Inflammation in the Neovaginal Microenvironment of Transfeminine Individuals

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology
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Abstract

Transfeminine individuals are assigned male at birth but do not identify as male. Some transfeminine individuals may choose to undergo the gender affirming surgery vaginoplasty to create a neovagina. There is a paucity of data on the neovaginal microenvironment to inform best gynecological practices. Vaginal and penile inflammation is modulated by local microbiota, but drivers of inflammation in the neovagina are poorly understood. The compositions of the neovaginal microbiota and immune milieu were elucidated from neovaginal swabs, using 16s rRNA gene sequencing and multiplex immunoassay, respectively. Immune data reduction and clustering was performed, and six unique immune profile types (IPTs) were found. Associations between IPTs and bacterial taxa were assessed using regression models. *Streptococcus*, *Atopobium deltea*, and *Prevotella buccalis* all trended toward significant associations with a proinflammatory IPT, however these associations were not statistically significant. Understanding the unique interplay between the neovaginal immune milieu and microbiota will inform trans-specific healthcare.

Keywords

Neovagina, Vaginoplasty, Transfeminine, Inflammation, Cytokine, Microbiota, Bacteria, Genital exposures, Hormone therapy
Summary for Lay Audience

Transfeminine individuals are those who are assigned male at birth but who do not have a male gender identity. Some transfeminine individuals may seek gender affirming care, such as hormone therapy and surgeries, to produce a physical appearance better aligned with their gender identity. Gender affirming surgery for transfeminine individuals can include vaginoplasty, which is the surgical creation of a neovagina, usually using penile and scrotal tissue. Many transfeminine individuals who have undergone vaginoplasty report gynecological symptoms such as odour, discharge, and bleeding. However, the underlying cause of these concerns is unknown. In the cisgender female vagina, these symptoms are often caused by disturbances in the vaginal microbiota (e.g., bacteria) that cause inflammation and increase susceptibility to sexually transmitted infections. Because the causes of vaginal symptoms are often known for cisgender females, targeted treatments are available; however, the relationship between bacteria and inflammation has not been researched in transfeminine people and thus no appropriate treatments are available.

Bacteria and immune factors in the neovagina were measured. Clustering analyses were performed to group the immune data into low, medium and high inflammation groups. Associations between bacteria and low, medium and high inflammation groups were then assessed using clustering tools and regression analyses. Three bacterial taxa trended toward being associated with a high inflammation group, however the association was not statistically significant. These taxa were *Streptococcus, Atopobium deltea, and Prevotella buccalis*. These findings are a critical step in developing a better understanding of the relationship between bacteria and inflammation. However, much more research is needed to inform developments of targeted treatments for gynecological healthcare for transfeminine individuals.
Co-Authorship Statement

All bacterial swab processing discussed in section 3.4.2 was completed by our collaborators in Dr. Jacques Ravel’s Lab at the University of Maryland. A clean raw counts dataset of unique bacterial taxa was produced by Bern Monari (PhD candidate, Program in Molecular Medicine, University of Maryland) and sent to me. All bacterial analyses presented in this thesis were completed by me.

Design and compilation of the questionnaire discussed largely in section 3.5.1 was completed by Dr. Jessica Prodger and Dr. Greta Bauer with the help of Songmiao Guan (past undergraduate student, University of Western Ontario).

Jason Hallarn (PhD candidate, Department of Epidemiology and Biostatistics, University of Western Ontario) was responsible for acquiring Research Ethics Board approval for the TransBiota study, and for programming the study questionnaire into a HIPAA-compliant REDCap secure server hosted at the University of Western Ontario. Jason was also responsible for producing the behavioural clusters discussed in section 3.6.3 and 6.1.1. The analysis discussed in this thesis, comparing cytokine concentrations between behavioural clusters, was completed by me.
Acknowledgements

I would like to acknowledge all the participants of the TransBiota initiative for dedicating their time to this study and providing samples. I would like to thank our collaborators at the University of Maryland, Dr. Jacques Ravel, Dr. Pawel Gajer and Bern Monari for their support and guidance with microbial data analysis. I would also like to thank Jason Hallarn for sharing his knowledge and being endlessly helpful throughout my time working on this project.

I would like to express my gratitude to my advisory committee members Dr. Jeremy Burton and Dr. Greta Bauer for their help in guiding me through this process and for their insightful comments.

I want to sincerely thank all my past and present colleagues from the Prodger Lab: Shirley Constable, Lane Buchanan, Eric Shao, Sarah Gowanlock, Reeya Parmar, Ainslie Shouldice, Omar Almomani, Victor Lam, Geoff Rempel and Aleena Ghafoor. Thank you for your advice, support, and friendship.

I would like to thank my parents who spent considerable time reading though and editing this thesis and much of my other written work produced throughout my time as a master’s student. Thank you so much for your support and encouragement.

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<td>ASV</td>
<td>Amplicon Sequence Variant</td>
</tr>
<tr>
<td>BV</td>
<td>Bacterial Vaginosis</td>
</tr>
<tr>
<td>CST</td>
<td>Community State Type</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes Simplex Virus 2</td>
</tr>
<tr>
<td>hDBSCAN</td>
<td>Hierarchical Density-Based Spatial Clustering of Applications with Noise</td>
</tr>
<tr>
<td>IPT</td>
<td>Immune Profile Type</td>
</tr>
<tr>
<td>PCA</td>
<td>Principle Component Analysis</td>
</tr>
<tr>
<td>PHATE</td>
<td>Potential of Heat diffusion for Affinity-based Transition Embedding</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually Transmitted Infection</td>
</tr>
<tr>
<td><strong>Terminology</strong></td>
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<tr>
<td><strong>Sex</strong></td>
<td>Biological attributes (e.g., chromosomal, anatomical and physiological factors) differentiating females, males, and intersex people</td>
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<tr>
<td><strong>Gender</strong></td>
<td>Socially constructed roles, behaviours, expressions, and identities that differentiate women/girls, men/boys and gender diverse people</td>
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<tr>
<td><strong>Cisgender</strong></td>
<td>A person whose gender identity is aligned with their sex assigned at birth</td>
</tr>
<tr>
<td><strong>Transgender</strong></td>
<td>A person whose gender identity is not aligned with their sex assigned at birth</td>
</tr>
<tr>
<td><strong>Transfeminine</strong></td>
<td>A person assigned male at birth who does not have a male gender identity</td>
</tr>
<tr>
<td><strong>Gender dysphoria</strong></td>
<td>Distress caused by incongruence between an individual’s gender identity and their assigned gender and/or primary or secondary sex characteristics</td>
</tr>
<tr>
<td><strong>Gender-affirming medical care</strong></td>
<td>A medical process through which individuals alter their primary and/or secondary sex characteristics to align with their gender identity</td>
</tr>
<tr>
<td><strong>Gender-affirming surgery</strong></td>
<td>A surgical procedure altering the physical appearance and sexual characteristics of an individual, to resemble those associated with their gender identity</td>
</tr>
<tr>
<td><strong>Vaginoplasty</strong></td>
<td>The surgical creation of a vulva and a neovaginal canal generally sought out by transfeminine individuals</td>
</tr>
<tr>
<td><strong>Vagina</strong></td>
<td>The muscular tube connecting the external genitals to the cervix of the uterus in the female genital tract. In the context of this thesis, this term refers to the vagina a person is born with.</td>
</tr>
<tr>
<td><strong>Neovagina</strong></td>
<td>A vagina that has been surgically constructed via vaginoplasty</td>
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Chapter 1

1 Introduction

1.1 The Genital Microenvironment Plays a Critical Role in Sexual and Reproductive Health

The immune milieu is a major determinant of sexual and reproductive health in both the vaginal and penile microenvironments. Inflammation in both female and male genital tracts is associated with increased proinflammatory cytokines, which can decrease epithelial barrier integrity. An intact epithelium is essential for defense against invading pathogens (e.g. Neisseria gonorrhoeae). Disruption to proper epithelial cell maturation in the vagina can prevent proper shedding of mature epithelial cells which helps to flush out pathogenic bacteria. Disruption to the epithelial barrier in both the penile and vaginal microenvironments allows for increased penetration of viral pathogens – HIV and HSV. Inflammation is also associated with immune cell recruitment and, in turn, increased risk of HIV acquisition. HIV infects immune cells in the genital mucosa, and an increased abundance of immune cells in the mucosa provides more targets for HIV. Therefore, inflammation is a critical determinant of whether sexual exposure leads to active infection.

Inflammation in genital microenvironments does not occur in isolation. The vaginal and penile microenvironments are homes to rich microbiota which can facilitate epithelial health and immune homeostasis or, in cases of microbial dysbiosis, can dysregulate these microenvironments. In a healthy genital microenvironment, the microbiota provides the first line of defense against pathogenic organisms and helps to regulate inflammation and epithelial integrity through production of metabolites with antimicrobial properties (i.e., lactic acid). When these local bacteria are removed or dysregulated in some way, these protective mechanisms break down, leaving individuals susceptible to STIs and other negative sexual health outcomes.
This complex interplay among immune factors, the microbiota, and the epithelium, has been extensively studied in the female genital tract, and to a somewhat lesser extent, the male genital tract. Symptoms such as odour, discharge and itching are associated with microbial dysbiosis in the vagina and targeted, evidence-based treatment options are available to help combat this\textsuperscript{15,16}. Circumcision has been shown to alter the penile microbiota, including reducing anaerobic bacteria and local cytokine levels, effecting a 60\% reduction in heterosexual acquisition of HIV in this population\textsuperscript{17}. There is evidence that these changes are a result of increased local oxygen levels and water loss on the penile coronal sulcus, demonstrating the profound effect that environmental changes can have on the microenvironment\textsuperscript{18–20}.

The vaginal microenvironment is also influenced heavily by sex hormones, vaginal exposures, and medications. Changes in local hormones regulate vaginal epithelial cell maturation, and vaginal exposures such as douching can physically disrupt the epithelial barrier resulting in proinflammatory changes. Penetrative sex can also introduce non-optimal bacteria into the vaginal microenvironment resulting in inflammation\textsuperscript{21–26}.

Although non-optimal bacteria in the vaginal and penile microenvironments are both associated with negative sexual health outcomes, some of which are the same (e.g., increased STI risk), it is important to note that what constitutes optimal and non-optimal bacteria differs between the vaginal and penile microbiota.

Bacteria characteristic of bacterial dysbiosis in the vagina include \textit{Gardnerella vaginalis}, \textit{Atopobium} spp. and other strict anaerobes, including \textit{Prevotella} spp.\textsuperscript{15,16,27}. In the penile microbiota, bacteria associated with inflammation, immune cell recruitment and increased HIV acquisition are \textit{Peptostreptococcus anaerobius}, \textit{Prevotella bivia}, \textit{Prevotella disiens}, \textit{Dialister propionicifaciens}, \textit{Dialister micraerophilus}, and a genetic near neighbor of \textit{Dialister succinatiphilus}\textsuperscript{14}. Although there is some overlap in non-optimal bacteria between these two environments, the dysbiotic communities are largely distinct (Figure 1).
Figure 1. The vaginal and uncircumcised penile microbiota. Bar plot of the relative abundances of the 19 most common bacterial taxa in the vagina (n=100) and the sub-preputial space of the uncircumcised penis (n=100). Composed of data from previous work by our lab (penile microbial data) and our collaborators (vaginal microbial data). Black boxes outline the high-risk vaginal microbiota (high relative abundance of Gardnerella and Atopobium), low risk vaginal microbiota (high relative abundance of Lactobacillus), high risk penile microbiota (high relative abundance of Prevotella, Peptoniphilus, Peptonaphilacea and Dialister) and low risk penile microbiota (high abundance of Corynebacterium)\textsuperscript{14-16}. (Bar plot created by collaborators at the University of Maryland)

1.2 Study Rationale

Transfeminine individuals are individuals who were assigned male at birth but do not have a masculine gender identity. Some transgender individuals experience gender dysphoria because of their gender identity being incongruent with their physical attributes. In response to this, they might seek out gender affirming medical care\textsuperscript{28,29}.

For transfeminine individuals, gender affirming care may include vaginoplasty, which is the surgical creation of a vulva and vaginal canal (referred to as a neovagina). The most common technique for vaginoplasty – and the only technique used in Canada – is penile-inversion vaginoplasty, which uses penile and scrotal skin to line the new vaginal canal. Many transfeminine individuals who have undergone vaginoplasty experience gynecological concerns. Symptoms such as odour, discharge, and itching (frequently associated with bacterial dysbiosis in cisgender women) are also reported by transfeminine individuals\textsuperscript{30}. However, the underlying cause of these symptoms among transfeminine individuals is largely unknown.

There is a paucity of data to inform gynecological health care for individuals who have undergone vaginoplasty, as research on the topic has been limited to a select
few studies and case reports\textsuperscript{31–35}. Many gynecological practices for transfeminine individuals with a neovagina are informed by practices tailored to the natal vagina (the vagina an individual is born with). This is problematic, as the neovagina has a unique microenvironment – a vaginal cavity lined with penile skin instead of vaginal mucosal epithelium.

As we know, environmental changes can have a profound effect on the genital microenvironment. It is therefore possible that invagination of penile skin to create a neovagina may have the opposite effect of circumcision; reduced oxygen levels and moisture loss may be associated with increased anaerobic bacteria and local inflammation.

A better understanding of the neovaginal microenvironment is necessary. Knowing what constitutes a healthy microenvironment associated with low inflammation, a healthy microbiota, and epithelial integrity is essential to inform tailored treatment for transfeminine individuals. To this end, this thesis aims to elucidate the microenvironment of the transfeminine neovagina and better understand what factors contribute to inflammation in it.
Chapter 2

2 Background

2.1 Gender Affirming Medical Care

Gender-affirming medical care for transfeminine individuals can include hormone therapy and surgeries. Hormone therapy often includes testosterone suppression, estrogen supplementation and, to a lesser extent, progestin supplementation, to develop secondary sex characteristics such as increased breast tissue, body fat redistribution and softening of the skin\textsuperscript{36–38}.

Gender affirming care is increasingly being recognized as a critical therapeutic intervention and medical necessity to combat gender dysphoria and improve quality of life.\textsuperscript{36,39}

2.1.1 Vaginoplasty and the Neovagina

Penile inversion vaginoplasty is the most common surgical technique used to create the neovagina\textsuperscript{39} and is currently the only vaginoplasty technique used in Canada (Figure 2).

The optimal outcome for vaginoplasty is a moist and hairless neovaginal canal with sufficient depth for penetration (if desired) and erogenous sensation all with concordant vulvar anatomy\textsuperscript{40}. Penile inversion vaginoplasty is complex and requires many steps as outlined below.

1. The anterior and posterior skin flaps are marked out on the scrotal tissue prior to the posterior aspect of the scrotum being removed as a full-thickness skin graft.
2. The neovaginal cavity is formed by blunt dissection of the prerectal space between the urethra and the rectum.
3. Orchiectomy (removal of the testicles) is performed.
4. The penile shaft is degloved and the penile glans with associated neurovascular bundle is separated from the penile skin.
5. The distal aspect of the penile tube skin is stitched closed.
6. From a part of the glans penis the neoclitoris and the labia minora are formed.
7. The full-thickness scrotal skin graft is then grafted to the distal aspect of the penile skin to create a tube of tissue that will become the neovaginal canal lining.
8. This grafted penile and scrotal tissue is inserted into the dissected space between the bladder and rectum to form the neovaginal canal.\textsuperscript{36,39,41}
Figure 2. Anatomy before and after penile inversion vaginoplasty. Penile inversion vaginoplasty involves dissection of the space between the bladder and the rectum, followed by degloving of the penile shaft and insertion if the penile shaft tissue into the dissected space. (Used with permission of Mayo Foundation for Medical Education and Research, all rights reserved) (Appendix 1)
2.2 The Vagina

Throughout an individual’s lifetime the physiology and morphology of the vagina changes. Mediated by hormonal changes through puberty, the menstrual cycle, childbirth, and menopause, the vaginal epithelium is altered. At puberty the vaginal epithelium matures and thickens, and intracellular glycogen content increases, in response to increasing estrogen. The vaginal pH also drops to approximately 3.5-4.5\(^{42,43}\). Throughout this thesis, the term “vagina” will be used to refer to the physiological and morphological aspects of the vagina of reproductive aged females who were born with a vagina.

2.2.1 The Vaginal Epithelium

The vagina is a muscular organ connecting the inner female genital tract (cervix and uterus) to the exterior female genitalia (vulva) (Figure 3) The outermost layer of the vagina is the mucosal layer which can be subdivided into the epithelial layer which lines the vaginal lumen, and the underlying lamina propria (Figure 4)\(^{44}\). The vaginal epithelium is a stratified squamous epithelium and the first line of contact with invading pathogens.\(^{44-46}\) The vaginal epithelium is made up of many strata which undergo continuous renewal. As vaginal epithelial cells mature, they move outward from the basal layer towards the apical layer where they are sloughed off into the vaginal lumen. This sloughing serves as an initial defense mechanism as it removes potential pathogens from the vagina.\(^{44}\)

Vaginal epithelial cells express cytokeratins K1, K4, K5, and K13, but tend not to form keratin bundles like those found in the skin. Additionally, unlike the outermost layer of the skin known as the stratum corneum, the apical layer of the vaginal epithelium is relatively permeable to water, microbes and immune mediators.\(^{44,46}\)
Figure 3. Representation of the female genital tract. The vagina connects the inner female genital tract (cervix and uterus) to the exterior female genitalia. (Created with BioRender.com)
Figure 4. Schematic of the vaginal mucosa. The vaginal epithelium is a stratified squamous epithelium that lies on top of the lamina propria. The vaginal epithelium lines the vaginal lumen. (Created with BioRender.com)
Estrogen and the Vaginal Microenvironment

The vaginal microenvironment shifts throughout an individual’s lifetime in response to hormonal changes. At puberty, estrogen increases and plays a critical role in shaping the vaginal microenvironment. Estrogen stimulates proliferation of vaginal epithelial cells and increases epithelial thickness, barrier integrity, cell differentiation and cytosolic glycogen. As epithelial cells ascend toward the vaginal lumen, the level of glycogen within the cytosol of the cells increases. Decreased estrogen in the vaginal microenvironment (as seen in menopause) results in a reduction in epithelial proliferation. The epithelium therefore thins and is more fragile, which can cause itchiness, dryness, and irritation. A decrease in estrogen is also associated with decreased glycogen deposition, decreased beneficial bacterial species, and increased pH.

The effect of estrogen therapy on the neovaginal microenvironment is poorly understood, however, estrogen therapy post-menopause is associated with reduced symptoms of vaginal epithelial fragility and restoration of beneficial bacterial species. These changes from hormone treatment post-menopause highlight estrogen’s importance in shaping the vaginal microenvironment and may play a critical role in gynecological health outcomes for transfeminine individuals who have undergone vaginoplasty and are on estrogen hormone therapy.

2.2.3 The Vaginal Microbiota

When shed into the vaginal lumen, glycogen from vaginal epithelial cells is catabolized into smaller polymers. These glycogen-derived polymers are the preferred carbon source for beneficial Lactobacillus spp., which optimally dominate the vaginal microbiota. These Lactobacillus spp. metabolize glycogen-derived polymers into lactic acid, lowering the vaginal pH. The low pH inhibits the growth of pathogenic organisms while promoting the growth of lactobacilli, thereby helping to perpetuate Lactobacillus spp. dominance in the vagina. Lactobacilli also produce antimicrobial compounds which further inhibit colonization by pathogenic organisms.
*Lactobacillus* dominance is associated with a lower risk of acquiring viral STIs HIV and HSV-2, as well as non-viral STIs such as *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. *Lactobacillus spp.* colonization is also associated with lower risk of pelvic inflammatory disease and preterm birth\(^{56-59}\).

### 2.2.4 Molecular Bacterial Vaginosis

It has been established there are five distinct vaginal microbiota referred to as community state types (CSTs): CST-I, CST-II, CST-III and CST-V dominated by *L. crispatus, L. gasseri, L. iners, L. jensenii*, respectively, and CST-IV composed of a diverse group of strict and facultative anaerobes, including species of the genera *Gardnerella, Atopobium, Mobiluncus, Prevotella* and other taxa in the order *Clostridiales\(^{21,22}\)*. This diverse set of strict and facultative anaerobic bacteria in CST-IV is responsible for Bacterial Vaginosis (BV), which is a common symptomology of pain, discharge and malodour. However, even in the absence of any symptoms CST-IV is associated with increased risk of pelvic inflammatory disease, preterm birth, low birth weight, and increased risk of STI and HIV acquisition\(^{15,23,60}\). CST-IVs therefore often referred to as “molecular BV”, and can be broadly defined as the loss of *Lactobacillus spp.* dominance and overgrowth of diverse strict and facultative anaerobes\(^{16,60}\).

BV is a highly prevalent condition and is clinically diagnosed with an Amsel or Nugent score. For diagnosis using Amsel score, three of the following four characteristics must be present for a positive diagnosis: vaginal discharge, amine odour, elevated pH and clue cells. Diagnosis using a Nugent score requires microscopy of a Gram-stained vaginal smear. The Nugent score reflects the abundance of large Gram-positive rods (*Lactobacilli*), Gram-negative and Gram-variable rods and cocci (including, *Gardnerella vaginalis, Prevotella, Porphyromonas* and *Peptostreptococcus*) and curved Gram-negative rods (*Mobiluncus*). A third and even more sophisticated and rigorous method to assess the vaginal microbiota and diagnose molecular BV, is using culture-independent approaches (16S rRNA gene sequencing or targeted PCR). These latter two
approaches can diagnose molecular BV in the absence of symptoms\textsuperscript{1,2,23}. This is critical to note, because, even in the absence of symptoms, vaginal dysbiosis is still associated with inflammation, and an increased risk of STI and HIV acquisition\textsuperscript{27}.

2.3 The Penile Epithelium

The penile epithelium, like the vaginal epithelium, is a stratified squamous epithelium. However, unlike the vaginal epithelium, the penile epithelium is thinner (100-200um vs 100-300um) and has a soft cornified outer layer made up of terminally differentiated, anucleated keratinocytes, called corneocytes. Corneocytes make up the outermost layer of the penile epithelium – the stratum corneum. The stratum corneum limits water loss and provides mechanical integrity via cell junctions called corneodesmosomes\textsuperscript{8,49,61}. The penile epithelium expresses different cytokeratins from the vaginal epithelium – K5/K14 in the intermediate layers and K1/K10 in the superficial layers\textsuperscript{62,63}. It also differs greatly from that of the vaginal epithelium in that it does not produce glycogen. Instead, corneocytes are packed with keratin bundles and, when these cells undergo desquamation through degradation of the corneodesmosomes, this frees that keratin along with other lipids and fatty acids, all of which are then used as food sources for local bacteria\textsuperscript{64–67}.

2.3.1 Circumcision and the Penile Microenvironment

Having an in depth understanding of the neovaginal epithelial microstructure and how it is affected by environmental changes (e.g., water loss, oxygen tension, hormones) is essential in determining best-practices to promote optimal gynecological outcomes. The effect of penile circumcision on the coronal sulcus illustrates the significant role the environment plays in shaping the local microbiota. Eliminating the foreskin results in increased oxygen tension and water loss on the coronal sulcus. This decreases the abundance of strict anaerobic bacteria and increases aerobic and facultative anaerobic bacteria considered to be more typical skin bacteria (i.e., \textit{Staphylococcus} and \textit{Corynebacterium}). The strict anaerobes reduced by circumcision include some implicated in molecular BV such as \textit{Prevotella}, \textit{Peptostreptococcus} and \textit{Finegoldia} \textsuperscript{14,15,17,39,68}. Invagination of penile
tissue may result in a microenvironment similar to the coronal sulcus of the uncircumcised penis, as the neovagina is exposed to less oxygen and experiences less water loss.

### 2.4 The Neovaginal Microenvironment: What is Currently Known

There is a paucity of data on the neovaginal microenvironment, with most existing research coming from a series of small case reports that utilized targeted PCR or culture-based detection methods, which capture only a tiny fraction of the bacterial species present.\(^{31-35}\)

One study of transfeminine individuals (n=9) who had undergone penile inversion vaginoplasty looked to see if, over time, the soft-cornified squamous epithelium of the penis changed histologically to resemble more the mucosal-type squamous epithelium found in the vagina. Two samples were taken from individuals who were undergoing vaginoplasty at the time of the study while the rest were from individuals who had undergone vaginoplasty 8 months to 14 years prior to biopsy. The researchers found that, even after 14 years, levels of keratinization did not change significantly and the neovaginal epithelium still resembled that of penile tissue. Additionally, there was no presence of glycogen. In the absence of glycogen, we can infer that it would be difficult for the neovaginal microenvironment to support the same *Lactobacillus* species that optimally dominate the vaginal microbiota\(^ {69}\).

Concordantly, a study examining the neovaginal microbiota of four transfeminine individuals who had undergone penile inversion vaginoplasty found *Lactobacillus spp.* in only one individual, and in low abundance. This study, which utilized 16S rRNA gene sequencing, found instead that the penile-derived neovaginal microbiota was colonized by genera found in the foreskin fold of the penis. These include, *Prevotella, Peptostreptococcus, Peptoniphilus, Porphyromonas, Campylobacter* and *Corynebacterium*\(^ {70}\). With the exception of Corynebacteri, this diverse set of anaerobes is associated with inflammation and increased STI risk in uncircumcised heterosexual men.\(^ {9,14,20}\)
2.5 Study Objective and Hypothesis

Previous research on the neovaginal microenvironment is limited; in all but one study the methods used to measure microbial data are limited and do not capture the majority of taxa present. To better understand the effect this microbiota has on inflammation and gynecological health in transfeminine individuals, it is crucial that we have a better understanding of the neovaginal immune milieu and how the microbiota and genital exposures modulate inflammation.

The objective of this study is to perform the first comprehensive description of the immune milieu of the surgically created neovagina, to better understand the interplay between immunology, the microbiota, and genital exposures (e.g., hygiene practices, behaviours, hormone therapy, and medications). This information will inform targeted treatment strategies and best practices to improve gynecological care for transfeminine individuals with vaginoplasty.

The overarching hypothesis of this thesis is that the penile-skin-lined neovagina will have unique bacterial communities with some similarities to the uncircumcised penile sub-preputial space. Additionally, a high abundance of anaerobes in this microenvironment will be associated with inflammation (Figure 5).

2.6 Individual Objectives and Hypotheses

**Objective 1:** Characterize the immune milieu of the transfeminine neovagina by performing hierarchical clustering on neovaginal immune analyte concentration data.

**Hypothesis:** The neovaginal immune milieu will fall into clusters defined by the expression of specific cytokines and will range from low inflammation to high inflammation.

**Objective 2:** Define the relationship between inflammation and bacterial communities present in the neovaginal microenvironment.
**Hypothesis:** The neovaginal microbiota will be more similar to the uncircumcised subpreputial penile microbiota than the vaginal microbiota. More diverse anaerobic bacterial communities will be associated with inflammation.

**Objective 3:** Explore the relationship between inflammation in the neovaginal microenvironment and exposures.

**Hypothesis:** Specific exposures such as unprotected sex, hygiene practices (e.g. douching), and probiotic use will alter the neovaginal microbiota and immune milieu.

**Figure 5. Thesis study conceptual model.** The genital microbiota influences inflammation in both the penile and vaginal microenvironment. Genital exposure such as penetrative sex, hygiene practices and hormone treatment may also modulate inflammation and the microbiota in the neovaginal microenvironment. The penile-tissue-lined neovagina presents a unique microenvironment that must be further studied to understand how these same microbial factors and exposures will affect inflammation in the neovagina.
Chapter 3

3 Methodology

3.1 Study Overview

This thesis examines data from the larger TransBiota study, a longitudinal study of both transfeminine and transmasculine individuals. The overarching goal of TransBiota is to gain a better understanding of how gender-affirming medical care influences the genital microenvironment.

Each participant in the TransBiota study provided swabs for microbial and immune analyses, and participated in a questionnaire, weekly for up to three weeks. TransBiota was a highly collaborative study, completed jointly between our group at Western University and colleagues at the University of Maryland. Jason Hallam helped design questionnaires and acquired Research Ethics Board approval. I managed sample collection and completed all immune analyses for both transfeminine and transmasculine samples. Bacterial characterization was completed by our collaborators at the University of Maryland for both transfeminine and transmasculine individuals.

All data (questionnaire, immune, and bacterial) was shared between investigators. Analysis was then divided between our groups, with the transmasculine analysis being completed at Dr. Ravel’s lab, largely by Bern Monari, and the transfeminine analysis being the subject of this thesis.

3.2 Clinical Study

3.2.1 Study Population

A total of 50 transfeminine individuals from across Canada are represented in this analysis. From these 50 transfeminine participants, we collected 147 samples for immune analysis and 138 samples for microbial analysis. Of the participants, 47 provided 3 weeks’ worth of samples, and 3 provided 2 weeks’ worth of samples. All
participants answered the weekly questionnaire when they provided their respective swabs.

### 3.2.2 Study Enrollment

Research ethics board approval was obtained from the University of Western Ontario prior to beginning the study. Recruitment occurred through collaborating clinicians and health care providers, through the community group TRANS+London, online via social media, and through recontact of consenting participants of the Trans PULSE Canada Survey. Interested potential participants were provided a link to an online screening questionnaire on a HIPAA-compliant REDCap secure server hosted at the University of Western Ontario.

Answers that deemed individuals ineligible for enrollment according to eligibility criteria (Table 1) led to termination of the screening questionnaire. Those that were eligible were led to the online Letter of Information where they could consent to participate in the study. The REDCap session was terminated for those who chose not to participate after reading through the Letter of Information. Participants that consented to participate in the study were prompted to enter their contact information (full name, email and mailing address).

### Table 1. Inclusion Criteria

1. Be living in Canada

2. Be 18 years of age or older

3. Identify as a gender that does not match their sex assigned at birth

4. Be able to complete the survey in English

5. Have undergone vaginoplasty surgery at least 12 months prior
3.2.3 Study Procedures and Sampling

Participants were mailed a study kit to the address they provided, which contained materials for at-home sampling (Table 2). The study kit was a cardboard box containing a booklet with illustrated self-sampling instructions, sample collection materials, and three postage-paid return envelopes. Participants were mailed the kit immediately after consenting and received an automatic email from the study’s University of Western Ontario-affiliated email address (tmistudy@uwo.ca) providing a link to the Week 1 Questionnaire one week after consenting to participate. The one-week allowed time to ensure that the participant received their study kit in the mail.

The participants were instructed to wait at least 12 hours after engaging in sexual activity and using vaginal products before completing study activities. The self-administered questionnaire captured detailed demographics (e.g., age and ethnicity), behaviours (e.g., sexual activity and hygiene practices), and gynecological symptoms experienced in the past year (e.g., malodour and discharge), focusing most on the past 7 and 30 days. At the beginning of the survey, participants were prompted to provide the language they prefer to use to refer their genitals so the survey could be tailored to each participant. After completing the Week 1 Questionnaire, participants were prompted to provide their biological sample using the materials in the study kit and following the illustrated instruction in the booklet provided.

Participants collected swabs for immune analysis, microbial analysis, pH estimate, and Gram staining. The swabs for immune and microbial analyses were collected and placed in sample tubes containing their respective validated collection media. Participants then collected the third swab to produce a smear on a glass slide for Gram staining, following the instructions provided. This same swab was then rolled on the pH strip, and participants documented the pH using the reference sheet provided. Gram staining of smears and information collected about pH will be used in future studies.
Both sample tubes, the glass slide and the pH reference sheet were then placed in the pre-paid envelope within a sealable plastic bag with an absorbent sheet. Participants were advised to deliver this envelope to a Canada Post mailbox within 24 hours of providing their sample. The samples were received and processed at the University of Western Ontario.

One week following the completion of the Week 1 Questionnaire and biological sample collection, participants received an email invitation containing a link to the Week 2 Questionnaire. The Week 2 Questionnaire asked participants about sexual activity, vaginal exposures, and gynecological symptoms experienced since completing the last questionnaire. Participants were then guided through the same vaginal swab and pH collection procedures as Week 1 mailed the swabs, glass slide, and pH reference sheet within 24 hours using the second pre-paid envelope. The week 3 data collection procedures were identical to those of week 2 data collection.

**Table 2. At home study kit contents**

1. 1 TransBiota Study instruction booklet
2. 10 swabs for genital sample collection
3. 3 bacterial sample tubes with orange caps
4. 3 immune sample tubes with grey caps
5. 3 glass slides in their cases
6. 3 pH strips
7. 3 pH colour reference sheets
8. 3 Ziploc bags with an absorbent sheet in each
9. 3 pre-paid envelopes
10. 1 pen for indicating pH on reference sheet
3.2.4 Study Design Limitations

This study employed a contact-free, self-sampling method to make the sample collection easier and more convenient for participants. However, self-sampling has limitations. Self-sampling brings into question the consistency of swab collection technique among participants. Each self-sampling kit gives clear instructions for how to self-swab but, some discrepancies are possible. Information about behaviours were self-reported and timelines were sometimes vague. For the purposes of this study, only behaviours that were reported in the week prior to sample collection were considered.

Sample shipping time can vary. The time between sample collection and being frozen at -80°C differed between samples. Samples may also be exposed to fluctuating temperatures during transport. Variability from storage time was mitigated by using collection media validated to preserve genetic material or a key cytokine (for bacterial and immunological analysis, respectively) for up to 2 weeks at room temperature.

It should also be noted that participants in this study may have a vested interest in the outcome. Individuals experiencing gynecological health concerns may have been more likely to sign up for the study. It therefore should be clearly stated that the participants in this study are not necessarily representative of the transfeminine people living in Canada as a whole.

3.3 Neovaginal Immune Milieu Characterization

3.3.1 Immune Swab Processing

Swabs for immune analyses were collected in 500ul of Immune Stabilization Media, a solution designed and validated by our group to stabilize immune analytes at room temperature for up to 14 days (Appendix 2).

The immune samples were received in Dr. Jessica Prodger’s lab and immediately aliquoted into 2 x 50ul aliquots and 1 x 120ul aliquot in low protein binding 1.5ml
cryovials, for a total of 4 aliquots including the original collection tube. Immune aliquots were then stored in a freezer at -80°C.

### 3.3.2 Immune Analyte Pre-Testing

Neovaginal immune swabs were collected in 500μl of phosphate buffered saline (PBS) mixed with a protease inhibitor (Complete Mini Protease Inhibitor Cocktail) and 10% bovine serum albumin (BSA). This solution was filtered, aliquoted, and stored at -80°C prior to being shipped to participants (at room temperature).

Twenty-six immune analytes were selected for pre-testing using microsphere- and antibody-based immunoassay techniques (Luminex and ELISA, respectively). Immune analytes were selected for:

1. Their previously published associations with bacterial dysbiosis, inflammation and HIV susceptibility in the vaginal and penile microenvironments (IL-1α, IL-1β, IL-6, IL-8, IL-13, IL-17A, IL-17E, IL-17F)\(^9,10,71–76\).
2. Associations with decreased epithelial integrity and epithelial remodeling (soluble E-cadherin and MMP-9)\(^77–82\).
3. Antimicrobial peptides (AMP) with bactericidal/viricidal activity and that are regulated by sex-hormones (Secretory Leukocyte Peptidase Inhibitor (SLPI), human β defensin 2 (HBD2), elafin, human neutrophil peptide (HNP)1–3)\(^83–85\).
4. Association with chronic and/or pathologic inflammation (IFN γ and TNF α)\(^26\).
5. Association with immune modulation / anti-inflammatory activity (IL-10)\(^86\).
6. Associated with epithelial modulation (IL-13, IL-22, GM-CSF)\(^87,88\).
7. Have chemotactic activity (IP-10, MIG, MIP-1α, MIP-1β, RANTES)\(^89\).

Eight samples were selected for pre-testing to ensure each analyte of interest was present at high enough concentrations to be detectable using the available kits: n=4
transfeminine and n=4 transmasculine genital immune swabs (neovaginal and vaginal, respectively). Analytes that were not detected robustly (i.e., majority of samples were below the standard 1) were excluded from further analysis (described in the Results section). Some immune analytes were also not included in the final analysis due to logistical barriers (i.e., cost, sample amount required for analysis). Of the twenty-six immune analytes chosen for pre-screening, nineteen of them were chosen for the analysis (Table 3).

Table 3. Immune Analytes

<table>
<thead>
<tr>
<th>GM-CSF</th>
<th>IP-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNG</td>
<td>IL-17A</td>
</tr>
<tr>
<td>IFNα2</td>
<td>IL-17E</td>
</tr>
<tr>
<td>IL-1α</td>
<td>IL-17F</td>
</tr>
<tr>
<td>IL-1β</td>
<td>MIG</td>
</tr>
<tr>
<td>IL-6</td>
<td>MIP-1α</td>
</tr>
<tr>
<td>IL-8</td>
<td>MIP-1β</td>
</tr>
<tr>
<td>IL-10</td>
<td>RANTES</td>
</tr>
<tr>
<td>IL-13</td>
<td>TNFA</td>
</tr>
<tr>
<td>IL-22</td>
<td></td>
</tr>
</tbody>
</table>

3.3.3 Immune Analyte Measurement

The concentrations of immune analytes were measured using a multiplex immunoassay (Millipore Sigma Multiplex Panel A kits measured on a Luminex MAGPIX system). Undiluted samples were plated at 25ul per well with internally color-coded magnetic microspheres each of which was coated with a specific capture antibody. Samples were left to incubate at 4°C for 16-18 hours. The plate was then washed 3 times with an automated plate washer (BioTek 405 TS washer) and a
biotinylated detection antibody was introduced and left to incubate for 1 hour. The reaction mixture was then incubated with Streptavidin-PE conjugate, the reporter molecule, for 30 minutes. Once the reaction was complete, the plate was washed while resting on a magnet to retain the magnetic microspheres inside the wells. The wells were then filled with 200ul of buffer solution and placed in the Luminex MAGPIX machine to be read. Each individual microsphere was identified, and the result of its bioassay was quantified based on fluorescent reporter signals.

3.4 Neovaginal Microbiota Characterization

3.4.1 Bacterial Swab Storage

Swabs for bacterial analysis were self-collected into 1ml of ZYMO DNA/RNA Shield, a validated nucleic acid preservative that affords stabilization of both DNA and RNA molecules at room temperature for several weeks. The samples for bacterial analysis were shipped at room temperature to Dr. Ravel’s lab for 16S rRNA gene sequencing.

3.4.2 Bacterial Swab Processing

All bacterial processing was completed by our collaborators at the University of Maryland, School of Medicine, Baltimore, USA according to the protocol described in Holm et al. 2019.

3.5 Neovaginal Exposures and Behaviors

3.5.1 Survey Data Collection

Once a week for three weeks, when participants took their genital swabs, they also completed an online questionnaire, as previously noted. Participants were asked questions regarding surgical details of their vaginoplasty, behaviors, and hygiene practices. Surgical variables included time of vaginoplasty, surgeon or surgical center, and tissue type used. Behavioral variables included hormone therapy (estrogen, progestin, antiandrogens and other), penetrative sexual activity, lubricant use, and medications or treatments. Participants were asked whether they had used
probiotics, antibiotics, steroid medications, or other prescription medications each week, and the administration route (oral vs. topical on the genitals). Hygiene practices included douching frequency, douche solution (e.g., water, saline), dilating frequency, exterior wash frequency and other hygiene practices (e.g., using exterior wipes).

3.6 Data Analysis

All statistical analyses were performed using R Statistical Software (v4.2.1; R Core Team 2022).91

3.6.1 Immune Data

3.6.1.1 Immune Data Imputation and Data Cleaning

The lower limit of quantification (LLoQ) was assigned for each analyte based on the assessment of standard curves using Belysa Immunoassay Curve Fitting Software92, which defines the LLoQ as the lowest concentration to satisfy three conditions:

a. The back calculated analyte concentration CV is less than or equal to 20%.
b. The recovery rate of back-calculated analyte concentration is between 80-120%.
c. All the standards above the LLoQ also satisfy conditions (a) and (b).

All samples that fell below the LLoQ were assigned a concentration of 0pg/ml for the purposes of analysis. If the majority of samples (>85%) fell below the determined LLoQ for a particular analyte, that analyte was removed from the final analysis (Table 4).

Analytes with >85% of samples above the LLoQ, but with (a) the majority of detectable samples very close in concentration to the LLoQ, and (b) poor CV (>20%) values for samples close to the LLoQ, were also assigned a lower limit of detection (LLoD). Samples above the LLoD (based on standard curves), but below the LLoQ (based on measurement replicability) were deemed detectable, but not quantifiable.
Samples that fell below the LLoD were assigned a value of 0pg/ml and samples that fell between the LLoD and the LLoQ were assigned the value halfway between the LLoD and the LLoQ.

Table 4. Immune Analyte LLoDs and LLoQs

<table>
<thead>
<tr>
<th>Immune Analyte</th>
<th>LLoD</th>
<th>LLoQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>IFNα2</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.6</td>
<td>8</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>IL-17A</td>
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</tr>
<tr>
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<td>IP-10</td>
<td>2.6</td>
<td>12.8</td>
</tr>
<tr>
<td>MIG</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>MIP-1β</td>
<td>1.9</td>
<td>9.6</td>
</tr>
<tr>
<td>RANTES</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>6.4</td>
<td></td>
</tr>
</tbody>
</table>
3.6.1.2 Immune Data Normalization

To account for variability in the active concentrations of each immune analyte, the immune data was transformed and normalized prior to analysis. Each concentration value was log10-transformed. Prior to log10 transformation, 1 was added to each value to avoid calculating the log of 0. Each value was then divided by the maximum concentration value observed for that immune analyte. The resulting log10 transformed and normalized concentration values were then multiplied by 100 to be expressed on a relative scale of 0-100.

3.6.1.3 Immune Data Dimension Reduction and Clustering

PHATE (Potential of Heat Diffusion for Affinity-based Trajectory Embedding)\(^3\) was used to reduce immune data dimensionality for visualization, interpretation and further exploration into possible clusters that exist within the dataset. PHATE was chosen over other more commonly used methods (e.g., PCA, k medoids), which did not capture the complexity of the dataset. PHATE preserves proregression structure in biological datasets resulting from biological processes (e.g., differentiation, signalling cascades), which was desirable for this dataset as we wanted to try and visualize the underlying cytokine signaling cascades occurring in the neovaginal microenvironment.

The basic steps of PHATE are as follows:

1. Input immune data matrix.
2. Compute the pairwise distances from the data matrix.
3. Transform the distances to affinities to encode local information.
4. Learn global relationships via the diffusion process.
5. Encode the learned relationships using the potential distance.
6. Embed the potential distance information into low dimensions for visualization\(^4\).

PHATE dimension reduction analysis was performed using the R package phateR v.1.0.7\(^4\) with the following parameters:
A three-dimensional embedding of the immune data was produced with each sample being assigned a 3D coordinate (Figure 6). Clustering was performed using hDBSCAN (within the R dbscan package\textsuperscript{95}, a hierarchical K nearest neighbors algorithm that scans the 3D embedding to determine clusters that exist within it. The minimum number of points that could be within a cluster was assigned to 8\textsuperscript{96}. This produced seven clusters and a zero group – points that do not fall into any specified cluster but are not a cluster in-and of-themselves. Soft clustering was then performed to assign the points within the zero group to a cluster.

Soft clustering is an algorithm (written by our collaborator Dr. Pawel Gajer) for post-processing of hDBSCAN output data that assigns samples within the zero cluster to one of the constructed hDBCSAN clusters. The assignment is based on the majority vote clustering using the K nearest neighbors of each sample and assigns x of cluster 0 to the cluster with the highest frequency of nearest neighbors among the constructed clusters.

### 3.6.2 Bacterial Data

#### 3.6.2.1 Combined Microbial and Immune Embedding and Clustering

PHATE was used to reduce microbial data dimensionality using the R package phateR v.1.0.7\textsuperscript{94} with the following arguments:

- ndim = 3 (the number of dimensions in which the data will be embedded)
- knn = 5 (number of nearest neighbors on which to build the embedding)
d. `knn.dist.method = “cosine”` (denotes the distance metric used to build the kNN graph)
e. `gamma = 0` (informational distance constant between -1 and 1 with 0 giving a square root potential)
f. `t = “auto”` (sets the level of diffusion)

A three-dimensional embedding of the microbial data was produced with each sample being assigned a 3D coordinate. The microbial embedding and immune embedding (described in section 3.6.1.3) were scaled to the same size using the function `loc.scale()` (written by our collaborator, Dr. Pawel Gajer). This function first computes the slopes of the K nearest neighbors of both the microbial and immune embeddings. The mode of these slopes is then calculated. The microbial embedding is then divided by the mode prior to the two embeddings being combined. The resulting combined dataset is run through PHATE to produce a 3D embedding of both the microbial and immune data.

As described in section 3.6.1.3, clustering was performed using hDBSCAN (within the R `dbscan` package), followed by the soft clustering algorithm, to determine if and how many biologically relevant clusters exist within the data.

### 3.6.2.2 Bayesian Regression Spline Modelling of Microbial Data

To explore associations between individual bacterial taxa and IPTs, the dependence of the probability of being in a certain IPT on the relative abundance of a specific bacterial taxa was analyzed using a Bayesian logistic regression nonparametric adaptive spline model.

Bayesian spline regression was performed with `rstan`, the R interface to Stan, using the function `spmrf.bernoulli.o2.comb.fn()` written by Dr. Pawel Gajer and previously validated for modeling microbial data.

Each IPT was coded as a binary outcome while the log10 relative abundance of each bacterial taxa was a continuous independent variable. Spline models were run for each IPT for the 89 most abundant taxa. Taxa were run at the lowest possible taxonomic level that was identifiable.
3.6.3 Behavioural Data

3.6.3.1 Behavioural Clusters and Inflammation

Behaviours and genital exposures do not occur in isolation so Jason Hallarn, a member of the TransBiota study team, performed hierarchical agglomerative clustering to develop unique behavioural clusters. Clustering was done on only the baseline questionnaire data, and included douching frequency, douche type, dilation frequency, penetrative sex, lubricant use, hygienic practices, and medications or treatments. Clustering was performed using the hcut function from the factoextra package\textsuperscript{100} with the following argument applied:

a. \texttt{hc\_func = “agnes”} (the hierarchical clustering function to be used)

b. \texttt{Hc\_method = “ward.D”} (the agglomeration method to be used)

c. \texttt{Hc\_metric = “Euclidean”} (the dissimilarity metric)

Four distinct clusters were identified, and the concentrations of each of the 13 cytokines were compared between each cluster. The Kruskal-Wallis test was used to determine significant differences in cytokine concentrations between each behavioural cluster.
Chapter 4

4 The Neovaginal Immune Milieu

4.1 Results: Immunology

Immune data consisted of 147 samples from 50 transfeminine individuals. Following clustering with hDBCSAN and soft clustering, discussed previously, seven total clusters were found (Figure 6) and of these seven clusters, we deduced there were 6 unique immune profiles which we called Immune Profile Types (IPTs). IPT1a/b (n=22) from clusters 5 and 6; IPT2 (n=13) from cluster 4; IPT3 (n=34) from cluster 3; IPT4 (n=35) from cluster 7; IPT5 (n=27) from cluster 1; and IPT6 (n=16) from cluster 2.

It should be noted that IL-22 displayed a near all-or-nothing expression, yielding individuals with relative expression of either 0 or 100, which strongly dictated clustering. To reduce the impact of IL-22 on overall clustering, IL-22’s maximum relative expression was set to 50, reducing the spread between people who did and did not have IL-22 and thus its impact on clustering.

Concentrations of all 13 cytokines/chemokines tended to trend upward moving from IPT-1 to IPT-6. An exception to this trend is the concentration of IL-1α which is expressed at a similar concentration across all IPTs (Figure 7).

IPT1a/b and IPT2 are characterized by relatively low concentrations of all cytokines/chemokines and make up the low inflammatory IPTs, accounting for 22 of samples and 15 individuals in the population. Individuals in IPT1a/b expressed IL-8 (48%; IQR 42.15%-5.27%), MIG (20.00%; IQR 13.18-23-13). IPT-2 has a similar profile to IPT1a/b with a slight increase in the concentration of IL-1β (27.24; IQR 20.49-34.57). IL-1β was only expressed in one sample in IPT1a/b)

IPT3 and IPT4 make up the middle inflammatory IPTs with n=69 samples representing n = 45 individuals. IPT3 and IPT 4 have a notable increase of IL-6, IL-8, RANTES and TNFα, as compared to the low inflammatory groups [(15.26%; IQR
However, IPT3 and IPT4 are distinct from one another: IPT3 has higher levels of IL-1β [(50.87%; IQR 36.40%-60.80%) vs. (17.75%; IQR 17.75%-28.12%)], while IP4 has higher levels of the chemotactic cytokine MIG [(46.24%; IQR 37.11%-57.92%) vs. 20.63%; IQR 16.49%-25.86%)].

IPT5 and IPT6 are the high inflammatory profiles, with the highest relative concentration of immune analytes, and account for n = 43 samples representing n = 23 individuals. These IPTs are defined by the presence of IFNγ (68.15%; IQR 62.47%-78.17%), which is absent in the low and middle inflammatory IPTs. There is also a notable increase in IL-1β (77.53%; IQR 65.44%-85.41%) and IL-8 (88.14%; IQR 82.74-97.07) across most samples in IPT 5 and 6. Although individuals in both IPT5 and 6 express similar levels of inflammatory cytokines, there are a few key differences between these two IPTs. IPT5 is unique in its abundance of IL-22, which was detected in nearly all samples within this IPT5 (21/27), but only one in IPT6. Conversely, IPT6 had higher concentrations of cytokines IL-6, IL-10 and TNFα [(72.25%; IQR 49.21%-82.77%), (79.26%; IQR 67.75%-90.01%), and (54.93%; IQR 47.93%-74.78%), respectively], as well as chemokines IP-10, MIG, MIP1α, MIP1β and RANTES [(43.63; IQR 43.63-90.15), (79.69%; IQR 57.58-89.63), (79.25%; IQR 62.71%-88.95%), (71.91%; IQR 62.03%-91.88%), and (79.00%; IQR 49.90%-90.22%), respectively].
Figure 6. Visualization of the 3D PHATE embedding of neovaginal immune analyte data Using the K nearest neighbors hDBSCAN 7 clusters were found within the 3D embedding. 6 unique IPTs were found within the 7 clusters: IPT1a/b, IPT2, IPT3, IPT4, IPT5 and IPT6. Figure generated with R package rgl().
Figure 7. Heatmap representing immune profile types (IPTs) within the neovaginal immune milieu. Each sample is represented by a single row (n = 147). The colour key indicates the relative concentration of each immune analyte (columns). The orange bars at the top of the heatmap represent the inflammatory group each IPT is associated with (i.e., low inflammation: IPT1a/b and IPT2; middle inflammation: IPT3 and IPT4; and high inflammation: IPT5 and IPT6). Generated with R package heatmap2().

4.2 Discussion: Immunology

Patterns of cytokine expression within the IPTs potentially highlight defined immune responses in the neovaginal microenvironment. Cytokines regulate a host of immune responses throughout the body and a proinflammatory response is crucial for effective clearance of pathogens. However, chronic high levels of pro-inflammatory mediators can cause tissue damage and pathologic inflammation103,104. Numerous mechanisms of genital tract inflammation have been elucidated in the vagina; however, we must consider the fact that most neovaginas are lined with penile tissue. As previously discussed, there are key differences between the vaginal and penile, or skin, epithelia. For this reason, it is important to consider the roles these cytokines are known to play in skin inflammation.

We observed a general ordinal increase of proinflammatory cytokine expression from IPT1a/b to IPT6 with a few exceptions. IL-1α expression was relatively similar across all IPTs. This may be attributed to the fact that IL-1α precursors are constitutively expressed by epithelial cells. IL-1α has profound effects on epidermal differentiation and can strengthen the epidermal barrier by influencing the mechanical attachment of cells and formation of the lipid envelope88. Expression must be tightly controlled, however, to avoid dysregulation and potential pathogenesis. Overexpression of the IL-1α precursor in keratinocytes has distinct consequences on the epithelial barrier and can promote an inflammatory skin phenotype by attracting other inflammatory cells18,87,104,105. As the levels of IL-1α
are similar across all IPTs, it is difficult to know if this level is normal or indicative of a dysregulated IL-1α response.

IL-1β which gradually increased between the low to high inflammation IPTs, like IL-1α, also plays a role in epidermal differentiation and might be highly relevant to mechanical injury of the skin and wound repair. IL-1β along with IL-1α was upregulated immediately after physical disruption of endocervical epithelial cells in an in vitro monolayer\textsuperscript{106,107}. IL-1β can also promote the infiltration of inflammatory cells from circulation into tissues resulting in chronic inflammation\textsuperscript{104}.

IL-8 also increases across the IPTs. IL-8 is a potent chemotactic cytokine associated with increased density of HIV-susceptible immune cells and increased risk of seroconversion in both the penile and vaginal microenvironment. IL-8 is also upregulated in numerous proinflammatory skin diseases like psoriasis\textsuperscript{14,108}.

IFNγ and TNFα – prototypic effector cytokines of the proinflammatory Th1 immune response – are expressed almost exclusively in the high inflammation IPTs. However, IPT6 is distinct in its elevated levels of IL-6 as well as the chemotactic cytokines MIG, MIP1α, MIP1β, IP-10 and RANTES.

Proinflammatory cytokine IL-6 has been shown to play a role in wound healing and in promoting keratinocyte proliferation and localizes to epidermal layers after barrier disruption. It also correlates with the severity of certain inflammatory skin conditions such as psoriasis and atopic dermatitis, potentially in relation to the epithelial barrier disruption associated with these diseases\textsuperscript{87,106}.

Chemotactic cytokines possess the ability to recruit distinct immune cell subsets and perform numerous functions in inflammatory disease. For example, upregulation of IP-10, MIG, MIP-1α, and RANTES signalling pathways is observed in skin affected by rosacea. Upregulation of MIG and IP-10 may also induce a positive feedback loop, where activation of Th1 immune cells leads to upregulation of IFNγ and TNFα production, which in turn further stimulate production of MIG and IP-10\textsuperscript{109}.
IL-10 is also expressed most strongly in IPT6. IL-10 is a potent anti-inflammatory cytokine and can augment the production of Th1 cytokines. Studies have found that IL-10 may inhibit proinflammatory cytokine synthesis and proliferation of keratinocytes. It has also been reported to assist in epithelial wound repair. Macrophage derived IL-10 was shown to activate signalling cascades that induce epithelial cell proliferation and wound closure.86,103,110

IL-22, which is almost exclusively expressed within IPT5, has a profound effect on epithelial differentiation and influences epithelial barrier function. IL-22 influences the expression of proteins involved in filaggrin processing and desquamation and, therefore, modulates key processes involved in maintenance of the stratum corneum. Although not proinflammatory, IL-22 may play a role in the pathogenesis of inflammatory skin conditions like psoriasis.87

The various functions described above highlight the potential roles these cytokines may be playing in the neovaginal microenvironment. Our next steps were to elucidate what factors in the neovaginal microenvironment may induce or modulate inflammation.
Chapter 5

5 The Neovaginal Microbiota: Implications on Inflammation

As previously discussed, the vaginal and penile microbiota play critical roles in modulating inflammation in their respective microenvironments. BV- and HIV-associated bacteria (e.g., *Gardnerella* and *Atopobium*) are associated with lymphocyte recruitment and proinflammatory cytokines release including IL-1α, IL-1β, TNFα and IFNγ. These cytokines can act to damage epithelial barrier integrity through disruption of tight junctions, increasing epithelial permeability. These dual actions are the proposed mechanisms by which the genital microbiota can increase an individual’s susceptibility to acquiring HIV. The next step was, therefore, to elucidate the neovaginal microbiota and determine if specific bacterial communities or species may be playing a role in inducing neovaginal inflammation.

5.1 Results: Microbiota and Immunology

5.1.1 Describing the Neovaginal Microbiota

16s rRNA gene sequencing of neovaginal swabs identified a total of 187 unique bacterial taxa belonging to 96 genera. On average, 37 unique bacterial taxa were found within each neovaginal sample (range 10-57). Not all sequences could be assigned taxonomy at the species level, so for the majority of analyses the dataset was collapsed to the genus level. The most abundant genera among the neovaginal samples were: *Prevotella* (18.85%; IQR 11.70%-26.94%), *Peptoniphilus* (10.38%; IQR 6.52%-14.08%), *Ezakiella* (6.14%; IQR 2.26%-12.42%), *Porphyromonas* (5.91%; IQR 1.65%-12.61%), *Fusobacterium* (0.99%; IQR 0.018% - 7.78%), *Anaerococcus* (3.00%; IQR 0.73%-6.20%), *Lactobacillus* (0.074%; IQR 0.00%-0.37%), *Dialister* (3.23%; IQR 1.69%-4.96%), *Finegoldia* (0.83%; IQR 0.11%-3.12%), *Varibaculum* (1.06%; IQR 0.16%-5.12%), *Streptococcus* (0.34%; IQR 0.015%-2.50%), *Campylobacter* (1.10%; IQR 0.54%-2.47%), *Parvimonas* (0.36%; IQR 0.00%-2.56%), *Murdochella* (0.39%; IQR 0.031%-1.94%), *Corynebacterium* (0.022%; IQR 0.00%-0.57%), *Lawsonella* (0.33%; IQR 0.082%-0.75%), *Mobiluncus* (0.42%; IQR 0.050%-1.43%), and *Atopobium* (0.26%; IQR 0.047%-1.66%). These
top 18 most abundant genera made up 81.50% of the total bacterial composition of neovaginal samples. 14 of these 17 genera were anaerobic, 2 were facultatively anaerobic and 1 was microaerophilic (Figure 8) (Table 5).
Figure 8. The neovaginal microbiota. Bar graph ordered by increasing abundance of Prevotella. Each vertical bar represents 1 of 138 microbial samples from 50 transfeminine individuals. Figure generated with R package ggplot2()\(^\text{114}\).

Table 5. The 18 most abundant bacterial genera in the neovagina.

<table>
<thead>
<tr>
<th>Oxygen Tolerance</th>
<th>Taxa</th>
<th>Median Relative Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>Prevotella</td>
<td>18.85%</td>
</tr>
<tr>
<td>AN</td>
<td>Peptoniphilus</td>
<td>10.38%</td>
</tr>
<tr>
<td>AN</td>
<td>Ezakiella</td>
<td>6.14%</td>
</tr>
<tr>
<td>AN</td>
<td>Porphyromonas</td>
<td>5.91%</td>
</tr>
<tr>
<td>AN</td>
<td>Fusobacterium</td>
<td>0.99%</td>
</tr>
<tr>
<td>AN</td>
<td>Anaerococcus</td>
<td>3.00%</td>
</tr>
<tr>
<td>FAN/AN/MAE</td>
<td>Lactobacillus</td>
<td>0.074%</td>
</tr>
<tr>
<td>AN</td>
<td>Dialister</td>
<td>3.23%</td>
</tr>
<tr>
<td>AN</td>
<td>Finegoldia</td>
<td>0.83%</td>
</tr>
<tr>
<td>FAN</td>
<td>Varibaculum</td>
<td>1.06%</td>
</tr>
<tr>
<td>FAN</td>
<td>Streptococcus</td>
<td>0.34%</td>
</tr>
<tr>
<td>MAE</td>
<td>Campylobacter</td>
<td>1.10%</td>
</tr>
<tr>
<td>AN</td>
<td>Parvimonas</td>
<td>0.36%</td>
</tr>
<tr>
<td>AN</td>
<td>Murdochiella</td>
<td>0.39%</td>
</tr>
<tr>
<td>AE</td>
<td>Corynebacterium</td>
<td>0.022%</td>
</tr>
<tr>
<td>AN</td>
<td>Lawsonella</td>
<td>0.33%</td>
</tr>
<tr>
<td>AN</td>
<td>Mobiluncus</td>
<td>0.42%</td>
</tr>
<tr>
<td>AN</td>
<td>Atopobium</td>
<td>0.26%</td>
</tr>
</tbody>
</table>

AN – Anaerobic
FAN – Facultative anaerobic
MAE – Microaerophilic
AE – Aerobic

5.1.2 Associations between the Neovaginal Microbiota and Immune Profile Types

A simple visualization of the data (sorting bacterial abundance bar plots by IPT) was produced to see if there were any obvious associations between bacterial community composition and IPTs. However, the bacterial communities within each IPT were diverse and largely indistinguishable from one another (Figure 9).
Relative Abundance
Figure 9. The neovaginal microbiota sorted by Immune Profile Types (IPTs). Each vertical bar represents 1 of 138 microbial samples from 50 transfeminine individuals. Each sample is assigned to its corresponding IPT. IPTs were generated with hDBSAN following embedding with PHATE using the dbscan() and phateR() packages respectively. Figure generated with R package ggplot2().

5.1.3 Combined Microbial and Immune Clusters

We postulated that less abundant taxa might be impacting inflammation, so more sensitive analyses were warranted.

Six clusters were found within the combined immune and microbial embedding (Figure 10). The immune data heavily influenced the clustering. For example, cluster 2 within the combined embedding contained the same samples as IPT6. The microbial communities within each cluster were diverse and did not display any distinct patterns within the clusters.

Although no specific bacterial communities clustered strongly with immune analytes, there were several specific bacterial genera that did appear to associate with certain clusters. *Lawsonella* was present in clusters 1 and 2 (high inflammation) and did not associate strongly with clusters 3-6 (low and medium inflammatory). *Bacteroides* was also largely absent from cluster 2, present only in three samples from two individuals. *Ezakiella* was also largely absent from cluster 2 but was present in all other clusters. *Escherichia shigella* was present in only a few samples, all of which were in clusters 4 and 5, which had the lowest inflammatory profiles.
Figure 10. Heatmap of combined immune and microbial PHATE embedding sorted by clusters. Each column represents 1 of 138 microbial samples from 50 transfeminine individuals. Each sample is assigned to a cluster. Clusters were generated with hDBSCAN following embedding with PHATE using the dbscan() and phateR() packages, respectively. Figure generated with R heatmap2().

5.1.4 Associations between Microbial Taxa and Immune Profile Types

To explore associations between individual bacterial taxa and IPTs, the dependence of the probability of being in a certain IPT on the relative abundance of a specific bacterial taxa was analyzed using a Bayesian logistic regression nonparametric adaptive spline model.

Of the top 89 most abundant taxa, 14 models were significant. Relative abundance of *Atopobium deltae*, *Prevotella buccalis* and *Prevotella oris* were all positively associated with the probability of being in IPT6. Conversely, the relative abundance of *Corynebacterium* was associated with a decreased probability of being in IPT6 (Figure 11a).

Higher relative abundance of *Streptococcus*, *Prevotella timonensis*, and *Poryphyromon* were associated with a decreased probability of being in IPT5, while the relative abundance of *Finegoldia* was positively associated with the probability of being in IPT5 (Figure 11b).

Higher relative abundance of *Campylobacter* was strongly associated with an increased probability of being in IPT4 (Figure 11c), while higher relative abundance of both *Gardnerella vaginalis* and *Prevotella disiens* were associated with a decreased probability of being in IPT3 (Figure 11d).

Finally, the relative abundance of both *Actinotignum* and *Negativicoccus* was negatively associated with the probability of being in IPT1a. There was a positive association between relative abundance of *Varibaculum* and the probability of being in IPT1b (Figure 11e).
a.
b.
c.

![Graph showing log10 Relative Abundance, C. Campylobacter vs. p(r3|4)].

d.

![Graph showing log10 Relative Abundance, G. Vaginalis vs. p(r3)].

![Graph showing log10 Relative Abundance, P. disiens vs. p(r3)].
e.
**Figure 11. Bayesian Splines Models.** The blue regression line represents the dependence of the probability of being in IPT6 on the log10 relative abundance of a bacterial taxa in all subjects. The grey area indicates the 95% credible region. The red line indicates the effect size which is the difference between the lowest and highest probability of being in a the specified IPT. Black tick marks along the x-axis represent all samples with a non-zero relative abundance and red tick marks represent samples that are within the IPT. **a.** Probability of being in IPT6 on the log10 relative abundance of *Atopobium deltae, Prevotella buccalis, Prevotella oris* (positive association) and *Corynebacterium* (negative association). **b.** Probability of being in IPT5 on the log10 relative abundance of *Prevotella timonensis and Porphyromonas* (negative association), and *Finegoldia* (slight positive association) and *Streptococcus* (positive association). **c.** Probability of being in IPT4 on the log10 relative abundance of *Campylobacter* (negative association). **d.** Probability of being in IPT3 on the log10 relative abundance of *Gardnerella vaginalis and Prevotella disiens* (negative association). **e.** Probability of being in IPT1a on the log10 relative abundance of *Actinotignum and Negativococcus* (negative association), and the probability of being in IPT1b on the log10 relative abundance *Varibaculum* (slight positive association).
5.2 Discussion: Implications of the Neovaginal Microbiota on Inflammation

The overall composition of the neovaginal microbiota is vastly different from the vaginal microbiota. *Lactobacilli* in our study was the seventh most abundant taxa present, making up 4.56% of the total bacterial abundance within the sample set. This was heavily influenced by samples from two individuals who were taking probiotics containing *Lactobacillus* (discussed in further detail in Chapter 6). After removing samples from these two individuals, the total bacterial abundance of *Lactobacillus* in the sample set dropped to 1.99%. This is in contrast to the vaginal microbiota of reproductive age females, where most individuals have near complete dominance by one *Lactobacillus spp.*\(^{22}\).

Instead, the composition of the neovaginal microbiota is more similar to that of the uncircumcised penis, sharing many of the same most abundant genera (i.e., *Prevotella, Finegoldia, Peptoniphilus, Anaerococcus, Dialister*, and *Streptococcus*)\(^{14,17,18,20}\). The neovaginal microbiota also has a notably low relative abundance of *Gardnerella*, the prototypic BV-associated bacteria in the vagina. *Gardnerella* was not present in the top 42 most abundant taxa and was present at a mean relative abundance of only 0.79% among all the samples. This is interesting to note, as many transfeminine individuals report BV-like symptoms, suggesting *Gardnerella* is not likely the underlying cause of these symptoms\(^{13}\).

The neovaginal microbiota is very diverse. On average, 37 (range: 10-57) genera of bacteria were present in each neovaginal sample. Although there were no obvious associations between IPTs and bacterial communities, there were a number of specific bacterial taxa associated with IPTs. It should be noted however, that these results must not be overinterpreted. A total of 623 models were run; At alpha=0.05, we would expect to have 32 statistically significant models if there were no association between IPT and log10 abundance of each taxon. Therefore, the frequency of statistically significant models is below the overall significance threshold.
Neovaginal microbial composition data was not sufficient to explain the distinct patterns of inflammation that are present within the neovaginal immune milieu and there were not enough statistically significant regression models to suggest an overall significant relationship between inflammation and bacterial taxa. It may be that there are other microbial factors at play (i.e., yeast/fungal infection) or the underlying cause of inflammation is an amalgam of different factors.
Chapter 6

6 Inflammation and Reported Genital Exposures in the Neovagina of Transfeminine Individuals

As bacterial data did not account for the distinct immune profiles that were present in the data, we next looked at reported behaviours and genital exposures to see if they were associating with heightened inflammation.

Behaviours and genital exposures of interest were those previously associated with inflammation in the vagina (penetrative sex with flesh genitals and douching), and medications which could potentially modulate microbiota and inflammation (probiotics, antibiotics, hormones and prescription medications)\(^{26,116}\).

6.1 Results: Behavioural Data

All but two of our participants reported having had their vaginoplasty done in Canada. Of the two participants who did not report having their surgeries in Canada, one reported having had their vaginoplasty in Thailand but specified that the operation had used scrotal tissue which is consistent with the penile inversion vaginoplasty technique. The other participant did not report any information regarding their vaginoplasty or where it was performed.

6.1.1 Behavioural Clusters and Inflammation

Four distinct behavioral clusters were previously identified by Jason Hallarn (Epidemiology and Biostatistics PhD Candidate) with agglomerative clustering. Cluster 1 (n=19) characterized by no douching but relatively high frequencies of dilation and penetrative sex; Cluster 2 (n =13) defined by limited exposures; Cluster 3 (n=7) characterized by frequent douching with water and frequent dilation; and Cluster 4 (n=11), characterized by diverse behaviors and exposures, including douching with solutions other than water, penetrative sex, and probiotic use.

The concentrations of each of the 13 cytokines were compared between clusters. A Kruskal-Wallis test was used to determined significance. No significant differences in cytokine concentration were found among the behavioural clusters (Figure 12).
Figure 12. Box plots of log10 cytokine concentrations (pg/ml) compared between behavioural clusters. The concentration of all 13 cytokines were compared between behavioural clusters (p-value of Kruskal-Wallis test in bottom left corner of each box plot). There were no significant differences in concentration between behavioural clusters for any of the cytokines.
6.1.2 Within-Participant Exploratory Analysis

Because behavioural practices were diverse among participants and the sample size was relatively small, a descriptive, hypothesis generating analysis of each participant was performed. Figures 13-34 display bacterial bar plots and immune heatmaps of each individual participant’s weekly neovaginal swabs. Bar plots were generated using ggplot2()\textsuperscript{114} and heatmaps were generated using heatmap2\textsuperscript{102}.

6.1.2.1 Probiotics

Four participants reported taking a probiotic in the week proceeding at least one of their sample collections.
A1.

A2.
Figure 13. Participant A (Panels A1 and BA2) reported taking an oral probiotic (Natural Factors) the day of the second swab collection and indicated they were taking it daily. The specific probiotic product is unknown; however the majority of Natural Factors’ products contain species of *Lactobacillus* or *Bifidobacterium*, or a combination of the two. There was a slight reduction in inflammation seen in the week 2 immune profile compared to week 1 and week 3. This aligned with an obvious shift in the microbiota at week 2. *Lawsonella* and *Bacteroides* are in high abundance at both week 1 and week 3 but were almost completely reduced at week 2 when the probiotic was taken. Although there was a slight reduction in inflammation at week 2 all samples fell within IPT6.
Figure 14. Participant B (Panels B1 and B2) reported taking a daily oral probiotic (Health Balance complete) everyday at each week. This specific probiotic is made up of six different species of *Lactobacillus* and two different species of *Bifidobacterium*. Full *Lactobacillus* dominance was present in each microbial sample. The most abundant species in week 1 was *Lactobacillus reuteri*, a species present in the probiotic itself. In weeks two and three *Lactobacillus gasseri* is the most abundant bacterial species and is not noted in the probiotic ingredient list. The type of vaginoplasty this person underwent was not reported and nor was the surgeon who performed it. Therefore, we cannot make inferences about whether a penile skin-lined neovagina can support full *Lactobacillus* dominance based on this individual case.
C1.

C2.
Figure 15. Participant C (Panels C1 and C2) reported taking a daily oral probiotic (Nature’s Bounty) at each sample collection, however they did not have *Lactobacillus* dominance as seen in Participant B. There was a small amount of *Lactobacillus* present in each sample (<5% relative abundance). The contents of this probiotic are unknown (the specific product was not noted by the participant). Week 1 and 2 samples fell into IPT5, and week 3 into IPT3. No other behaviours in weeks 1 and 2 were reported to potentially explain the increased inflammation.
D1.

D2.
Figure 16. Participant D (Panels D1 and D2) reported using a topical probiotic (Probacal) in week 1 and topical lactic acid gel (Gynelac) in weeks 2 and 3 and reported using these products on a weekly or multi-month basis. Inflammation decreased from week 1 to weeks 2 and 3, moving from IPT4 to IPT1a/b (D2). Lactobacillus dominance is present in Week 1 and Week 3 but lost in week 2. Week 2 had a high abundance of Finegoldia, Peptoniphilus and Veillonella with only a very small amount of Lactobacillus (<5%).
6.1.2.2 Antifungal Medication

E1.

Figure 17. Participant E (Panels E1 and E2) reported taking Nystatin, an antifungal treatment, at week 1 and week 3, four and seven days prior to taking their sample, respectively. Inflammation was reduced at week 1 and 3 which were both in IPT3 while week 2 was in IPT5. All other behaviours were consistent week-to-week.
6.1.2.3 Antibiotics

F1.

F2.
**Figure 18. Participant F** (Panels F1 and F2) used Nidagel, six days prior to the week 1 swab and intermittently in the month prior. Nidagel – a common treatment for BV – is topical metronidazole inserted with an applicator. Participant F provided only 2 swab samples, and both clustered in the middle-inflammation groups (IPT4 and IPT3 for weeks 1 and 4, respectively). There was a noticeable increase in IL-1α, IL-1β, IL-22 and TNFα from week 1 to week 2. This corresponded with a shift in the microbiota; week 1 had higher relative abundances of *Lactobacillus, Corynebacterium* and *Staphylococcus*, while in week 2 *Prevotella, Peptoniphilus, Ezakiella* and *Porphyromonas* were dominant.
**Figure 19. Participant G** (Panels G1 and G2) reported using oral minocycline, a broad-spectrum antibiotic, one day prior to week 1, and had been taking it daily for the last month. This antibiotic is commonly used for treatment of *acne vulgaris* and STIs, however, this individual did not report having any diagnosed classical STI. This individual had a very consistent and inflamed immune milieu at each week, with all samples falling into IPT5. There was a large shift in the microbiota at week 3 with an increase in the abundance of *Gardnerella, Lawsonella, Sneathia, Atopobium*, and *Bifidobacterium*. Minocycline has been shown to reduce IL-8 and in turn neutrophil chemotaxis thus attenuating the proinflammatory process in epidermal keratinocytes. These affects, however, are not seen in this individual as IL-8 levels are very high and stay high each week even after the person stops reporting taking the antibiotic.
6.1.2.4 Penetrative Sex

H1.

H2.

IFNG
IL-1α
IL-1β
IL-6
IL-8
IL-10
IL-22
IP-10
MIG
MIP-1α
MIP-1β
RANTES
TNFα
Figure 20. Participant H (Panels H1 and H2) reported having condomless penetrative sex with an uncircumcised partner at week 2 and week 3, five and two days prior, respectively. It was noted the partner ejaculated in the individual’s neovagina at week 2 but not week 3. This individual has low inflammation at each week, with their samples falling into IPT1, IPT2 and IPT1 at weeks 1, 2 and 3, respectively. There was however a massive shift in the microbiota between week 1 and 2. The microbiota in week 1 is dominated by Lactobacillus but in weeks 2 and 3 it is highly diverse, and Lactobacillus was almost completely absent.
I1. 

Figure 21. Participant I (Figure 13, Panels I1 and I2) reported condomless penetrative sex with an uncircumcised partner 4 days prior to week 1, with ejaculation. This was the only reported event of penetrative sex. Inflammation was consistently high at all three weeks (all in IPT 6). All other reported behaviors were consistent week-to-week, however, the microbiota shifts quite obviously between weeks 1 and 2. There is an increasing abundance of *Streptococcus* from weeks 1-3. Weeks 1 and 2 also have a much higher abundance of *Fusobacterium* and *Finegoldia*...
Figure 22. Participant J (Panels J1 and J2) reported condomless penetrative sex with an uncircumcised partner six days prior to week 2. No ejaculation was reported, and all other behaviours reported were consistent week-to-week. There is increased inflammation at week 2 which could be a result of penetrative sex, however as it was reported as happening 6 days prior, the association is quite weak. The microbiota between weeks 2 and 3 is stable however, there is a decrease in the less abundant bacteria denoted by ‘Other’ and Gallicola from week 1 to week 2.
6.1.2.5 Hormone Therapy

K1.

K2.
Figure 23. Participant K (Panels K1 and K2) reported that they stopped taking estrogen nearly 2 years prior to enrolling in the study, after having been on it for 6 years. They did not report taking any other exogenous hormones. They had high inflammation with both samples in in IPT6. More than 50% of the total bacterial abundance from week 1 was from the bacteria outside of the top 42 most abundant taxa. The most abundant bacteria in week 1 is Bacteroides, Bifidobacterium, Faecalibacterium, Agathobacter, Ruminococcus and Blauthia. These taxa tend to be associated more so with the gastrointestinal tract. The microbiota shifted between weeks 1 and 2 and this was accompanied by a slight decrease in IL-1β, IL-6, MIP-1α, MIP-1β, RANTES and TNFα. In week 3 there was a high abundance of Streptococcus, along with a lesser abundance of Lactobacillus, Anaerococcus and Peptoniphilus.

Participant B (Figure 14, Panels B1 and B2, pg. 61) reported never having been on estrogen. They did not report taking any other hormone therapy. This same individual reported taking a weekly probiotic and had full Lactobacillus dominance and a medium inflammatory profile at each week. With the use of a probiotics, it is hard to assess whether or not lack of estrogen is playing a major role in this case.
Figure 24. Participant L (Panels L1 and L2) reported being on testosterone and had been on it for 5 months at the time of enrollment. All other reported behaviours were consistent between weeks. They had medium inflammation with samples landing in IPT 3, IPT4 and IPT 4 for weeks 1, 2 and 3, respectively. The microbiota had a high abundance of *Prevotella* (>30% abundance) and *Streptococcus* (8-10% abundance) each week.
6.1.2.6 Douching

An average of 16 participants reported douching with water each week. 6 participants (M, N, O, P, Q, R: Figure 25 -Figure 32) douched with water every week and did not report any other outlined behaviours. 2 participants (S, T: Figure 33 and Figure 34) reported douching at only one week and behaviours and genital exposures were otherwise consistent. Douching with water did not appear to be universally associated with increased inflammation. Of the individuals that reported douching each week, most samples were in the middle and low inflammatory IPTs. Week 1 of participant N was in IPT 5 and all three weeks of participant M’s samples fell into IPT6. Participant M also had a very unstable microbiota that fluctuated each week.

Participant S who only reported douching at week 3 had a stable immune milieu with each sample falling into IPT 4. Similarly participant T shifted from IPT 2 at week 1, to IPT 3 at weeks 2 and 3 but no changes in inflammation were observed at week 3 when they reported douching.
Figure 25. Participant M (Panels M1 and M2)
Figure 26. Participant N (Panels N1 and N2)
Figure 27. Participant O (Panels O1 and O2)
Figure 28. Participant P (Panels P1 and P2).
Figure 29. Participant Q (Panels Q1 and Q2)
R1.

Figure 30. Participant R (Panels R1 and R2)
Figure 31. Participant S (Panels S1 and S2)
Figure 32. Participant T (Panels T1 and T2)
Figure 33. Participant U (Panels U1 and U2) was the only participant to report douching with a commercial product (Summer’s Eve Douche). This individual had consistently high inflammation at each week with all samples being in IPT5. This individual reported similar behaviours each week. The inflammation may be related to the douching product, but it is difficult to deduce this as they are using it consistently.
Figure 34. Participant V (Panels V1 and V2) reported douching with diluted vinegar in week 1 and boric acid in week 2 and 3. They also specified in their survey that the boric acid was being used to lower their neovaginal pH. Each sample was in the middle inflammatory IPTs and the only obvious change in the microbiota is a decrease in *Corynebacterium* between weeks 1 and 2 and a concurrent increase in *Gardnerella*. 
6.2 Discussion: Behavioural Data

Penetrative sex with flesh genitals and seminal fluid are both identified inflammatory factors in the vagina. Physical disturbance of the vaginal epithelium can induce inflammation, and seminal fluid also contains proinflammatory cytokines. Despite this, there were no noticeable associations between increased inflammation and the occurrence of penetrative sex. The minimum amount of time between reporting having penetrative sex and taking the immune swab was 2 days. It may be that penetrative sex does result in increased inflammation however, the transient increases in inflammatory cytokine may be resolved within 2 days.

Douching with water was reported among many participants and did not appear to associate heavily with inflammation. Of the participants who reported douching with water at each week, there was only one that had consistently elevated inflammation with all samples being in IPT5. There was also not a notable change in between weeks for those who reported douching at only one week. A similar trend was seen in the individual who reported douching with vinegar and boric acid. Their immune milieu was largely stable with their samples being in the middle inflammatory IPTs.

Probiotics may impact the neovaginal microenvironment. Along with changes seen in the microbiota following probiotic use, it appeared in some cases there were associated changes in inflammation. Although no two participants reported taking the same probiotic, three participants reported taking similar standard oral probiotics containing various species of Lactobacillus and Bifidobacterium. The microbiota of these participants were very different from each other but largely unique compared to the average neovaginal microbiota found in this study (i.e. high abundance of Prevotella, Peptoniphilus, Ezakiella and Porphyromonas) leading us to postulate that probiotics could potentially be used to alter the composition of the neovaginal microbiota in a treatment setting. However, further controlled studies would be needed to see if these changes were in fact due to the use of probiotics.
Much like the vagina, it may be that estrogen plays a key role in shaping the neovaginal microenvironment. One participant who reported not being on estrogen had very high inflammation which was associated with major shifts in unique microbial composition. More than 50% of the total bacterial abundance in their week 1 swab was from bacterial genera that were outside the top 42 most abundant genera reported in this thesis. It should be noted that many of the most abundant genera of bacteria in this sample were characteristic of the gut microbiota and it is possible that the sample was contaminated with fecal matter. However, there was also a large corresponding increase in inflammation in this sample which suggests that the presence of these bacteria may not be transient or due to contamination. It may be that estrogen plays a critical role in determining what taxa can thrive in the neovaginal microbiota.

There were a number of instances of the microbiota changing week to week but not accompanied by a matched shift in the immune milieu. This leads us to believe other factors within the neovaginal microenvironment, such as yeast, may also be playing a role in modulating inflammation.
Chapter 7

7 Overall Summary and Future Directions

7.1 Summary

This thesis described the neovaginal immune milieu and microbiota from a cohort of 50 transfeminine individuals who participated in the larger TransBiota study. It focused on characterizing the neovaginal immune milieu and exploring potential causes of inflammation in the neovaginal microenvironment.

The vaginal and penile microbiota play a major role in modulating genital inflammation and sexual and reproductive health in cisgender men and women. To determine if this could also be true of the neovaginal microenvironment of transfeminine individuals, we collected weekly immune and microbial swabs over the course of 3 weeks from 50 transfeminine participants.

The 13 cytokines were quantified in neovaginal swabs using a multiplex immunoassay (Luminex MAGPIX). Cytokine concentration data dimensionality was reduced – while retaining important information about cytokine cascades – using the PHATE algorithm. The embedding generated with PHATE was then scanned with a k-nearest neighbours algorithm, and six unique immune profile types (IPTs) were identified.

Microbial data was generated by our collaborators at the University of Maryland School of Medicine via 16s rRNA gene sequencing. A simple visualization of the relative abundances of the most abundant bacterial taxa stratified by IPT was completed, but no obvious associations between bacterial community composition or structure and IPT were observed.

For a more thorough examination of potential associations between IPT and bacterial community composition or structure, immune and bacterial data were embedded together using PHATE, and this embedding was clustered. Seven clusters were identified, but there were no clear associations between microbial communities and inflammation. The combined clustering was driven primarily by the immune data.
and the microbial communities were diverse and largely indistinguishable among the clusters.

Associations between specific bacterial taxa and IPTs were then analyzed using Bayesian spline models. Increased abundance of *Streptococcus*, *Atopobium deltae*, *Prevotella buccalis* and *Prevotella oris* were all associated with an increased probability of being in a high inflammation IPT. Increased abundances of *Corynebacterium*, *Porphyromonas* and *Prevotella timonensis* were all associated with a decreased probability of being in a high inflammatory IPT. There were no bacterial taxa for which an increase in their relative abundance was associated with increased probability of being in a low inflammation IPT. The total number of associations found between bacterial taxa and inflammation was within a range that would be expected due to chance and therefore the results of this analysis should not be overinterpreted. Overall, the microbial composition of the neovagina could not explain differences in immunology between participants.

We next looked at survey data to see if reported behaviours (penetrative sex and douching) and neovaginal exposures (probiotics, antibiotics, medications, hormones) contributed to variability in inflammation. No significant associations were identified between behavioural groups (previously identified by another graduate student) and cytokine expression.

Finally, an exploratory descriptive analysis was performed on each participant to generate hypotheses for future research. Individual immune heatmaps and bacterial bar plots were generated for each participant, and behaviours and exposures reported at each sample were noted. Of all the exposures, probiotics and local topical antimicrobial agents were identified as potentially modulating the neovaginal microbiota, with some shifts in microbial composition aligning with changes in inflammatory profiles.
7.2 Future Directions

As vaginoplasty becomes an increasingly popular surgery for transfeminine individuals, it is critical that further research be undertaken to better inform best practices and targeted treatment development. Although there were no strong associations made between bacteria and inflammation in this thesis, there is a substantial body of literature from other anatomical sites that suggest it is very likely that bacteria are playing a role in modulating inflammation.

As the neovagina is surgically created with varying amounts of penile and scrotal tissue, and occasional augmentation with intestinal, peritoneal, or other skin grafts, it is possible that there is substantial heterogeneity between individuals. Our participants reported diverse vaginal practices, and many lamented the paucity of data on best practices (in open write-in sections). Many participants used home or internet remedies to help alleviate symptoms (e.g., homemade lubricants from xanthan gum and methyl cellulose, or douching with boric acid and commercial products touted to reduce pH). This leaves many potentially confounding variables at play when trying to parse out what may be causing inflammation in a relatively rare population.

Many participants within this study also reported gynecological symptoms such as odour, discharge, and bleeding. These same symptoms in the vagina are often a result of bacterial dysbiosis and are associated with inflammation and increased risk of STI acquisition. Future studies should elucidate whether these symptoms are indicative of bacterial dysbiosis in the neovagina or if they may be a symptom of something else, such as yeast. Previous studies on the vagina and several case reports on the penile skin-lined neovagina have shown that the presence of Candida species (yeast) is associated with symptoms such as discharge and itching. Yeast infection is also associated with increased inflammation. It is therefore possible that the inflammation seen in these neovaginal samples is due in part to yeast infection.

Future studies by our group will focus on longitudinal sampling with strict behavioural protocols and structured interventions to reduce variability. The penile
microbiota present prior to vaginoplasty may also play a large role in determining what bacteria initially seed the neovaginal microenvironment. Our group is initiating a study following participants through the vaginoplasty process to better understand the influence of the pre-existing microbiota and how the neovaginal microbiota and inflammation shift during healing. These next steps are critical in determining what constitutes a healthy neovaginal microenvironment with low inflammation and low risk of STI and HIV acquisition. They will also aid in our understanding of what microbial factors may play a proinflammatory role in the neovaginal microenvironment, with the goal of using this information to direct better gynecological health care for transfeminine individuals.
8 References


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Appendix 1. Permission letter from Mayo Clinic for use of Figure 35. Anatomy before and after penile inversion vaginoplasty.
AOV – 0.192

AOV – 0.492
AOV = 0.465
Appendix 2. Line graph of log10 cytokine concentrations (pg/ml) stored in Immune Stabilization Media, over the course of 2 weeks. The concentrations of all 13 cytokines from 3 vaginal secretion samples stored in Immune Stabilization Media were measured. Samples were left at room temperature for increasing periods of time and snap frozen at -80°C. A total of 18 samples representing 3 individuals and 6 time points were measured in duplicate on a multiplex immunoassay to determine if the concentrations of each cytokine depreciated with time. A repeated measures ANOVA test was completed to compare the means of each analyte at each time point. IL-8 was found to be significantly different following a repeated measures ANOVA. However, a post hoc paired T-Test showed that the differences in concentrations between 1hr and 24hrs were not significant. There was no MIP-1α present in any of the samples. There were no significant differences in concentration between time points for any of the other 11 cytokines.