duplicate in a 96 well plate. The fluorescence of fluorescein isothiocyanate–dextran was measured using a microplate reader (SpectraMax i3 plate reader; Molecular Devices; Sunnyvale, CA) at an excitiation of 490nm and emission of 520nm. The dextran leakage in the basolateral compartment is expressed as a percentage of dextran added to the apical compartment. Calculation:

(dye in basolateral well/dye in apical well)*100 = % of dextran leakage

6.15 Glycogen Assay

Primary GECs grown in the presence or absence of physiological concentrations of female sex hormones (E2 and P4) or hormonal contraceptive (MPA) for 10 days. Apical and basolateral supernatants were collected. Cells were collected by adding 200uL of 1X Tryspin to the apical

compartment of the transwell system. The cells were incubated for 4 minutes at 37 degrees Celsius. Cells were collected and placed in media containing FBS and centrifuged at 10,000 rpm for 5 minutes to remove trypsin. The cells were lysed using ddH_2O and boiled. Glycogen was measured using a Glycogen Assay Kit (Bio Vision K646-100). Each sample (cell lysates and cell supernatants) was added into 8 wells of a 96 well plate and diluted 1:2 with hydrolysis buffer. Hydrolysis enzyme was added to 4 wells of each sample. The wells containing no hydrolysis enzyme were used a glucose background. The relative fluorescence was measured using microplate reader. To determine glycogen concentration the fluorescence of the glucose background wells was subtracted from fluorescence of the wells with the added enzyme.

6.16 Hydrogen Peroxide Assay

Primary GECs were grown to confluence in the presence or absence of physiological concentrations of female sex hormones (E2 and P4) or hormonal contraceptive (MPA). Lactobacilli (GR-1 and RC-14) were added to the apical compartment of the transwell system for 6 hours at 100 CFU/cell. Hydrogen peroxide was measured using a Hydrogen Peroxide Detection Kit (Bio Vision K265-200). Samples were collected from each treatment (50uL) on ice and plated in a 96 wells plate. A reaction mixture containing assay buffer, OxiRed Probe, and HRP solution was added to each sample for 10

minutes at room temperature. Optical density (570nm) was measured using a microplate reader.

6.17 Statistical Analysis

Statistical analysis and graphical representation was performed using GraphPad Prism 6.0d (GraphPad Software). To compare ≥3 mean values, the Kruskal–Wallis nonparametric analysis of variance test was used. When an overall statistically significant difference was detected (defined on the basis of a P value of <.05), the Dunn test was used to correct for multiple comparisons. A one-way analysis of variance (ANOVA) using the Bonferonni post-test was also used for analysis of experiments. When comparing two treatment groups, an unpaired t-test was used for analysis.

CHAPTER 8: REFERENCES

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