Carbohydrates and Fungal Toxin Exposure Influence the Vaginal Microbiota, Metabolome, and Reproductive Health of Women

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Abstract

The reproductive health of women is influenced by microorganisms and their metabolites, namely those representing the vaginal microbiota and those producing toxins that are ingested. To manipulate the vaginal microbiota toward a health-associated, *Lactobacillus*-dominant state, an approach adopting prebiotic lactulose was taken. Using batch culture, lactulose supported *Lactobacillus* maintenance and positively altered metabolites, while not disrupting indigenous *L. crispatus* epithelial adherence. The vagina also harbours abundant glycogen, but initial assumptions that lactobacilli utilize it were incorrect. I have now shown that glycogen selectively stimulates organisms associated with dysbiosis and *L. iners* in a self-limiting manner. Other compounds such as environmental toxins have not previously been shown to impact vaginal health. In a cohort of Rwandan women, I found that they were indeed exposed to fungal toxins. In summary, I have shown that microbes, carbohydrates, and mycotoxins can play a significant role in the health of women in Canada and beyond.
Keywords
Vaginal microbiota, metabolomics, liquid chromatography-mass spectrometry, lactobacilli, prebiotics, lactulose, glycogen, mycotoxin, Rwanda, aflatoxin, fumonisin, ochratoxin A, zearalenone.
Co-Authorship Statement

The investigations presented in this thesis were predominantly carried out by Stephanie Collins under the supervision of Dr. Gregor Reid. Details regarding additional authorship contributions are listed below:


SLC designed and carried out all experiments and wrote the paper. SS recruited healthy patients. AM developed the method for LC- and GC-MS metabolomics of vaginal swabs. AM and SLC analyzed the data. GR supervised the study and edited the manuscript. MS provided instrumentation and intellectual support for metabolomics. CvdV and RK collected and provided 16 *L. crispatus* strains. All authors read and approved the final manuscript.

Chapter 5: Collins SL, Renaud J, McMillan A, Walsh J, Miller JD, Sumarah M, and Reid G.

Chapter was written in its entirety by SLC. SLC, JR and JW carried out all experiments. AM designed original study and collected blood and urine samples and clinical data from patients in Rwanda. Urine processing method was designed by SLC and JR. AM and JR developed method for plasma aflatoxin analysis. GR, MS, and JDM provided intellectual support and/or supervised the study.
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</tr>
</thead>
<tbody>
<tr>
<td>2-HV</td>
<td>2-hydroxyisovalerate</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AF</td>
<td>aflatoxin</td>
</tr>
<tr>
<td>AGC</td>
<td>automatic gain control</td>
</tr>
<tr>
<td>BCP</td>
<td>bromocresol purple</td>
</tr>
<tr>
<td>BV</td>
<td>bacterial vaginosis</td>
</tr>
<tr>
<td>CBA</td>
<td>Columbia blood agar</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CLR</td>
<td>centered log-ratio</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>F</td>
<td>fumonisin</td>
</tr>
<tr>
<td>FA</td>
<td>formic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FOS</td>
<td>fructo-oligosaccharides</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GHB</td>
<td>(\gamma)-hydroxybutyrate</td>
</tr>
<tr>
<td>GOS</td>
<td>gluco-oligosaccharides</td>
</tr>
<tr>
<td>(\text{H}_2\text{O}_2)</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HESI</td>
<td>heated electrospray ionization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</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>ISU</td>
<td>individual sequence unit</td>
</tr>
<tr>
<td>KSFM</td>
<td>keratinocyte serum-free medium</td>
</tr>
<tr>
<td>LEA</td>
<td>Lactobacillus epithelium adhesin</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>MOX</td>
<td>methoxyamine-HCl</td>
</tr>
<tr>
<td>MRS</td>
<td>de Mann, Rogosa, and Sharpe</td>
</tr>
<tr>
<td>MSTFA</td>
<td>N-methyl-N-(trimethylsilyl)-trifluoroacetamide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NYC III</td>
<td>New York City III media</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OTA</td>
<td>ochratoxin A</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PRM</td>
<td>parallel reaction monitoring</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SCFA</td>
<td>short-chain fatty acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
</tr>
<tr>
<td>VDMP</td>
<td>vaginally defined media + proteose peptone</td>
</tr>
<tr>
<td>VK2</td>
<td>vaginal keratinocyte 2</td>
</tr>
<tr>
<td>ZEN</td>
<td>zearaleneone</td>
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</table>
Chapter 1

Introduction
1.1 Preamble

Microbes are present everywhere—from our bodies to our environment. Even in tissues and body cavities that were previously considered sterile in healthy people, such as breast tissue (1) and the upper urinary tract (2), there reside notable communities of bacteria. In fact, the number of non-redundant bacterial genes in any individual far outweighs their own genetic content (3). Together, the microbes and their genetic potential that populate an environment are termed the microbiome (4), while their cumulative secreted metabolic by-products are referred to as the metabolome.

Several hypotheses have arisen to try to explain how specific and distinct bacteria enter and populate these sites. Some organisms are transferred from mother to baby during development, whether in utero through amniotic fluid (5–7) or the umbilical cord (8), during vaginal childbirth (9), or through breast milk (10, 11). Others are acquired from the environment through ingestion, inhalation, or direct contact.

Most of the billions of microbes that humans are exposed to daily are allochthonous, only colonizing transiently, while a small proportion reside autochthonously. Dictating this are the numerous host and microbial factors that influence their interaction. First, the human genome largely influences tolerance to certain microbes (12) because of variants in immune responses such as leptin and chemokine signaling, and pattern recognition receptors (13). Other host factors include bile acid composition, which bidirectionally influences and is modified by gut microbiota (14), and epithelial structure. Regardless, resident microorganisms must also possess the necessary machinery to obtain nutrients such as carbon and nitrogen from their niche, which varies from site to site. Additionally, attachment mechanisms such as adhesin expression, membrane hydrophobicity, and the ability to form biofilms can be essential for long-term habitation by microbes. Of course, even if all these factors are in place, microbe-microbe competition can also exclude organisms from a given site.

Human health is to a large extent dictated by microorganisms and their products, whether it be through pathogen exposure or symbiotic relationships. This has formed the basis of the hygiene hypothesis, which underlines the importance of early life contact with a
diversity of microbes that shape the immune response, without which allergic diseases have become prevalent (15). Furthermore, imbalances of our bodies’ microorganisms influence the onset or prevention of many conditions, from irritable bowel syndrome (16), to kidney stone formation (17) and anxiety (18). Certain microbial metabolites relate directly to disease symptomology, such as the amines secreted by pathogenic microbes to cause odour in vaginal dysbiosis (19), or the production of butyrate and its anti-inflammatory effects in the gut (20).

Microbes are especially known to influence women’s health. In breast tissue, a distinct microbiota is associated with cancer compared to healthy tissue, possibly due to production of DNA damage-causing metabolites (21). Similarly, a woman’s risk for contracting sexually transmitted Neisseria gonorrhea or Chlamydia trachomatis infections varies depending on the composition of the vaginal microbiota (22, 23). In the case of women who become pregnant, microbes are known to influence the pregnancy itself, as well as outcomes for the fetus. As an example, miscarriage can be induced by persistent colonization with vaginal opportunists (24, 25). Toxins released by food-contaminating microorganisms that are consumed by women are also known to have detrimental effects on fetal development. For example, zearalenone, a compound secreted by fungi from the Fusarium genus and a common food contaminant, mimics estrogen to cause developmental delays and death in pregnant rats (26, 27). Although many regulations are in place to limit excessive ingestion of such toxic microbial metabolites, little is still known about exposure levels in developing nations where food is often scarce, or what the potential negative consequences of chronic low dosage are on women’s health.

Improved techniques for studying bacterial composition and their metabolites have provided burgeoning insight into host-microbe interactions. These studies have elucidated the tight relationship between microbiome structure and their metabolome, allowing for integration of community-function relationships (28). Molecular techniques for measuring bacterial populations, such as metagenomics and 16S rRNA sequencing, have increased the ability to identify bacteria and their functions, something more difficult using culture-based methods. These advances have especially broadened the repertoire of organisms thought to be present in various sites including the vagina (29), urinary tract (2), and gut
Although 16S rRNA sequencing is limited by its inability to observe the genetic potential of a bacterial community, and the observed abundances are proportional, this technique is effective at monitoring conditions where there are large-scale, defined changes to the microbiota. An outstanding example of this is the vaginal microbiome, which is dominated by relatively few species in health, and dysbiosis is represented by significantly increased diversity and a number of known bacterial genera.

To gain a better understanding of microbial activity, the use of analytical methods to identify metabolic products provides a valuable tool. In comparison to proteomics or transcriptomics, metabolomic methods measure all the small molecule metabolites that are present in a system. Since these are the end-products of bacterial processes, quantification of metabolites is the most accurate reflection of bacterial activity and communication. The predominant instruments used are nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS). NMR-based methods provide highly reproducible results, but lack sensitivity compared to mass spectrometry. Since gas chromatography is limited to measuring compounds that effectively volatilize, liquid chromatography is the most valuable tool for measuring low abundance and a broad range of compounds in metabolomics experiments. With these novel technologies, huge advances in research on the intersection of microbes and women’s health have been achieved.

1.2 The vaginal microbiome

In most healthy individuals, the vaginal microbiota is dominated either by *Lactobacillus iners*, *L. crispatus*, *L. jensenii*, or *L. gasseri* (32), with variations in predominance depending on several factors. While Caucasian women are more often *L. crispatus*-dominant, women of Asian descent are more commonly populated with *L. iners* (32, 33). Circulating estrogen levels also significantly impact the composition of the vaginal microbiota. Following menopause, estrogen and *Lactobacillus* levels in the vagina concomitantly decrease (34, 35). Hormone replacement therapy, involving estrogen and progesterone administration to reduce unwanted symptoms of menopause, can restore *Lactobacillus* dominance (36). On a smaller scale, estrogen also causes microbiome fluctuations throughout the menstrual cycle, with the lowest *Lactobacillus* levels occurring
during menses (37). This has been linked to glycogen production by vaginal epithelium, which is stimulated by elevated estrogen, leading to more abundant carbohydrate sources, supposedly for resident lactobacilli (38).

Not all lactobacilli are considered to equally contribute to a healthy vagina. For example, a *L. crispatus*-dominated microbiota is strongly associated with health, while *L. iners* is found in healthy women and those with bacterial vaginosis (BV). This is supported by the findings that *L. crispatus*-dominant women have more acidic pH (32) and experience less instability in microbiota composition (39). On this basis, studies have identified positive characteristics of *L. crispatus*, such as the ability of vaginal secretions from a *L. crispatus* dominant microbiota to kill *Escherichia coli* (40) and *in vitro* assays showing inhibition of pathogen growth (41). The species has also been shown to confer direct benefits to the host, by exerting minimal pro-inflammatory and cell stress responses to the vaginal epithelium compared to *L. iners* (42, 43); promoting thickening of cervicovaginal fluid that better defends against HIV-1 (44); and blocking pathogen adherence (45). In contrast, *L. iners*-dominated microbiotas tend to have higher pH and are more unstable (32, 39, 46). However, recent evidence suggests that *L. iners* may simply be better suited than *L. crispatus* to adapt to the varied conditions of the vaginal environment, allowing it to persist throughout dysbiosis and antibiotic therapy (47, 48).

### 1.2.1 Protective mechanisms of vaginal lactobacilli

Vaginal lactobacilli possess a vast repertoire of functions that are thought to impart their dominance in this niche. Many lactobacilli produce antimicrobial compounds such as hydrogen peroxide (H$_2$O$_2$), lactic acid, and bacteriocins (49). Early *in vitro* studies emphasized H$_2$O$_2$ as a major defense mechanism of lactobacilli due to its ability to kill *Gardnerella vaginalis* and sequester HIV-1 through the H$_2$O$_2$-peroxidase-halide pathway (50, 51), leading to the screening of vaginal probiotics on the basis of H$_2$O$_2$ production (52). However, the validity of this has come into question since H$_2$O$_2$ is ineffective in vaginal conditions, namely oxygen-depletion and acidic pH below 4.5, and it can also be toxic to lactobacilli (50, 53, 54). Furthermore, the activity of H$_2$O$_2$ is sequestered by the proteinaceous environment imparted by male ejaculate and vaginal secretions (55). Given
this, the biological impact of $\text{H}_2\text{O}_2$ in exerting *Lactobacillus* dominance is no longer seen as well founded.

The greatest body of evidence exists for lactic acid fermentation as the primary defense for vaginal lactobacilli. By definition, lactobacilli are fermentative and release lactic acid as a metabolic by-product, giving the vaginal ecosystem a distinctly acidic pH that is not tolerated by many pathogens (56, 57). Both acidic pH (<4.5) and elevated lactic acid in cervicovaginal fluid are strongly associated with health, and the former is often used diagnostically (19, 56, 58). O’Hanlon et al. (57) demonstrated physiological concentrations of lactate in healthy women at 1.0% (w/v), which is a sufficient concentration to kill common pathogenic bacteria, but not lactobacilli (53, 59). Lactic acid acts through disruption of the membrane of Gram-negatives (60) to kill organisms associated with vaginal dysbiosis (53), and it is also directly viricidal to HIV-1 (59). In addition, lactic acid influences the host’s response to pathogens, where physiological levels induce an anti-inflammatory immune response in vaginal epithelial cells (61) that limits the potential for HIV acquisition (62). Ultimately, extensive research on lactic acid has clarified the need for this *Lactobacillus* metabolite and acidic pH for vaginal health.

Vaginal lactobacilli have also been observed to secrete bacteriocins, a structurally diverse group of antimicrobial peptides that target physiologically similar microbes to the producer. Bacteriocin activity against the dysbiotic organism *G. vaginalis* has been reported by many vaginal lactobacilli (49, 63, 64), including predominant species *L. gasseri*, *L. jensenii*, and *L. vaginalis*. Further characterization of bacteriocin gene clusters has been performed on *L. crispatus* and *L. gasseri* strains, revealing their encoded gassericins (64) and putatively identified bacteriocins with similarity to helveticin J and enterolysin A (65). Antimicrobial proteins are also secreted by exogenous probiotic lactobacilli, such as the soluble antimicrobials found in *L. rhamnosus* GR-1 supernatant (66). This has implicated bacteriocins as an alternative treatment for dysbiosis of the vagina as their activity is specific to disease-associated strains and not lactobacilli. Unfortunately, pathogenic *G. vaginalis* strains have already developed resistance against various bacteriocins (48, 62), and antibiotic-resistant strains are even more likely to have
bacteriocin resistance (68). Since the in vivo effect of these bacteriocins on the bacterial composition in the vagina is still unknown, further studies are needed to assess their impact.

Biofilm formation is a major virulence factor in a variety of human pathogens. It relies initially on bacterial adherence to a surface, followed by secretion of exopolysaccharides and other factors, to form a structure that protects contained bacteria from invading microbes and antibiotics (69). Although vaginal biofilms are often considered a characteristic of pathogens such as G. vaginalis and Atopobium vaginae (70), lactobacilli can also utilize biofilms to maintain dominance in this niche (71). Furthermore, Lactobacillus-derived biosurfactants can disrupt pathogenic biofilms and change epithelial cell surface tension to make it less receptive to pathogen adherence (72, 73). Combined with the observation that adherent vaginal lactobacilli (74) utilize surface glycoproteins and saccharides to displace Candida albicans and G. vaginalis from epithelial glycolipids (66, 75, 76), adherence and biofilm production are major beneficial characteristics of lactobacilli.

Nonetheless, when pathogenic colonization persists, Lactobacillus adhesion can still contribute to the removal of invaders through coaggregation (67). This is initiated by the adhesion of bacteria to one another, and it is frequently observed between urogenital species (77, 78). Notably, vaginal lactobacilli adhere strongly to pathogenic Staphylococcus aureus to displace it from the ecosystem (79). However, this system works both ways, as bacterial coaggregation also dislodges Lactobacillus spp. from the vaginal epithelia. Thus, strains being considered as candidate probiotics are screened for aggregative properties, with a view that administering them will release adherent pathogens from native lactobacilli (78). In support of this, probiotic L. reuteri RC-14 was observed to have stronger adhesion to S. aureus than common indigenous lactobacilli (79) and has been shown to incorporate into pathogen biofilms and subsequently kill encapsulated pathogens (80).

1.2.2 Bacterial vaginosis

Despite abundant antimicrobial mechanisms innate to vaginal lactobacilli, nearly one in three women experience a microbial imbalance of the vagina referred to as BV (81). A
recent review has critiqued the terminology surrounding BV, whereby it appears to encompass several inflammatory and non-inflammatory phenotypes (Reid, 2017). In the currently accepted, non-inflammatory form of the condition, lactobacilli are outgrown by pathogenic anaerobes, especially *Prevotella, Atopobium, Mobiluncus,* and *Gardnerella* species (82). Around 80% of BV-positive women are asymptomatic (81) and not treated, and it is thought that up to one quarter without symptoms have latent BV, characterized by a vaginal microbiome consisting of these diverse fastidious anaerobes (32). This explains why so many undetected cases appear in such studies of so-called “healthy” women, thereby limiting physicians’ ability to best manage these individuals. It also adds confusion because symptoms are derived from bacterial metabolites that are not directly related to the target therapy.

The emergence of new and diverse pathogens in BV chemically modifies the vaginal environment compared to healthy women. Lactic acid production by lactobacilli is reduced so pH rises above 4.5, and pathogens upregulate enzymes whose activities release short-chain fatty acids (SCFAs) such as succinate, acetate, and butyrate (19, 83, 84). For this reason, a succinate:lactate ratio of >0.4 has been used as a marker for BV. But this method lacks sensitivity (80%) and specificity (83%) (85), possibly because *L. crispatus* also produces succinate (19). McMillan *et al.* (80) utilized mass spectrometry to improve detection of metabolic markers of BV, and in doing so, identified 2-hydroxyisovalerate (2-HV) and γ-hydroxybutyrate (GHB). Improved diagnostic capabilities are possible by using 2-HV:tyrosine ratios (sensitivity of 89% and specificity of 94%), which also eliminates the need for normalizing to sample weight (19). Another BV-specific SCFA, butyrate, reduces gut inflammation and could be acting similarly to prevent inflammation in BV (86), potentially limiting the ability to sense the condition. Several BV-associated species and genera such as *P. amnii, Dialister, Mobiluncus, Prevotella, Megasphaera,* and *Mycoplasma* (83, 84) also produce amines (putrescine, cadaverine, and spermidine) responsible for the “fishy odour” of symptomatic BV (58). Overall, chemical changes in BV are responsible for many characteristic symptoms and signs, and certain profiles have the potential to improve diagnostic techniques for identifying overgrowth of pathogenic anaerobes.
Historically, two methods have been the standard for BV diagnosis: Amsel criteria and Nugent scoring. Under the former, clinical diagnosis requires at least three of four Amsel criteria: Elevated pH, creamy discharge, malodor, and the presence of “clue cells”—vaginal epithelial cells coated with short, Gram-variable rods (58). The Nugent scoring technique is widely adopted for laboratory diagnoses of BV, and uses Gram-staining methods to score Lactobacillus-dominated (0 to 3), intermediate (4 to 6), and BV organism dominated (7 to 10) microbiotas (87). Gram-stain based methods appear to more accurately detect dysbiosis associated with BV (84), as they rely on an observer to note Lactobacillus reduction in place of pathogenic bacteria. However, bacteria can take on many shapes and sizes, so the reliability of a Gram stain is questionable. Other laboratory techniques such as 16S rRNA sequencing of the V6 or V4 regions (88) are more effective in identifying BV organisms, and specific microarrays have been developed on this basis (89). As the cost of molecular techniques reduce, it is hoped that these sensitive and more effective ‘omics’ methods will reach standard clinical diagnostic settings.

Despite the lack of apparent symptoms and signs in most cases, BV increases the risk for additional infections and adverse reproductive health. Numerous studies in pregnancy have reported associations with preterm labour and low birth weight, with BV patients at a 1.4–6.8 times greater risk of giving birth prematurely (90–92). In the first trimester of pregnancy, BV also doubles the risk for miscarriage in otherwise healthy individuals (24). Though it is unknown exactly how this occurs, speculation has been on upper genital tract inflammation due to bacterial colonization. This is somewhat surprising considering that BV is a relatively non-inflammatory condition lower in the vagina. A study by Hillier et al. (93) demonstrated that, among the 61% of premature births with bacterial colonization of the chorion and amnion, the most commonly infecting pathogens were BV-associated bacteria Ureaplasma urealyticum and G. vaginalis. Furthermore, BV organisms penetrate and infect amniotic fluid (23). Their subsequent release of virulence factors such as sialidase (91), mucinase (91), and endotoxins (94) may induce inflammatory cytokines (94, 95) to trigger premature delivery and spontaneous abortion (96). However, additional investigations are needed to fully elucidate the mechanism of the labour response in these women.
BV conditions can also reduce the presence of health-promoting lactobacilli, increasing susceptibility to sexually transmitted bacterial and viral infections. Comorbidity with BV increases viral load (97), shedding (98), and contraction (99) of HIV-1, leading to a 3-fold increased risk for transmitting the virus to male partners (100). Enhanced susceptibility of BV-affected women to contracting HIV-1 might be explained by the influx of CCR5+ T cells to the vagina (101), the cellular target for HIV (102). Upregulated interleukin-1β from BV infection may also enhance the transcriptional activity of HIV, further exacerbating the infection (103). Thus, the overall mechanism by which BV-infected women become susceptible to adverse HIV outcomes is multifaceted and still poorly understood.

Lactobacilli help the host defend against *Neisseria gonorrhoeae* (22, 99), *Chlamydia trachomatis* (22), pelvic inflammatory disease (104), and cervicitis (105), but this is lost during BV onset. In addition, BV is strongly associated with *Trichomonas vaginalis* (99) and herpes simplex virus 2 (HSV-2) (106) infections, though HSV-2 is likely the cause of BV co-occurrence rather than the result (107). One potentially confounding issue in these associations is that there are shared risk factors between STI and BV contraction and unfortunately it is difficult to ascertain which variables are responsible.

Given the complications associated with BV, researchers have directed their efforts toward identifying its risk factors. Many otherwise dangerous sexual behaviours like having multiple partners, group sex, and sexual abuse are correlated with BV (108, 109), but condom and hormonal contraceptive use are protective (110, 111). As BV is a condition and not an infection by any single organism, it has not been considered a sexually-transmitted disease. However, BV bacteria may be transmitted directly by women having sex with women (112, 113). Other sources of these microbes may provide external reservoirs for BV organisms, such as those found in urine (114), anal and oral cavity (115), or partner’s penile microbiota (116), potentially leading to persistent re-colonization.

The treatment of BV typically involves a 7-day regimen of either oral or local metronidazole or oral clindamycin (117). Both drugs quickly deplete pathogenic anaerobes in the vagina, with early efficacy as high as 94-96% (118–120), yet BV organisms often recover once the antibiotic regimen ceases (119, 121). Thus, BV recurrences are common,
being 20% after one month (120) and as high as 58% one year after metronidazole treatment (121). Risk behaviours for relapse include having a consistent sexual partner throughout treatment and sex with women (121), which supports a mechanism where infected women recirculate BV organisms between their partner and themselves. *Gardnerella vaginalis* is also highly adaptive against metronidazole, considering its many antibiotic-resistant strains (68) and rapid recolonization after treatment (119). Bacterial biofilms of *G. vaginalis* and *A. vaginae* contribute to bacterial resistance in BV, and are present in 90% of cases (69, 70, 122). Since *G. vaginalis* is the most effective primary colonizer of vaginal surfaces (123), it is thought that its initial attachment paves the way for additional pathogens to adhere (124). Finally, some researchers attribute antibiotic failure to collateral damage to native lactobacilli (125), since neither metronidazole nor clindamycin promote recovery of indigenous lactobacilli. Regardless, there is clearly a need for alternative treatment methods, particularly those that treat BV as a dysbiotic condition that requires restoration of lactobacilli, rather than an infection.

1.2.3 The potential for vaginal probiotics and prebiotics

There has already been a great deal of promise for BV treatment using natural remedies such as probiotics, as these focus on the replenishment of health-associated vaginal lactobacilli. The United Nations/World Health Organization defines probiotics as “live microorganisms, which when administered in adequate amounts, confer a health benefit on the host” (126). *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 are the most documented for use in treating and preventing BV, especially since they persist for some time in the vagina (127). In several studies, oral supplementation of GR-1 and RC-14 in conjunction with oral metronidazole improved *Lactobacillus* restoration in BV-affected women by 10–39% (128–130). This was not recapitulated in a separate cohort of HIV-positive women, suggesting that intact immunity is still required for recovery (131).

Alternatively, prebiotics, defined as “substrates that are selectively utilized by host microorganisms conferring a health benefit” (132, 133), have been adopted to increase the proportion and activity of lactobacilli in other microbiotas (133, 134). Lactobacilli in the vagina are also feasible targets for prebiotic stimulation, however, few studies have been performed to this end. Compounds such as lactoferrin, not a traditional prebiotic, were
found to prevent BV onset and its associated negative reproductive outcomes in a woman with recurrent miscarriages (92). Lactoferrin is an antimicrobial compound found in bodily fluids and is not fermented by vaginal bacteria. Other researchers have demonstrated the potential for various fructo-oligosaccharides (FOS) and gluco-oligosaccharides (GOS). Rousseau et al. (135) isolated vaginal lactobacilli and tested these and pathogens (C. albicans, E. coli, and G. vaginalis) against several FOS and GOS compounds, finding that only lactobacilli were stimulated by FOS Actilight® and α-1,6/α-1,4 GOS. A locally administered gel formulation containing these α-1,6/α-1,4 GOS prebiotics improved Nugent scores following metronidazole treatment of BV (136). However, key experiments are still required to determine which prebiotics safely stimulate lactobacilli growth without also stimulating BV organisms and other pathogens, as well as produce metabolic activity associated with a healthy vagina.

1.3 Fungal toxins and women’s health

Unlike probiotics and prebiotics, other microbes and microbial products can have serious detrimental effects to the health of women. Much research has been dedicated to the consequences of fungal colonization in a woman’s own ‘mycobiome’, particularly due to the reported 75% incidence of vulvovaginitis in women, caused by yeasts from the genus Candida (137). However, contamination of food by fungi can also result in the ingestion of toxic products. Of these, mycotoxins are of particular concern due to their extremely high prevalence, known serious adverse health effects, and inability to be detoxified by regular cooking practices. Mycotoxins are secondary metabolites secreted by various Aspergillus, Fusarium, and Penicillium spp., which commonly contaminate crops destined for animal or human consumption. The World Health Organization outlined major mycotoxins of concern in humans in a 1999 bulletin, which included the classes aflatoxins, ochratoxins, zearalenone, and fumonisins (138). In general, the effects of mycotoxin exposure can be delineated by mycotoxicosis, or acute exposure, and chronic long-term effects, of which there is much less research.
1.3.1 Aflatoxins (AF)

The most thoroughly documented mycotoxins belong to a group called aflatoxins, which are produced by *Aspergillus* fungi. Specifically, aflatoxin B$_1$ and B$_2$ are secreted by *Aspergillus flavus* and *A. parasiticus*, and G$_1$ and G$_2$ only by *A. parasiticus*. These molds contaminate nuts and seeds (139), especially peanuts, and cereals such as maize, millet and wheat. Aflatoxin production is induced in a wide range of environmental conditions, but most favourably in humid climates with consistent temperatures above 24°C (140). Thus, the effective management of aflatoxin production involves fungicide application (141), drying of crops, storage in refrigerated conditions, and frequent testing. In the US, these extensive measures result in an estimated $52.1$ million – $1.68$ billion cost to the corn industry alone (142).

Of the different aflatoxins, B$_1$ is one of the most carcinogenic compounds known to humans (143), due to its bioactivation during metabolism into a DNA-reactive epoxide. Upon ingestion and absorption, AFB$_1$ is metabolized by detoxification enzymes in the liver, namely cytochrome P450 (CYP) 3A4 and 1A2 (144). Oxidation can result in two major products, the *exo* and *endo* isomers, of which the former is a highly genotoxic compound and the latter is sequestered. While CYP1A2 metabolism can produce either metabolite, most aflatoxins are degraded by CYP3A4 preferentially into the bioactivated *exo*-epoxide. CYP1A2 can also detoxify AFB$_1$ into AFM$_1$, a metabolite which is subsequently excreted in urine and is a sensitive biomarker for short-term AFB$_1$ exposure. Conversely, the highly reactive structure of AFB$_1$-*exo*-epoxide conjugates DNA and forms an adduct with guanine, which results in consistent mutations during DNA replication. Since most of this reactive product is produced in the liver, this is the primary target for mutation, and causes the associated high risks for liver cancer in exposed populations. In addition, AFB$_1$-*exo*-epoxide may also react with positively-charged lysine residues in circulating albumin. This adduct represents another sensitive biomarker for AFB$_1$ ingestion, but due to the long half-life of albumin, reveals longer-term exposure.

Toxicity due to acute high doses of aflatoxin is termed aflatoxicosis, which often presents as liver failure. Most cases of aflatoxin exposure are chronic low dose, which leads to liver cancer, as described above, as well as developmental delays and growth stunting in children.
Recently, aflatoxin B\textsubscript{1} was associated with more circulating virus in people infected with HIV, suggesting an immuno-suppressant effect (146).

Due to the globalization of food, aflatoxin exposure occurs pretty much worldwide, including non-tropical climates; however, countries in Africa appear to be disparately affected. Even in Kenya, where a serious outbreak of aflatoxicosis in 2004 resulted in over 200 deaths, contamination still occurs because of failures to implement adequate safety procedures (147). Reports of AFB\textsubscript{1} across Africa have shown that exposure is common in women. Levels of the plasma albumin adduct biomarker in women from Kenya, Ghana, and Egypt were a median of 5.0, 7.47, and 4.9 pg/mg albumin, respectively (148–150), while urinary AFM\textsubscript{1} was found at a median level of 162 and 19.7 pg/mg creatinine in Zimbabwean and Egyptian women (150, 151). AFM\textsubscript{1} can also be passed to infants through breastfeeding, with African mothers’ breast milk concentrations ranging from 4.2–497 pg/mL (152, 153).

No studies, to date, have measured aflatoxin exposure levels in Rwandan populations, but food contamination appears to be widespread in this country. The European Commission (EC) and US Food and Drug Administration (FDA) regulations for total aflatoxins in food are 4–10 and 20 ppb, respectively, and the EU also specifies a limit of 2–8 ppb for AFB\textsubscript{1}. Observations of aflatoxin contamination above these regulatory limits in Rwandan maize flour have frequently occurred (154–156), as well as lower quantities in cassava (156, 157). Given the mass popularity of maize and cassava consumption in Rwanda, this population is at elevated risk for AFB\textsubscript{1} toxic effects and further work must address this.

1.3.2 Fumonisins (F)

Fumonisins B\textsubscript{1} and B\textsubscript{2} are mycotoxins produced by \textit{Fusarium} spp., namely \textit{Fusarium verticillioides}, previously known as \textit{F. moniliforme} (158). \textit{F. verticillioides} has the highest expression of FB\textsubscript{1} and FB\textsubscript{2} in humid conditions and suboptimal growth temperatures of 20°C and 20–30°C, respectively (159). Under these conditions, FB\textsubscript{1} and FB\textsubscript{2} have been commonly associated with improper handling of maize and cereals, such as wheat and millet. Short-term acute exposure to fumonisins causes a form of mycotoxicosis characterized by diarrhea and stomach cramping (160), while chronic intake in adults has
been associated with esophageal (161) and liver cancer (162). Furthermore, exposure in pregnant women is associated with developmental defects in their fetus, namely neural tube defects (163).

The toxicity of these compounds has been traced mainly to their ability to replicate the structure of cellular sphingosine and sphinganine. Both FB₁ and FB₂ bind the active site of enzymes required for the degradation of these sphingolipids, thereby preventing their catalysis and causing cellular accumulation. Since increased levels of sphingolipids are thought to reduce folate uptake in utero, and folate depletion increases risks for neural tube defects, this has been proposed as a mechanism of teratogenicity by fumonisins (164). However, there has been no correlation observed between cancer incidence and elevated sphingolipids (165), suggesting that there may be other mechanisms directing fumonisin’s carcinogenic effects, such as induction of apoptosis or increased reactive oxygen species (166).

In humans, measured urinary levels of FB₁ and FB₂ represent validated biomarkers for exposure (167). Urinary FB₁ is typically found in significantly greater abundance than FB₂, reflecting similar proportions in contaminated crops. For example, in adult cohorts from South Africa, Cameroon, and Nigeria, reported urinary FB₁ concentrations were 470–1520 pg/mg creatinine, 330 pg/mg creatinine, and 4.6 ng/L (167–170), while the same Cameroonian and Nigerian individuals had FB₂ concentrations below the limit of quantification and at 1.0 ng/L, respectively (169, 170). One country whose population which has been neglected in human exposure studies has been Rwanda, where elevated FB₁ and some FB₂ have been found in numerous food sources. Average detectable levels of FB₁ in Rwandan maize flour and cassava are similar, and range from 21.9–31.1 µg/kg, and for FB₂ are slightly lower at 10.1–17.0 µg/kg (156, 157). FB₁ was also found to contaminate banana beers brewed in Rwanda at a consistent level around 32 µg/kg (171). Although these are below the limits set by the EC and US FDA, which are 200–1000 ppb and 2000–4000 ppb, respectively, elevated concentrations could still be acquired from consuming these products in excess and the detriments of chronic low doses are still applicable.
1.3.3 Zearalenone (ZEN)

Zearalenone (ZEN), sometimes referred to as F-2 toxin, is a heat-stable mycotoxin that is also predominantly produced by the *Fusarium* fungi, making it a popular contaminant of cereals and grains. Like fumonisins, ZEN is secreted differentially by various species of *Fusarium*, and in greatest amounts in a warm, damp climate (172). Its negative effects, particularly for females, are due to its ability to act as an estrogen agonist, mediated by its structural similarities to the hormone (173). Thus, women with elevated ZEN are at greater risk for breast cancer (174, 175) and in pregnant women, fetal abnormalities may develop (27).

Human exposure to ZEN across African nations appears to be particularly prevalent. In several studies quantifying ZEN in Cameroon and South Africa, this compound was present in nearly all individuals, with the average urinary concentrations at 210 and 529 pg/mg creatinine, respectively (168, 169). In Rwanda, zearalenone is among one of the most commonly found mycotoxins in cassava, contaminating nearly 50% of this popular crop (157), though it was absent in another study of cassava flours (156). Beer brewed in Rwanda also reportedly contains 0.66-2.2 µg ZEN/kg (171). With the widespread and abundant contamination of crops with ZEN, as well as its clear influence on women’s health, additional studies are essential for understanding its impact in women in the developing world.

1.3.4 Ochratoxin A (OTA)

Ochratoxin A (OTA) contamination of food is perhaps the most widespread of all mycotoxins. This toxin is secreted by *Aspergillus ochraceus*, *A. carbonarius*, and *Penicillium verrucosum* into a variety of foods including coffee (176), grapes, and various grains. Its ingestion from these crops is often associated with various kidney diseases (177), including Balkan endemic nephropathy; however, research on the mechanism of this pathogenicity is limited to animals and correlative studies (178). With the seriousness of multiple other mycotoxins in African countries, as described above, OTA was not historically considered a major threat to human health. However, several studies have reported OTA commonly in Africa, such as in Cameroon, South Africa, and Nigeria where
urinary levels were 70 pg/mg creatinine, 41 pg/mg creatinine, and 0.2 µg/L, respectively (168, 169). Specifically in Rwanda, both cassava and peanut crops are reportedly contaminated with OTA at levels above the regulatory limits (156, 157). Considering the disparity between the lack of research in Rwanda and other nations in Africa, and the apparent prevalence of OTA contamination, there warrants additional studies in this area.

1.4 Rationale and Specific Aims

1.4.1 Rationale

Microorganisms and their metabolites significantly influence the health of females, with an outcome of health or disease being mediated by the genetic capabilities of the species present. The vaginal microbiota is shaped by several host factors, including carbohydrates, and it can either support vaginal homeostasis through the production of antimicrobial and anti-inflammatory lactic acid (62, 179), or form an environment conducive to pathogen infiltration (22). The healthy state is characteristic of a Lactobacillus-dominated vaginal microbiota, while the latter represents a form of dysbiosis currently termed BV, whereby bacterial diversity increases and lactobacilli and vaginal acidity are depleted (19, 32, 180). Treatment of this condition, typically a regimen of antibiotic metronidazole, is often ineffective at restoring vaginal lactobacilli and results in high rates of BV recurrence (121, 181). Thus, prophylactics and treatments are necessary to prevent remission and treat BV, but the ultimate goal should be to recover and enhance native lactobacilli and their beneficial properties.

The mono-culture methods that are utilized to evaluate interventions to the vaginal microbiota, and the animal models used prior to human trials are unsatisfactory to recapitulate the complexity of bacterial interactions in the vaginal microbiota that direct responses to a new carbohydrate source (182). The vaginal environment is not naturally devoid of sugars that might themselves contribute to microbial homeostasis. In particular, glycogen, released by vaginal epithelium, has long been thought to preferentially supplement lactobacilli due to a strong correlation between the two (34). Given its abundance, yet lack of studies demonstrating its metabolism by many of the most common vaginal bacteria, glycogen’s role in shaping the vaginal microbiota remains to be
determined. I hypothesize that carbohydrate sources, both intrinsic and external to the vaginal environment, can be manipulated to favour *Lactobacillus* dominance and activity in the vagina.

The negative impact of microbial products can also derive from sources external to the female microbiota. Mycotoxins are heat-stable compounds secreted mainly by *Aspergillus*, *Fusarium*, and *Penicillium* molds that have a range of carcinogenic, teratogenic, and toxic effects (138). Foods from countries in the developing world, such as Rwanda, are disparately contaminated with mycotoxins because the warm, humid climate is conducive to fungal growth, and there is limited information about how to recognize and prevent exposure. Though several studies have identified aflatoxins, fumonisins, ochratoxin A, and zearalenone in Rwandan crops (154–157), the biological exposure and toxic effects in women are still unknown, despite them being the predominant cultivators in Rwanda (183). I hypothesize that urine and blood mycotoxins will reflect levels in contaminated foods in Rwanda, and higher exposure will correlate with adverse health outcomes in women.

1.4.2 Aim 1: Evaluate lactulose, and a panel of prebiotics, for their ability to selectively stimulate vaginal lactobacilli and improve the metabolic profile

In studies on the gut microbiota, the ability of unique compounds to selectively stimulate lactobacilli and other beneficial organisms has been adopted with significant success (184). These compounds, called prebiotics, modulate the composition and activity of the microbiota toward a state that benefits the health of the host (132). In the vagina, dysbiosis is extremely common and involves significant reductions in the *Lactobacillus* population and disruptions to the metabolic profile. Several studies have evaluated prebiotics for stimulating lactobacilli in the vaginal microbiota to prevent or treat BV; however, none have examined their effects on the microbiota as a unit, or their metabolites (92, 135, 136, 185). As microbial products are the cause of malodour, inflammation, and other symptoms in vaginal dysbiosis (19, 186), they must be measured alongside evaluations of vaginal prebiotics.
In Chapter 2 of this thesis, I develop an effective polymicrobial batch culturing model to evaluate the potential of lactulose as a vaginal prebiotic, to stimulate lactobacilli, restore acidity, and reduce markers of BV.

1.4.3 Aim 2: Examine additional mechanisms through which lactulose improves the beneficial properties of vaginal epithelium and *Lactobacillus crispatus*

The consensus definition for prebiotics was recently expanded to encompass a broader repertoire of compounds, potential active sites, as well as their mechanism of action (132). Importantly, one must consider the impact prebiotics have on bacterial-bacterial interactions, as well as the host tissues they contact. Since *Lactobacillus crispatus* is most associated with feminine health, additional implications for its stimulation by prebiotics should be considered (32, 39). We previously observed that vaginal *Lactobacillus crispatus* strains ubiquitously fermented lactulose, resulting in significant pH decline and elevated lactic acid production. However, additional properties of lactulose stimulation may alter properties of the vaginal microbiota and host. To benefit the microbial ecosystem, lactulose should impart an advantage to *L. crispatus* against organisms involved in dysbiosis, which may be mediated by acid production, or secretion of the putatively identified antimicrobial peptide (bacteriocin) present in its genome (65). Conversely, integrating sugars into a new environment can disrupt polysaccharide-dependent attachment interactions between *L. crispatus* and the vaginal epithelium (187). Before taken into human trials, lactulose must also be tested for antagonistic effects on the vaginal epithelium, as its integrity is essential for vaginal health.

Therefore, Chapter 3 will address these additional properties and the effect lactulose has on bacterial and host interactions.

1.4.4 Aim 3: Evaluate how glycogen influences the growth and activity of vaginal microbes

Glycogen, a highly branched oligosaccharide consisting of glucose monomers, is the major carbohydrate source available in the female genital tract, and therefore an important component in shaping the resident microbiota. It is freed in large abundance from vaginal
epithelial cells, particularly when elevated estrogen levels stimulate its production (188). Initially, glycogen was thought to supplement vaginal lactobacilli because of the association between high glycogen levels, a Lactobacillus-dominant microbiota, and an acidic vaginal pH (34, 188, 189). However, most commonly dominant species such as L. crispatus, L. jensenii, and L. gasseri, as well as L. plantarum, have been observed to not utilize glycogen (190). This paradigm was seemingly resolved with the discovery that human α-amylases are also released into vaginal fluid from the epithelia, where they can metabolize glycogen into component sugars that are fermented by lactobacilli (191). However, these metabolites, mostly glucose and maltose, are almost ubiquitously utilized by bacteria and would not specifically benefit lactobacilli. Upon comparison of the vaginal transcriptome during health and BV, Macklaim et al. (83) observed that bacterial α-amylases are upregulated when lactobacilli are in low abundance and that these genes originate from BV-associated bacteria. Furthermore, the L. iners dominated microbiota has higher α-amylase activity than the L. crispatus dominated microbiota (43). It is reasonable to postulate then, that glycogen may stimulate the growth of unwanted BV organisms rather than the previously considered lactobacilli.

In Chapter 4, I therefore directly evaluate the glycogen fermentative capacity of vaginal lactobacilli and BV isolates, and its influence on the interaction between microbiota members.

1.4.5 Aim 4: Report on exposure levels of important mycotoxins to address how they may influence risk to women’s health in Rwanda

Mycotoxins are a serious concern for human health, especially in the developing world where an estimated 4.5 billion people are repetitively exposed (192). Acute exposure to these compounds results in mycotoxicosis, while chronic low doses have been linked to liver and kidney damage, cancer, growth stunting, and adverse fetal development (138). As weaker regulations on crops exist in developing countries and their climate is conducive to fungal growth, contamination with mycotoxins in these countries remains consistently high. Despite several studies observing aflatoxin, fumonisin, ochratoxin A, and zearalenone contamination in Rwandan crops (154–157), few studies have reported on the human exposure levels. As women represent both the primary contact to farmlands (183)
and targets for many of the health effects, specifically related to infant health, their exposure to these mycotoxins is important to quantify.

In Chapter 5, I report the development of a urinary multi-mycotoxin method using LC-MS/MS and measured biomarkers in a high-risk cohort of Rwandan women, and determine their impact on fertility and general health outcomes.
Chapter 2

Evaluation of prebiotics for maintenance and restoration of a Lactobacillus-dominated vaginal microbiota establishes lactulose as a promising candidate.

Parts of this chapter have been published in:
2.1 Abstract

Perturbations to the vaginal microbiota can lead to dysbiosis, including bacterial vaginosis (BV), which affects a large portion of the female population. In a healthy state, the vaginal microbiota is represented by low diversity and colonization by *Lactobacillus* spp., whereas in BV, these species are displaced by a highly diverse population of bacteria associated with adverse health outcomes. Since prebiotic ingestion has been a highly effective approach to invigorate lactobacilli for improved intestinal health, we hypothesized that these compounds could stimulate lactobacilli at the expense of BV organisms to support vaginal health. Monocultures of commensal *Lactobacillus crispatus*, *L. vaginalis*, *L. gasseri*, *L. johnsonii*, *L. jensenii*, and *L. iners* in addition to BV-associated organisms and *Candida albicans* were tested for their ability to utilize a representative group of prebiotics: lactitol, lactulose, raffinose and oligofructose. The disaccharide lactulose was found to most broadly and specifically stimulate vaginal lactobacilli, including the strongly health-associated *L. crispatus*, and importantly, not BV organisms or *C. albicans*. Using batch cultured vaginal samples, we showed that exposure to lactulose promoted *Lactobacillus* maintenance in healthy samples, but not restoration from dysbiosis. Regardless of initial health status, lactulose stimulated healthy acidity through lactic acid production, while suppressing malodor-associated putrescine and cadaverine. This provides support for further testing of lactulose to prevent dysbiosis, and potentially reduce the need for antimicrobial agents in managing vaginal health.

2.2 Introduction

In most healthy reproductive-age women, *Lactobacillus* spp. are the main constituents of the vaginal microbiota. Their ability to acidify this niche through lactic acid production (193), and otherwise beneficially modulate the environment (194), is profoundly important for female health and reproduction (195). However, in a condition known as bacterial vaginosis (BV), anaerobes such as *Gardnerella vaginalis*, *Atopobium vaginae*, *Prevotella* spp., and *Mobiluncus* spp. proliferate, sometimes causing aberrant symptoms and signs (89, 180). These organisms displace *Lactobacillus* spp., causing an elevation in vaginal pH and predisposing women to pelvic inflammatory disease, HIV-1 transmission, and infection by *Trichomonas, Chlamydia trachomatis* and *Neisseria gonorrhoeae* (22, 99,
Recurrent BV patients also have higher rates of vulvovaginal candidiasis, caused by overgrowth of the opportunistic *Candida albicans* (196). The condition is extremely common, with a reported prevalence of 30% in the United States (81), leading to a significant burden to both women and the health care system (197).

In recent years, researchers have identified metabolic changes that provide insight into BV symptomatology and a basis for novel detection methods. Lactic acid, produced as a metabolic by-product mainly by *Lactobacillus* spp., has long been understood to pose health benefits by acidifying the vagina (56, 57) and limiting pathogen viability (53, 59, 60). Thus, its absence has been associated with diversity and BV (19). One of several diagnostic criteria for BV includes odour presentation (58), which has been linked to biogenic amine production, namely putrescine and cadaverine (19, 84, 186, 198), by BV-associated *Dialister* spp. and *Prevotella* and *Porphyromonas* spp., respectively (84). Conversely, tyrosine levels are reduced in BV, likely because of their use as precursors for amine production (19, 198). Amine detection has been taken into spectrometry-based diagnostics for BV with some success (199), though there may be better alternatives. McMillan *et al.* (19) showed that gamma-hydroxybutyrate (GHB) was a reproducible, sensitive, and selective biomarker for BV and traced its origins to *G. vaginalis*, a common microbe associated with dysbiosis. Together, these reflect overall modifications of the metabolic profile during BV onset that are important in understanding its pathogenesis.

The treatment for BV has for decades been administration of metronidazole or clindamycin targeting anaerobes. However, in addition to side effects and failure to restore microbiota homeostasis, large numbers of women fail to respond or relapse within months of treatment (121, 200). While some women maintain a stable vaginal microbiota, most show fluctuating patterns due to antimicrobial agents, menstruation, and sexual activity, as well as unknown factors (39). Too often, restoration of a *Lactobacillus*-dominant microbiota does not occur. This has led to interventions containing probiotic therapies, such as *L. reuteri* RC-14 and *L. rhamnosus* GR-1, which help the indigenous lactobacilli recover (128–130).
Another approach worth considering is the use of a prebiotic, defined as a substrate that is selectively used by beneficial host microorganisms to confer a health benefit (132). These are generally food-grade compounds including inulin and other fructo-oligosaccharides (FOS), sugar alcohols, galacto-oligosaccharides (GOS), lactulose, and raffinose. They are known to stimulate native bifidobacteria and lactobacilli in the intestine, leading to a range of favourable gastrointestinal outcomes including reduced inflammation (201). The concept of using selective nutrients to supplement vaginal microbes was first introduced by our group in 1995, where we showed that skim milk improved vaginal *Lactobacillus* numbers in women with recurrent urinary tract infections (202). Several prebiotics have since been evaluated for their efficacy in stimulating vaginal lactobacilli both *in vitro* and in small human trials, including GOS (135, 136), FOS (135, 203), and glucomannan hydrolysates (185, 204). The assessment of these and other formulations, however, has been based on testing against single strains or characterizing dysbiosis using combinations of culture- and microscopy-based techniques, which lack the sensitivity needed to detect the numerous low abundance anaerobes present in BV. For example, organisms denoted BVAB1-3 have been deemed critical for BV onset and pathogenesis, yet have been unculturable to date (115, 205, 206). Instead, molecular techniques such as 16S rRNA sequencing and metabolomics (used in this study) should be adopted to accurately observe modifications to the vaginal microbiota and metabolome.

Therefore, the objective of the present study was to develop a method for testing the effects of prebiotic compounds against a range of biologically relevant vaginal lactobacilli and bacteria associated with dysbiosis, and to apply 16S sequencing and mass spectrometry-based targeted metabolomics to assess their impact.

### 2.3 Results

#### 2.3.1 Fermentation profiles of prebiotics by vaginal microbes

Following bacterial culture in 0.5% prebiotic-supplemented, dextrose-free media, pH was measured post-incubation to indicate whether prebiotics were fermented and if this process could restore acidity, an essential component of vaginal health. Lactulose increased the maximal growth of *L. crispatus* (*p*<0.0001), *L. gasseri* (*p*<0.05), *L. vaginalis* (*p*<0.01), and
L. jensenii RC-28 (p<0.0001) compared to no prebiotic media (Figure 2.1). Lactulose treatment also correspondingly lowered the pH compared to no prebiotic media for L. crispatus (p<0.001) and L. jensenii RC-28 (p<0.001) (Figure 2.1). Since a L. crispatus-dominated vaginal microbiota is most strongly correlated to positive health outcomes (25, 40), and strains of the same species can vary greatly in their ability to degrade sugars, the metabolism of lactulose by fifteen clinical L. crispatus isolates was demonstrated (Figure 2.2). Although lactulose did not significantly elevate the viable counts of L. iners (p=0.50), the pH was lowered compared to no prebiotic media (p<0.01) (Figure 2.1). This suggests that L. iners produces fermentation products upon lactulose inoculation, despite not growing. Lactulose limited the growth of C. albicans (p<0.001) and A. vaginae (p<0.05) compared to media without prebiotic, while not affecting the growth of G. vaginalis, P. bivia, or M. curtisii (Figure 2.2). Overall, lactulose was broadly and effectively used by vaginal Lactobacillus spp., while not stimulating potential pathogens.

Oligofructose, raffinose and lactitol were less preferred than lactulose by vaginal lactobacilli in culture. Oligofructose improved the growth of L. gasseri (p<0.001) and L. jensenii 25258 (p<0.0001) compared to no prebiotic control, though acidic pH was only reached by L. jensenii 25258 (p<0.001) (Figure 2.1). However, oligofructose antagonized L. iners (p<0.01) (Figure 2.1) and attenuated the growth of C. albicans compared to no prebiotic media (p<0.0001) (Figure 2.3A). Raffinose elevated the maximal growth of L. crispatus (p<0.0001), L. gasseri (p<0.05), and L. vaginalis (p<0.01), causing media acidification in L. vaginalis culture only (p<0.05) (Figure 2.1). Conversely, raffinose also lowered L. iners viability compared to media without prebiotic (p<0.01) (Figure 2.1). Although lactitol only stimulated L. crispatus growth (p<0.0001), acidic products were produced by both lactitol-inoculated L. crispatus (p<0.001) and L. jensenii RC-28 (p<0.05) (Figure 2.1). Furthermore, lactitol reduced C. albicans density relative to no prebiotic media (p<0.0001) (Figure 2.3A). No notable growth of G. vaginalis, A. vaginae, M. curtisii, or P. bivia was observed in oligofructose, raffinose, or lactitol (Figure 2.3B).

2.3.2 Bacterial composition of vaginal samples incubated with prebiotics

Having established that lactulose most effectively stimulated Lactobacillus spp. in monoculture, we developed a method to culture the community of vaginal microbiota from
Figure 2.1: Growth and pH of vaginal lactobacilli cultured in prebiotics

Individual cultures of vaginal lactobacilli inoculated in 0.5% prebiotic media prior to pH testing. For growth curves, points represent mean (OD$_{600}$) or geometric mean (CFU/mL) of 3-7 replicates ± SEM, with differences determined using 2-way ANOVA with Dunnett’s multiple comparisons test. For pH measurements, bar heights are mean pH ± SD, with differences to control determined using Kruskall-Wallis’ with Dunn’s multiple comparisons tests.
Figure 2.2: Lactulose is ubiquitously utilized by vaginal \textit{L. crispatus} clinical isolates

Individual cultures of vaginal \textit{L. crispatus} isolates obtained from women with or without BV were inoculated in dextrose-free MRS supplemented with 0.5\% (w/v) lactulose. Points represent the mean OD$_{600}$ ± SD.
Figure 2.3: Growth and pH of *C. albicans* and BV organisms cultured in prebiotics

(A) *C. albicans* supplemented with 5% prebiotic. (B) BV-associated bacteria inoculated in 0.5% prebiotic media prior to pH testing. For growth curves, points represent mean (OD$_{600}$) or geometric mean (CFU/mL) of 3-7 replicates ± SEM, with differences determined using 2-way ANOVA with Dunnett’s multiple comparisons test. For pH measurements, bar heights are mean pH ± SD, with differences to control determined using Kruskall-Wallis’ with Dunn’s multiple comparisons tests.
swabs collected from healthy women. These were then inoculated in media containing 0.5% prebiotic, or control media without prebiotic. Initially, all four healthy swabs were dominated by *Lactobacillus* spp., with those from Subjects 1, 3, and 4 mostly composed of *L. crispatus*, and Subject 2 mostly with *L. iners* (Figure 2.4). The microbial community of each subject reacted variably to prebiotic treatment. Notably, each prebiotic appeared to improve the resilience of lactobacilli. In Sample 1, lactobacilli increased in abundance in each prebiotic relative to control media by 48 hours (Figure 2.4). Lactulose particularly maintained *Lactobacillus* dominance, as noted by its restoration to above 90% abundance by 48 hours (Figure 2.4). In Sample 2, lactulose, as well as lactitol and raffinose, improved *Lactobacillus* maintenance, while oligofructose supplementation caused a relative abundance shift toward *Staphylococcus* spp. compared to the no-prebiotic control (Figure 2.4). Sample 3 microbiota responded similarly to treatment with lactulose, lactitol, and oligofructose, with early time-points showing elevated *Lactobacillus* abundance compared to control before 48 hours post-inoculation (Figure 2.4). Raffinose supplementation, on the other hand, improved *Lactobacillus* abundance throughout 48 hours (Figure 2.4). Only lactitol induced prolonged maintenance of *Lactobacillus* spp. in Sample 4, compared to no-prebiotic media and lactulose, raffinose, and oligofructose supplementation (Figure 2.4).

To elucidate whether these proportional changes were due to increased numbers of beneficial lactobacilli, rather than a loss of aerobic organisms, we next used qPCR for total *Lactobacillus* numbers. Lactulose treatment increased *Lactobacillus* abundance by 48 hours in *L. crispatus*-dominant Samples 1, 3, and 4, and in early time-points for Sample 2 (Figure 2.5A). Oligofructose consistently stimulated lactobacilli, increasing its abundance in all four swabs after 48 hours; though the mean change was not significant (p=0.2388) (Figure 2.5A). Raffinose slightly increased total lactobacilli after 48 hours in Samples 1 and 3, while decreasing their numbers in Samples 2 and 4 compared to no-prebiotic media (Figure 2.5A). Lactitol increased *Lactobacillus* levels in Samples 1 and 4, while decreasing them in 2 and 3 (Figure 2.5A).

Since BV-related organisms were in low relative abundance and therefore were not observable in the context of the whole microbiota, their abundances, as a centered-log ratio, were monitored individually. Notably, none of the detectable BV-associated organisms
Figure 2.4: Swab microbiota dynamics throughout inoculation in prebiotics

Vaginal swab microbiota from four separate donors were cultured in dextrose-free vaginally-defined medium with 0.5% (w/v) prebiotic. Bar heights represent the fraction of the microbiota taken up by each genus at the designated sampling point, as determined by 16S rRNA sequencing of the V4 region.
A  Total *Lactobacillus* (16S rDNA copies/mL)

B  *M. curtisi* (cells/mL)

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<table>
<thead>
<tr>
<th></th>
<th>No prebiotic</th>
<th>Lactitol</th>
<th>Lactulose</th>
<th>Raffinose</th>
<th>Oligofructose</th>
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Average

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Time (h)
Figure 2.5: Growth of lactobacilli and *M. curtisii* in prebiotic-supplemented vaginal swab microbiota

Concentration of (A) *Lactobacillus* spp., and (B) *M. curtisii* 16S rDNA from vaginal swab microbiota grown in 0.5% prebiotic. Points are individual measures of growth for each individual swab, and are the geometric mean ± SEM for the average of all swabs. Geometric means were analyzed for significant differences using two-way ANOVA with Dunnett’s multiple comparisons test.
present in the swab communities were stimulated by prebiotic treatment (Figure 2.6). Two commonly characterized BV organisms, *A. vaginae* and *M. curtisii*, were not proportionally abundant enough to be identified by sequencing, so absolute quantification qPCR was adopted to specifically target these bacteria. In further support of its absence, *A. vaginae* was not detected with this technique (Data not shown). *M. curtisii* levels were elevated to the greatest extent by lactitol and oligofructose in Samples 1 and 4, though neither reached significance overall (Figure 2.5B).

### 2.3.3 Metabolite changes in prebiotic-cultured healthy vaginal swab communities

Several other important indicators of vaginal health include acidity and metabolites from actively fermenting commensal lactobacilli (53, 57). To determine whether acidity could be restored by prebiotic treatment in the healthy vaginal microbiota model, swab samples were pH tested following 48 hours of incubation with the prebiotics. Lactulose was found to significantly lower the pH (p<0.05), a phenomenon not observed with any other prebiotic (Figure 2.7A). Since *in vivo* vaginal acidification in healthy women is predominantly due to lactate production by lactobacilli (56), LC-MS quantification of this metabolite was performed. After 48 hours, lactulose and oligofructose increased lactate production in the cultures of Samples 1, 2 and 4 compared to no-prebiotic media, but only increased lactate at early time points in Sample 3 (Figure 2.7B). Lactitol also increased lactate abundance in Samples 2, 3, and 4, while raffinose lowered media lactate compared to no-prebiotic control in Samples 1, 2, and 3 (Figure 2.7B).

To confirm that prebiotic utilization by bacteria in the human samples was the cause of media acidification and lactate production, GC-MS of spent media was used to detect levels of prebiotics following incubation. Both lactulose (p<0.05) and lactitol (p<0.01) were significantly lowered in the media following 48 hours of swab bacterial growth. Conversely, raffinose levels remained static (Figure 2.8), indicating that changes caused by raffinose treatment were not due to bacterial metabolism of this prebiotic. Oligofructose’s highly polymerized and variable structure prevented accurate detection of this prebiotic, and therefore it was eliminated from this analysis.
Figure 2.6: Abundance of various BV-associated organisms from healthy vaginal swab consortia grown in prebiotics
Mean centered log-ratios ± SD of BV-associated bacterial OTUs from 16S rRNA sequencing of the V4 region. Samples were taken throughout growth of batch cultured healthy swab bacteria in 0.5% (w/v) prebiotic or no prebiotic control. There were no significant differences (p>0.05) in proportional abundance between any prebiotic treatment and control media, according to two-way ANOVA with Dunnett’s multiple comparisons test (n=4).
Figure 2.7: Metabolic analysis of healthy vaginal swab consortia in prebiotics
(A) Mean pH ± SD of swab communities following 48 hours of growth in prebiotics. Statistical significance determined according to the Kruskall-Wallis test with Dunn’s multiple comparisons (*p<0.05). (B) Peak area of LC-MS-detected lactate, log2 corrected, in swab bacterial supernatant. Dots are individual measures of growth over time.
Figure 2.8: Abundance of lactitol, lactulose, and raffinose following healthy vaginal swab consortia growth

Peak area of GC-MS-detected prebiotics, log2 transformed, in swab bacterial supernatant before addition of bacteria (media) and after 48 hours of growth. Mean quantities are significantly different from baseline, according to the one-sample t-test (*p<0.05, **p<0.01).
Since GHB and 2-hydroxyisovalerate are specific biomarkers of BV, and cadaverine, putrescine, and tyramine are responsible for vaginal odor (19, 84), we measured these metabolites in healthy vaginal samples inoculated with prebiotics, but did not detect them in any growth condition (Data not shown).

2.3.4 Lactulose does not restore a Lactobacillus-dominated vaginal microbiota in an in vitro co-culturing model of dysbiosis

Nugent scoring of swabs upon collection identified samples DES123, NVP548, RAC123, SAO123, SHA966, and XYZ456 as BV positive, and samples 999ZZZ, ALT519, CUB452, and LIS123 as intermediate. Using 16S rRNA sequencing of the V4 region, changes to the bacterial composition of batch cultured swabs upon lactulose treatment were monitored. For samples ALT519, DES123, and NVP548, there were few, if any, differences in the bacterial profile of lactulose-treated and control swab consortia (Figure 2.9). Swabs 999ZZZ, CUB452, SHA966, an XYZ456 inoculated with lactulose were nearly dominated by Streptococcus by 48 hours, at the expense of Proteus, Escherichia coli, Finegoldia/Gardnerella vaginalis, and Peptostreptococcus/Proteus/Dialister in the respective controls (Figure 2.9). Despite a transient shift to Streptococcus at 24 hours, the population in swab LIS123 shifted almost entirely to L. crispatus in lactulose-treated media by 48 hours, while its control counterpart retained greater diversity and a population consisting of Porphyromonas, L. jensenii, and L. crispatus (Figure 2.9). Control- and lactulose-treated sample RAC123 achieved similar diversity at 48 hours, though some of the genera were dissimilar, including increased Streptococcus and Sutterella and decreased Parvimonas and Howardella spp. with lactulose treatment (Figure 2.9). Lastly, lactulose-supplemented SAO123 had a much lower proportion of undefined Lactobacillus spp. after 24 and 48 hours, and increased relative abundance of Streptococcus, E. coli, and Proteus (Figure 2.9).

To narrow down the impact of lactulose on Lactobacillus abundance within the swab consortia, CLR s of OTUs representing predominant lactobacilli were plotted over time. Overall, lactulose had no significant impact on the three major species detected (L. crispatus, L. iners, and L. jensenii), though a slight trend toward increased total
Figure 2.9: 16S rRNA microbiota composition of aberrant vaginal consortia inoculated with 1% (w/v) lactulose

Bacterial collected from vaginal swabs of 10 separate donors with an intermediate or BV-associated Nugent score were cultured in dextrose-free vaginally-defined medium with 1% (w/v) lactulose (Lac) or no prebiotic (Ctrl). Bar heights represent the fraction of the microbiota taken up by each genus at the designated sampling point, as determined by 16S rRNA sequencing of the V4 region.
*Lactobacillus* was observed (Figure 2.10). Multivariate analyses of the aberrant microbiotas in lactulose and control media did not detect any overarching community differences in the two treatment groups (Data not shown).

### 2.3.5 Lactulose-induced metabolic changes of dysbiotic vaginal swab microbiota.

As in the previous exploration of prebiotic effects on the healthy vaginal metabolic profile, pH and metabolites associated with BV were quantified throughout aberrant swab inoculation in lactulose. Following 48 hours of growth in lactulose, the overall mean pH of BV positive and intermediate swab batch cultures was lowered significantly compared to control (p<0.01, Figure 2.11A). This response was bimodal, with four samples having substantially lowered pH compared to others, corresponding to samples CUB452, LIS123, RAC123, and SHA966 (Figure 2.11A). Apart from LIS123, these samples were related in that their *Streptococcus* population was elevated by 48 hours in lactulose (Figure 2.9). In contrast, lactulose-treated LIS123 was nearly dominated by *Lactobacillus crispatus*, compared to approximately 10% abundance in control (Fig. 2.9). Lastly, acidic response was not directly related to the patient’s original Nugent score (Figure 2.11A). Upon measuring levels of common acidic bacterial metabolites in the vaginal microbiota, a significantly elevated (p<0.001) concentration of lactic acid was observed in the media following 48 hours of aberrant swab inoculation (Figure 2.11B).

Several additional metabolites of importance, apart from acids, were quantified throughout the growth period. Surprisingly, lactulose was not depleted from the media after 24 hours or 48 hours of bacterial growth (p>0.05, Figure 2.12A). There were also no differences in tyrosine production or consumption during the incubation period (p>0.05, Figure 2.12B), and gamma-hydroxybutyrate was not detected in any sample (Data not shown). However, lactulose treatment significantly lowered secretion of putrescine (p<0.001) and cadaverine (p<0.05) (Figure 2.12C).
Figure 2.10: Mean abundance of *Lactobacillus* spp. in aberrant swab microbiotas treated with lactulose

Mean centered log-ratios of 16S rRNA sequences representing total *Lactobacillus*, *L. crispatus*, *L. iners*, and *L. jensenii* from swab batch cultures grown in dextrose-free VDMP ± 1% (w/v) lactulose. Genus and species were identified by comparison to the Silva reference database. *Lactobacillus* abundance was only included if read counts were greater than blank 16S extractions. Points represent mean abundance ± SD (n=10). Significance between control and lactulose treatment at each time point was determined using two-way RM ANOVA with Sidak's multiple comparisons test (p>0.05).
Figure 2.11: Treatment with lactulose triggers acid production by aberrant vaginal swab microbiotas in vitro

Acidic characteristics of aberrant swab microbiotas throughout growth in 1% (w/v) lactulose-supplemented or control dextrose-free VDMP. Following growth, (A) Mean pH ± SD of supernatants were measured. Significance was determined using Kruskall-Wallis with Dunn’s multiple comparisons test (*p<0.05). Points are coloured according to the Nugent score of the associated swab. (B) Concentration (in µg/mL) of lactic and succinic acids in supernatants at various time points, determined using targeted LC-MS with external standards. Significant differences between lactulose-treated and control media at each time point were determined using two-way RM ANOVA with Sidak’s multiple comparisons test (***p<0.001).
Figure 2.12: Levels of lactulose, tyrosine, and production of odor-associated BV metabolites by aberrant vaginal swab microbes

Metabolic characteristics of aberrant swab microbiotas throughout growth in 1% (w/v) lactulose-supplemented (●) or control (○) dextrose-free VDMP were determined using targeted LC-MS with external standards. Points represent concentration (in µg/mL) of (A) lactulose, (B) tyrosine, and (C) odor-associated amines in each supernatant at the indicated time following inoculation. Significant reductions in lactulose from initial measurement were determined using one-way ANOVA with Dunnett’s multiple comparisons test (p>0.05), while differences in tyrosine and amine concentrations between lactulose-treated and control media at each time point were determined using two-way RM ANOVA with Sidak’s multiple comparisons test (*p<0.05, ***p<0.001).
2.4 Discussion

Herein we have characterized a candidate prebiotic, lactulose, for its ability to promote microbial and metabolic homeostasis in healthy and dysbiotic vaginal microbiota models, independent of antibiotic use. We showed that supplementation of bacterial monocultures with lactulose stimulated *L. gasseri*, *L. vaginalis*, *L. jensenii* RC-28 and 16 strains of *L. crispatus*, while antagonizing *C. albicans* and *A. vaginae*. Additionally, although lactulose did not improve the viability of *L. iners*, the drastically lowered media pH suggests that it was fermented by *L. iners*. These initial experiments indicate that lactulose selectively propagates a range of commensals and not vaginal pathogens. In a healthy model, lactulose promoted maintenance of a *Lactobacillus*-dominated community and production of acidic metabolites, namely lactate. Despite its inability to restore lactobacilli in a similar *in vitro* model of dysbiosis, lactulose again effectively increased overall lactate secretion and acidity, while also lowering levels of vaginal odor-associated putrescine and cadaverine.

Variation in the positive, *Lactobacillus*-stimulating response by lactulose between the healthy and aberrant swab models likely reflects difficulty lactobacilli experienced in outcompeting a whole community of bacteria and the challenges associated with finding a completely selective prebiotic. As we are taking the community from a likely homeostatic state and delivering fresh nutrients, initial bacterial abundance will weigh greatly on its ability to outcompete other organisms. Despite the apparent preference of lactulose for lactobacilli, both models showed a notable proportion of aerobes, especially *Streptococcus* in the aberrant population, that gained dominance. There are very few studies that have monitored vaginally-relevant aerobic microbes’ growth in lactulose since this is the first case utilizing it for vaginal health; however, others have shown stimulation of oral and enteric *Streptococcus* spp. by lactulose (208–210). Weakening this argument is the observation that lactulose was not spent from the media inoculated with aberrant microbiotas, suggesting that it is not consumed by these bacteria. Furthermore, since these organisms are often not colonizers of the vaginal niche and are likely only present due to unavoidable exposure to aerobic conditions, this may not reflect *in vivo* efficacy of the tested compounds. Regardless, further considerations might be of benefit prior to taking this into humans. A greater understanding for the lactulose-metabolizing potential of the
dysbiotic vaginal microbiome, perhaps through uncovering specific enzymes required for its degradation and analyzing the transcriptome in health and disease for such genes (83), might better predict its efficacy in humans.

Across both healthy and dysbiotic in vitro models, lactulose induced beneficial changes to the metabolic profile. Its pH-lowering effect is largely attributable to increased secretion of lactic acid, the predominant fermentation metabolite of lactobacilli. Importantly, lactic acid and low pH are indicative of vaginal health and inhibit the development of BV, HIV-1 infectivity, and other dysbioses (53, 59, 60). Lactulose has also been reported to rapidly increase media acidity in non-vaginal Lactobacillus cultures (211, 212), suggesting that it might provide a broad and immediate advantage to acid-tolerant, commensal lactobacilli over pathogens. In our model of dysbiosis, lactulose significantly attenuated putrescine and cadaverine production, amines that are associated with vaginal microbiota diversity (186, 199) and BV-related malodor (19, 84, 198). More recently, these metabolites have been observed to enhance pathogen biofilm formation (213), an important component of BV pathogenesis and antibiotic resistance (122, 124, 214). As lactulose is not digested in the simulated aberrant microbiotas, the mechanism of action might be through inhibition of lysine and ornithine decarboxylases, the major enzymes responsible for cadaverine and putrescine synthesis in vaginal microbes (186). However, without controlled studies this is largely speculative. Considering its beneficial effects on the bacterial metabolome, lactulose is well founded for use as a vaginal prebiotic.

Of the other prebiotic compounds assessed, lactitol was also fermented by vaginal lactobacilli in monoculture and increased the proportional abundance of Lactobacillus spp. in the vaginal sample consortium. However, despite an increase in media lactate, this compound did not restore an acidic pH. This suggests that the quantity of lactate produced by the lactitol-treated consortium was not sufficient to lower media pH. In practice, lactitol could be administered with pH-lowering reagents in a two-pronged approach, a concept that has been taken into product development but not yet evaluated. Lactitol administered with lactobacilli following clindamycin treatment has been shown to improve recovery from BV (215), but the role of lactitol alone was not delineated, leaving questions about its contribution to this therapy.
In culture, raffinose was fermented by several *Lactobacillus* strains, but it appeared to antagonize *L. iners*, a common vaginal commensal found in women with and without BV (46, 193). Although raffinose positively altered the composition of the vaginal swab consortium, it did not directly stimulate lactobacilli. This suggests that raffinose inhibits the growth of bacteria such as *Staphylococcus* or *Enterococcus* spp., thereby allowing commensal lactobacilli to propagate. This is further supported by the lack of fermentation products produced and high levels of raffinose remaining in the spent media of the vaginal sample. However, other researchers have also observed that raffinose can be used by *Trichomonas vaginalis* (216), the vaginal parasite responsible for trichomoniasis, further underscoring its unsuitability as a vaginal health agent.

Oligofructose was utilized by *L. gasseri* and *L. jensenii* 25258 in our study, and *L. acidophilus* in a previous investigation (203). However, despite its *Lactobacillus*-stimulating properties in our vaginal microbiota model, oligofructose did not increase the proportion of lactobacilli, and it antagonized *L. iners* AB-1 in culture. Oligofructose also stimulated growth of *C. albicans*, the causative agent of vulvovaginal candidiasis, and did not acidify the swab sample despite doing this in mono-culture. Since polymerized oligosaccharides such as oligofructose tend to be fermented slower and to a lesser degree than short oligosaccharides (217), it may have taken longer than the allotted 48 hours to produce sufficient metabolic products to lower the pH. It is important to note that the retention time of prebiotics in the vagina is likely much shorter than colonic transit time and therefore quick-releasing effects would be optimal for this niche. Others have observed that FOS with lower degrees of polymerization than oligofructose are used by vaginal *Lactobacillus* isolates and not *C. albicans* or *G. vaginalis* (135), thus this does not rule out the potential for all oligosaccharides in vaginal application. However, only a single strain of each lactobacilli was tested in monoculture in these studies, so their effect on a complex culture system remains to be investigated. For the reasons stated above, our results suggest against the use of oligofructose for vaginal health.

The model developed herein for studying the effects of prebiotics on the vaginal microbiota uses the organisms already active within the vaginal ecosystem of each subject. Although less reproducible than a defined steady-state culture, utilizing samples taken from
numerous donors allows improved insight into the breadth of responses that could be observed in vaginal microbiota interventions. As we observed here, despite genus-level similarities in microbiota composition, the selective nature of prebiotics exerts variable effects on bacterial proportions because of differences in bacterial expression of carbohydrate metabolism genes, even at the strain level (218, 219). As any candidate intervention must eventually be applicable to women, a method that more closely predicts diversity in responses has merit. Ultimately, lactulose must be taken into a randomized clinical trial to prove its functionality in humans.

In summary, we have demonstrated that lactulose stimulates vaginal lactobacilli, but not common dysbiotic organisms, promotes a healthy acidic environment, and limits the production of BV odor-associated metabolites. Confirmation in this novel human model provides a basis for further exploration of lactulose as a vaginal prophylactic. We have observed the greatest benefit for maintenance of a eubiotic microbiota, which is broadly applicable due to the frequent microbial fluctuations women experience throughout the menstrual cycle (37) and from sexual activity (109, 113). As a treatment for dysbiosis, lactulose may be valuable in conjunction with antibiotics or more Lactobacillus-stimulating therapeutics because of its positive effects on pH and the metabolic profile. Another forthcoming potential application may include conditioning an individual’s own aberrant microbiota ex vivo with lactulose for beneficial reintroduction into the vagina, a concept that has been coined TripleA therapy (220). Ultimately, this inexpensive compound may reduce the necessity for antibiotics, including their side effects and inability to prevent dysbiotic recurrences.

2.5 Materials and Methods

2.5.1 Strains, growth conditions, and prebiotics used

Vaginal Lactobacillus strains (L. crispatus ATCC 33820T, L. vaginalis NCFB 2810, L. gasseri ATCC 33323T, L. jensenii RC-28 and L. jensenii ATCC 25258) and L. johnsonii ATCC 20553 were cultured on MRS agar (Becton, Dickinson & Company, Sparks, MD) anaerobically in jars at 37°C. L. iners AB-1 and BV-associated bacteria Gardnerella vaginalis ATCC 14018, Atopobium vaginae ATCC BAA-55, Prevotella bivia ATCC
29303, and *Mobiluncus curtisii* ATCC 35241 were cultured on Columbia Agar with 5% sheep’s blood (CBA) (Becton, Dickinson & Company) anaerobically at 37°C in a controlled chamber (80% N₂, 10% CO₂, 10% H₂). *Candida albicans* TIMM 1768 was aerobically cultured on Lysogeny Broth (LB) agar at 37°C. Prebiotics selected were lactitol monohydrate (Alfa Aesar, Ward Hill, MA), lactulose (Alfa Aesar), D (+)-raffinose (Sigma, St. Louis, MO), and Orafti P95 oligofructose (Beneo, Oreye, Belgium).

OD₆₀₀ measurements were taken for *Lactobacillus* growth curves, excluding *L. iners*, in dextrose-free MRS with 0.5% (w/v) prebiotic using the MultiSkan Ascent (Thermo-Fisher, Waltham, MA). Due to their inability to be cultured in a microaerophilic environment, growth of *L. iners*, *G. vaginalis*, *A. vaginae*, *P. bivia*, and *M. curtisii* was monitored using drop plating in an anaerobic chamber (80% N₂, 10% CO₂, 10% H₂) in dextrose-free New York City (NYC) III broth with 0.5% prebiotic. Growth was determined by comparison to control media without dextrose. Once the growth media was spent, cultures were subjected to pH fermentation testing (VWR International, Radnor, PA). *C. albicans* viability in dextrose-free Tryptic Soy Broth (TSB) with 5% prebiotic was measured by optical density during constant aerobic agitation at 37°C.

2.5.2 Confirmatory analysis of clinical *L. crispatus* strains

The clinical *L. crispatus* strains were isolated from vaginal swabs used to remove abundant mucus during routine cervical examinations at the sexually transmitted infections (STI) clinic in Amsterdam, the Netherlands. These swabs, which are normally discarded, were collected in June and July 2012 and immediately placed in transport media with additional 15% glycerol and stored at -80°C. As visitors to the STI clinic were informed that their samples may be used for scientific research after anonymization and no extra clinical procedure was performed, no official approval under the Dutch Medical Research Involving Human Subjects Act (WMO) was required (reference number W12_86 # 12.17.0104). For this study, vaginal swabs from women with or without BV, according to the Nugent score, were plated on TSB agar supplemented with 5% serum and 0.25% lactic acid and pH set to 5.5 and grown under micro-aerobic conditions (6% O₂) for 2-3 days (221). *L. crispatus* strains were identified based on colony morphology and their 16S rRNA sequence. To measure growth in lactulose, *L. crispatus* strains were inoculated, as above,
in dextrose-free MRS supplemented with 0.5% (w/v) lactulose, and OD_{600} measurements were taken every 30 min for 24 hours.

2.5.3 Healthy subjects and vaginal swab collection

Four healthy premenopausal women (aged 25 to 30) volunteered for the study, approved by the Health Sciences Research Ethics Board at Western University (HSREB#106089, Appendix A.1). After signing informed consent, women self-swabbed the lateral vaginal walls five times using separate sterile Dacron® swabs. A healthy vaginal pH <4.5 was confirmed by participants using pHem-Alert® applicator keys (Gynex Corporation, Redmond, WA). Confirmation of healthy status was performed by smearing a swab on a microscopic slide and Gram staining (Becton, Dickinson & Company), and scored according to the Nugent system (87). Subjects were included only if a normal Nugent score of 0-3 was observed, indicating the presence of large Gram-positive rods. Independent swabs were used for initial microbiota analysis and assessment of prebiotic effects over time.

2.5.4 Incubation of healthy vaginal samples in prebiotics

Each vaginal swab was placed in 1 mL of PBS and vortexed for 5 min to release the bacteria and cellular contents. The liquid was then equally distributed into dextrose-free Vaginally-Defined Media + Peptone (VDMP) (222) with or without 0.5% (w/v) lactitol, lactulose, raffinose, or oligofructose. Individual time points and prebiotic growth conditions had separately designated tubes. Growth of swab bacteria was performed in anaerobic jars at 37°C until designated time points, when tubes were centrifuged for 10 min at 21 000 × g to separate cells from media. The pellet was subjected to microbiome analysis (see below), and supernatant assessed for metabolites by mass spectrometry, and for acidification using pH 2.0-9.0 test strips (VWR).

2.5.5 Non-healthy subjects and vaginal swab collection

Ethics approval for collection of vaginal swabs was obtained (HSREB#106089, Appendix A.1). Ten pre-menopausal women (aged 18 to 55) were recruited at Victoria Family Medical Centre in London, Canada. After signing informed consent, vaginal swabs were
obtained by a medical professional in addition to routine cervical examinations. Immediately upon collection, swabs were placed in liquid Amies media as a part of the ESwab™ anaerobic transport system (Copan Diagnostics Inc., Murietta, CA) and processed in the laboratory. A second swab was obtained to determine health status of the microbiota using the Nugent scoring system.

2.5.6 Incubation of non-healthy vaginal samples in lactulose

Vaginal swabs suspended in liquid Amies media were vortexed on low for 5 min to extract bacterial cells. The liquid was then equally distributed into pre-reduced dextrose-free VDMP with or without 1% (w/v) lactulose. Growth of swab bacteria was performed in an anaerobic chamber (80% N₂, 10% H₂, 10% CO₂) at 37°C until 24 and 48 hours post-inoculation, when separate designated tubes were removed. Cells were separated from supernatant by centrifugation for 10 min at 21 000 × g and stored separately at -80°C. The pellet was subjected to microbiome analysis (see below), and supernatant assessed for metabolites by mass spectrometry, and for acidification using pH 2.0-9.0 test strips (VWR).

2.5.7 Microbiota analysis by 16S rRNA sequencing

2.5.7.1 DNA extraction

Microbial DNA was extracted from swab microbiota using the Powersoil®-htp 96-Well Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA), according to the manufacturer’s instructions.

2.5.7.2 PCR amplification and sequencing

The V4 variable regions of 16S ribosomal RNA were amplified in a PCR reaction containing 50 µL light mineral oil, 1 µL sample DNA, and 10 µL each of barcoded primers at 3.2 pMol/µL, which together were set to 85°C before adding 20 µL of GoTaq master mix (Promega, Madison, WI). Reactions were primed at 95°C for 3 min, followed by 25 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min. Samples, media blanks, extraction blanks, and amplification blanks, were subsequently prepared and sequenced at the London Regional Genomics Centre (lrgc.ca, London, Ontario, Canada). First, DNA was quantified using a Qubit 2.0 Fluorometer (Thermo-Fisher), pooled at an equal volume
of each sample, and purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany). Purified amplicons were then paired-end sequenced with 250 cycles on an Illumina MiSeq (San Diego, CA) in 5% Phi X.

2.5.7.3 Data analysis

The protocol for initial processing of reads was adapted from the dada2 workflow by Dr. Greg Gloor (github.com/ggloor/miseq_bin), using the “dada2” and “ShortRead” packages in R version 3.2.2 (r-project.org). Read quality was determined by plotting quality profiles for both the forward and reverse reads. Filtering was performed based upon quality profiles and reads were trimmed to the expected length of 183 and 174 for V4 with paired-end 2x250 cycle sequencing using 12-mer barcodes. Dereplication was performed to summarize individual sequence units (ISUs) by abundance in each sample. Reads derived from PCR or sequencing errors were detected and removed using joint sample inference and error rate estimation on dereplicated ISUs. Pairs of forward and reverse reads were then overlapped and merged into complete sequences. By summarizing overlapping sequences by length, outliers were removed: five 200 bp sequences, one 201 bp sequence, and two 209 bp sequences were identified and removed, leaving only sequences of 238-240 bp. Chimeric sequences were also identified and removed. The final output of 139 sequences at this stage can be found at DOI:10.6084/m9.figshare.4657192 (figshare.com).

Taxonomy was generated to the genus level by comparison of best hits to the Silva rRNA database v123, and to the species level, where possible, according to the Silva species assignment database v123 (www.arb-silva.de/silva-license-information). Taxonomy was designated when sequences matched the species with 100% identity and there were no other matches above 97% identity. OTUs were then created by grouping at the genus level. Only OTUs with an abundance of at least 1% across all samples were included for further analysis, leaving only 10 unique genera. To calculate centered log-ratios for compositional analysis (223), zero-value OTU counts were replaced with an estimate value, then subjected to CLR calculation. Resulting CLRs were graphed in stacked barplots using R (r-project.org).
2.5.8 Absolute quantification RT-qPCR

Real time (RT)-qPCR was performed on swab gDNA for 16S or 16S-23S rRNA gene of *A. vaginae*, *G. vaginalis*, *L. crispatus*, *L. gasseri*, *L. iners*, *L. jensenii*, *L. vaginalis*, and total *Lactobacillus*, and the mucin-desulfatating sulfatase *mdsC* gene from *P. bivia*, as previously described (224, 225). Concentrations of forward and reverse primers in each reaction were as follows: 200 nM (LgassF/LgassR, InersFw/InersRev, LV16s_23s_F/LV16s_23s_R3, *M. curtisii*F/R, PBsulF/PBsulR), 150 nM (LBF/LBR), 100 nM (LcrisF/LcrisR), 300 nM (LjensF/LjensR), 700 nM (AV-F/AV-R), 1250 nM (F-GV1) and 625 nM (R-GV3). Reactions were performed in triplicate with 5 µL of 100-fold diluted gDNA, 5 µL of primers at their optimal concentration in ddH$_2$O, and 10 µL Power SYBR® Green PCR Master Mix (Life Technologies, Warrington, UK). Amplification was mediated by the 7900HT Fast Real-Time PCR System (Thermo), with cycling parameters indicated in Table 2.1. To detect primer dimers and other non-target double-stranded DNA, cycling was followed by melt curve analysis. No significant contaminant annealing was detected (Data not shown).

### Table 2.1: Primer and thermal cycling parameters for RT-qPCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Thermal cycling parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td><strong>LBF:</strong> ATGGGAAGAACACCAGTGGCG</td>
<td>15 min 37x 15s 95 °C, 45s 50 °C, 45s 72 °C</td>
</tr>
<tr>
<td><strong>Lactobacillus</strong></td>
<td><strong>LBR:</strong> CAGCACTGAGAGGCAGAAAC</td>
<td></td>
</tr>
<tr>
<td><em>L. iners</em></td>
<td><strong>InersFw:</strong> GTCTGCGCTTGAAGATCGG</td>
<td>15 min 35x 15s 95 °C, 55s 60 °C, 60s 65 °C</td>
</tr>
<tr>
<td></td>
<td><strong>InersRev:</strong> ACAGTGTAGGATGATCATC</td>
<td></td>
</tr>
<tr>
<td><em>L. crispatus</em></td>
<td><strong>LcrisF:</strong> AGCGAGCGGAACTAACAAGATTAC</td>
<td>15 min 40x 15s 95 °C, 60s 60 °C, 20s 72 °C</td>
</tr>
<tr>
<td></td>
<td><strong>LcrisR:</strong> AGCTGATCATGCATGTGT</td>
<td></td>
</tr>
<tr>
<td><em>L. gasseri</em></td>
<td><strong>LgassF:</strong> AGCGAGCGTGCCTGATGAAATTTC</td>
<td>15 min 40x 15s 95 °C, 60s 57 °C, 60s 65 °C</td>
</tr>
<tr>
<td></td>
<td><strong>LgassR:</strong> TCTTTAAGCTGTCATTG</td>
<td></td>
</tr>
<tr>
<td>Bacterial Species</td>
<td>Primer Set</td>
<td>Time</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------</td>
<td>------</td>
</tr>
<tr>
<td>L. jensenii</td>
<td>LjensF: AAGTCGAGCGAGCTTGCCTATAG</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>LjensR: CTTCTTTCATGCGAAAGTAGC</td>
<td></td>
</tr>
<tr>
<td>L. vaginalis</td>
<td>LV16s_23s_F: GCCTAACCATTGGAGGG</td>
<td>15 min</td>
</tr>
<tr>
<td></td>
<td>LV16s_23s_R: CGATGTGATGTTTCG</td>
<td></td>
</tr>
<tr>
<td>A. vaginae</td>
<td>AV-F: CCCTATCCGCTCTGATACC</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>AV-R: CCAATATCTGCACATTCA</td>
<td></td>
</tr>
<tr>
<td>G. vaginalis</td>
<td>F-GV1: TTACTGATGTATCACTGTAAGG</td>
<td>10 min</td>
</tr>
<tr>
<td></td>
<td>R-GV3: CCGTCACAGGCTGAACAGT</td>
<td></td>
</tr>
<tr>
<td>M. curtisii</td>
<td>F: GCGATGGTTCCAGAGATGGGCCA</td>
<td>2 min</td>
</tr>
<tr>
<td></td>
<td>GCCTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CACGAGTCCCGACGCTGAAGA</td>
<td></td>
</tr>
<tr>
<td>P. bivia</td>
<td>PBsulF: ACGTTGGGCAAAGCTCCTTTGTCT</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>PBsulR: GCGTGTACGCCAGTTGCAAGA</td>
<td></td>
</tr>
</tbody>
</table>

Bacterial quantities (in cells/mL) were then calculated by comparison to a standard curve of genomic DNA from pure culture of the reference species (see bacterial strains above). Isolated DNA from L. iners was used as a standard for total Lactobacillus, due to the common abundance of this organism in the vaginal microbiota (180). Total copies of the gene of interest in standards were calculated using total DNA content from measurements by the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and known gene copies per genome by searching the organism in the NCBI Genome database. Non-detects were treated as containing no DNA and averaged with concentrations of other replicates to reduce positive bias (226).

2.5.9 Metabolomics on vaginal swab supernatants

2.5.9.1 Targeted gas chromatography-mass spectrometry

To precipitate proteins, 100 µL of supernatant from swabs grown in prebiotics (above) were diluted with 200 µL of 1:1 methanol:H₂O, vortexed for 10 seconds, then centrifuged
for 10 min at 10 000 × g. Half (150 μL) of the supernatant was collected into a vial, along with 2 μL of 1 mg/mL ribitol internal standard, where they were dried in a speedvac without heat. Samples were derivatized with 50 μL of 2% methoxyamine-HCl in pyridine (MOX) at 50°C for 90 min, followed by 50 μL of N- Methyl-N- (trimethylsilyl)-trifluoroacetamide (MSTFA) at 50°C for 30 min. To prepare for injection, samples were transferred into micro-inserts.

One μL of sample was injected into an Agilent (Santa Clara, CA) 7890A GC, 5975 inert MSD with triple axis detector. Samples were injected using pulsed splitless mode using a 30 m DB5-MS column with 10 m Duraguard, diameter 0.35mm, thickness 0.25 μm (J&W Scientific, Folsom, CA). Helium was used as the carrier gas at a constant flow rate of 1 mL/min. Oven temperature was held at 70°C for 5 min then increased at a rate of 5°C/min to 300°C and held for 10 min. Solvent delay was set to 7 min, and total run time was 61 min. Masses between 25 m/z and 600 m/z were selected by the detector. Samples were run in random order, and one specific sample was included in every batch as a quality control for machine consistency.

Chromatogram files were deconvoluted and converted to ELU format using AMDIS Mass Spectrometry software (227) with the sensitivity set to low, resolution to medium, and support threshold to high. Chromatograms were aligned using Spectconnect (http://spectconnect.mit.edu) (228) with the support threshold set to low. The integrated signal (IS) matrix output was used for all further analysis. Zeros were replaced with two thirds the minimum detected value on a per metabolite basis followed by a log base 2 transformation. All further analyses were performed using these log-transformed values. Identities of known metabolites of interest were confirmed by comparison to standards.

2.5.9.2 Targeted liquid chromatography-mass spectrometry

Due to poor separation of lactate peaks using GC-MS, liquid chromatography was used to quantify lactate. Samples were vortexed for 15 sec, then transferred to micro-inserts and directly injected into an Agilent 1290 Infinity HPLC coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo-Fisher) with a HESI (heated electrospray ionization) source. For HPLC, 2 μL of each sample was injected into a ZORBAX Eclipse plus C18 2.1 × 50
mm × 1.6 micron column. Mobile phase (A) consisted of 0.1% formic acid in water and mobile phase (B) consisted of 0.1% formic acid in acetonitrile. The initial composition of 0% (B) was held constant for 30 sec and increased to 100% over 3.0 min. Mobile phase B was then held at 100% for 1.5 min and returned to 0% over 30 sec for a total run time of 6 min.

Full MS scanning between the ranges of m/z 50-750 was performed on all samples in negative mode at 140 000 resolution. The HESI source was operated under the following conditions: nitrogen flow of 25 and 15 arbitrary units for the sheath and auxiliary gas respectively, probe temperature and capillary temperature of 425°C and 260°C respectively and spray voltage of 3.9 kV. The automatic gain control (AGC) target and maximum injection time were $3 \times 10^6$ and 500 ms respectively. For molecular characterization, every tenth sample was also analyzed with a data dependent MS/MS method where a 35 000 resolution full MS scan identified the top 12 signals above a threshold of $8.3 \times 10^4$, which were subsequently selected at a 1.2 m/z isolation window for MS/MS. Normalized collision energy for MS/MS was 28, resolution 17 500, AGC target $10^5$ and maximum injection time was 60 ms. Blanks of pure methanol and water were run between every sample to limit carryover, and a single sample was run multiple times with every batch to account for sample variation. After data acquisition, Thermo .RAW files were converted to .MZML format and centroided using ProteoWizard (229). Files were then imported into R using the XCMS package (230) for chromatogram alignment and deconvolution. Features were detected with the “xcmsSet” function using the “centWave” method and a 1 ppm tolerance. Prefilter was set to 3-5000, noise $10^3$, and signal to noise threshold was set to 5 in negative mode. Retention time correction was conducted using the “obiwarp” method, grouping included features present in at least 25% of samples, allowable retention time deviation was 5 seconds, and m/z width set to 0.015. Areas of features below the signal to noise threshold in the data were integrated using the “fillPeaks” function with default settings. Any remaining zeros in the data were then replaced with two-thirds the minimum value on a per mass basis before log base 2 transformation. The log-transformed mass list was then exported as a single .txt file and used for all further analyses. For principle component analysis, data were pareto scaled using the “MetabolAnalyze” package in R. Principle components were then plotted using the “pca” function from the “FactoMineR” package.
in R, and no grouping was observed (Data not shown). Identities of metabolites were determined by authentic standards based on accurate mass, retention time and MS/MS spectra.

2.5.10 Statistical analysis

Growth curves and pH bar plots were graphed and statistically analyzed using GraphPad Prism 6 (La Jolla, CA). Significantly enhanced growth compared to no prebiotic media control was identified using repeated measures two-way ANOVA with Dunnett’s multiple comparisons test. Kruskall-Wallis’ with Dunn’s multiple comparisons test was used to identify differences in pH compared to no prebiotic media control. Figure 2.3 was prepared using R.
Chapter 3

Effects of *in vitro* lactulose administration on vaginal epithelial viability and *Lactobacillus crispatus* antimicrobial properties
3.1 Abstract

Resident microbes and the epithelial lining form the first line of defense against microbial pathogens in the vagina. In healthy women, lactobacilli are the main colonizers and secrete abundant antimicrobial lactic acid to contribute to acidic pH. We previously showed that the prebiotic lactulose ubiquitously stimulated vaginal *Lactobacillus crispatus* strains to grow and produce acidic metabolites, but no research to date has examined how selectively utilized compounds affect bacterial-bacterial and bacterial-host interactions in the vagina. Herein, we show that acids produced by *L. crispatus* fermentation of lactulose are toxic to common dysbiosis-associated organisms *Gardnerella vaginalis*, *Atopobium vaginae*, and *Prevotella bivia*, and that this activity could be abrogated with supernatant neutralization. Although lactulose treatment was detrimental at high doses to epithelial activity, it did not disrupt *L. crispatus* attachment to these host cells. With growing interest in prebiotic utilization at sites distant to the gut, properties additional to fermentation should be considered.

3.2 Introduction

Vaginal health is dependent on the barrier properties imparted by the vaginal epithelium and the resident microbes lining it (231–233), as well as their various secretions (49). This barrier forms the primary defense system against not only dysbiosis (53, 179), but also invading pathogens such as *Candida albicans* (190, 234–236), *Trichomonas vaginalis* (99, 237), *Chlamydia trachomatis* (22, 23), and HIV-1 (44, 62, 97). The tightly enmeshed stratified squamous epithelium of the vagina limits the passage of harmful bacteria and protects from shear forces, while also providing a surface for selective bacteria to adhere (238). In healthy women, this microbial population is dominated by *Lactobacillus* species, with few other organisms present (180, 193). Along with their metabolites, this community of bacteria is termed the vaginal microbiome, and forms a protective layer upon the vaginal epithelium. Acidic vaginal pH, formed by *Lactobacillus*-derived lactic acid, is particularly essential for maintaining microbial symbiosis (56, 60). However, additional secretions from vaginal lactobacilli are thought to impart defensive properties to the environment, including surfactants that inhibit attachment and colonization of pathogens (72), and bacteriocins that selectively kill similar bacteria (239).
Not all lactobacilli are created equal. Depending on the predominant *Lactobacillus* species, women vary in vaginal pH, stability during temporal fluctuations of the microbiome, and susceptibility to infection (39). Of the common species, *L. crispatus* provides the most beneficial properties to the vaginal niche. Compared to other lactobacilli, individuals colonized by *L. crispatus* have more acidic vaginal pH, microbiome stability throughout the menstrual cycle, and are less likely to be co-colonized by BV-causing organisms (39, 193). Because of this, research has been conducted to elucidate properties unique to *L. crispatus* that make it well-adapted to support vaginal health and dominate this niche. The ability of *L. crispatus* strains to readily adhere to the vaginal epithelium, through the expression of fibronectin-binding proteins, and produce exopolysaccharides (EPSs) necessary for biofilm formation (66, 79) represent adaptation traits in this bacterium. In addition, *L. crispatus* secretes more acidic by-products than other vaginal lactobacilli and contain genes for bacteriocin production (65, 66). These beneficial properties make *L. crispatus* an ideal target for stimulation by prebiotic interventions.

Prebiotics, which are compounds that selectively stimulate beneficial bacteria to cause a health benefit to the host (132), are a promising therapeutic to this end. Although this newly recognized definition of prebiotics may encompass compounds with several benefits to the host and host microbiome, little research has evaluated their effects outside of their ability to selectively stimulate commensal bacteria. Our previous work demonstrated that lactulose ubiquitously stimulated vaginal isolates of *L. crispatus* obtained from women both with or without BV, resulting in production of acidic by-products, namely lactic acid (Collins *et al.*, 2018). However, other benefits of prebiotic therapeutics may include improved antagonism of *L. crispatus* against dysbiotic organisms, stronger adhesion of commensal *L. crispatus* to vaginal epithelium, and enhanced viability of the epithelial layer itself. These have not, so far, been explored.

Recently, an increased interest in developing novel antibiotics derived from human commensals has led to the discovery of several new potential antimicrobials, including peptides with a narrow range of activity termed bacteriocins. Although these compounds vary greatly in their structure, they tend to target similar microbes to the producer to provide an advantage in their niche. Considering the propensity of *L. crispatus* to retain
dominance in the vaginal microbiota, bacteriocins may play a role in preventing the colonization of BV organisms. The core genome of *L. crispatus* contains two conserved putative bacteriocins with genetic similarity to enterolysin A and helveticin J (66), though their actual expression and spectrum of activity remains unknown. Other bacteriocins derived from vaginal *Lactobacillus* species have been characterized for their ability to target urogenital pathogens such as *Escherichia coli* and *Enterococcus faecalis* (65), providing a basis to explore this concept for the prevention of BV.

Lactulose fermentation may also affect the adherence potential of vaginal lactobacilli. Sugar availability is known to alter the expression of adherence-related proteins in bacteria, and is a direct substrate for the synthesis of EPS (240). More indirectly, at a low pH, fermentation in lactobacilli can change surface properties to promote adhesion to intestinal epithelium (187). Although this has not been tested in the vaginal niche, lactulose will undoubtedly influence protein expression and/or secretion in the lactobacilli that ferment it. Direct application of oligosaccharides can also disrupt carbohydrate-dependent adhesion of bacteria by blocking adherence protein interactions to host receptors (241). Therefore, it is important to evaluate the effects lactulose might have on attachment of commensal *L. crispatus* to vaginal epithelium to determine whether it may have detrimental or beneficial properties in this regard.

Although human cells do not possess the metabolic capacity to degrade lactulose, research has yet to evaluate its effects when applied to human tissues. One study observed that lactulose has a protective effect in colonic HT-29 cells against bile acids and fecal water (242), though no mechanism was established. With the ultimate goal of human application, lactulose must be thoroughly assessed on its interacting host tissue. Our aim was to measure vaginal cell viability upon lactulose treatment. These experiments were designed to provide insight into lactulose’s effects on the viability of vaginal epithelium and *L. crispatus*, and uncover how it regulates interactions with organisms involved in BV.

3.3 Results

To determine how by-products from *L. crispatus* fermentation of lactulose affect the viability of BV organisms, *G. vaginalis*, *A. vaginae*, and *P. bivia* were inoculated in
lactulose- or control-treated *L. crispatus* supernatants. Compared to control-treated supernatants, lactulose-treated *L. crispatus* supernatants had a significant killing effect to *G. vaginalis* (p<0.0001) and *P. bivia* (p<0.0001), and a similar, but not significant trend for *A. vaginae* (Figure 3.1). However, neutralizing these supernatants to pH 8.0 restored the viability of inoculated BV organisms to levels comparable to control (Figure 3.1).

Using the vaginal epithelial cell line VK2/E6E7, *L. crispatus* adherence upon lactulose treatment was evaluated. Despite glucose-treated *L. crispatus* having greater attachment to vaginal cells, lactulose growth had no effect on attachment (Figure 3.2). Further to this, lactulose was directly tested for effects on VK2/E6E7 viability. In a dose-dependent manner, lactulose lowered the viability of vaginal epithelial cells (Figure 3.3).
Figure 3.1: Lactulose-treated *L. crispatus* has pH-dependent antagonism against BV organisms

Time-kill assay of BV organisms anaerobically inoculated in spent supernatants from *L. crispatus* in 0.5% lactulose-supplemented or control dextrose-free MRS. Supernatants were neutralized using HCl and NaOH and sterilized using 0.2 µm filters. Dotted line represents the limit of detection for viable bacteria. Significant differences in viability between treatments at 48 hours determined using RM two-way ANOVA with Sidak’s multiple comparisons test (****p<0.0001).
Figure 3.2: Lactulose does not affect *L. crispatus* attachment to vaginal epithelium

Approximately $10^6$ *L. crispatus* cells were incubated for 1 hour on fully differentiated VK2/E6E7 vaginal cells. Adherence expressed as a percentage of *L. crispatus* bacteria seeded onto VK2/E6E7 cells. Statistical significance was determined according to one-way ANOVA and Dunnett’s multiple comparison’s test (*p*<0.05).
Figure 3.3. Lactulose is inhibitory to VK2/E6E7 cell viability in a dose-dependent manner

Viability of the vaginal epithelium (VK2/E6E7) cell line determined using the Vybrant® MTT cell proliferation assay kit (ATCC). Various concentrations (% w/v) of lactulose were added to KSFM and incubated for one hour before readout. Significant differences to control determined using one-way ANOVA with Dunnett’s multiple comparisons test (****p<0.0001).
3.4 Discussion

This short report has demonstrated the potential mechanisms of action that lactulose has on the barrier properties of the vaginal ecosystem. Importantly, fermentation of lactulose stimulates *L. crispatus* to produce abundant acidic products that are detrimental to the viability of BV organisms and could fundamentally alter the selective environment present. However, it does not alter the ability of this bacterium to attach to vaginal epithelium, and may have a directly detrimental effect on tissue proliferation.

Direct antagonism of lactobacilli against dysbiotic organisms is a well-studied, essential feature of lactobacilli for maintaining vaginal homeostasis. We report here a stimulation of this activity in the strongly health correlated *L. crispatus* against key microbes involved in BV, which is abolished at neutral pH. Although it could be posited that this pH-dependent secretion from *L. crispatus* is a protein whose action is inactivated at neutral pH, several features suggest otherwise. Firstly, *L. crispatus*-dominated vaginal microbiotas have a significantly lower pH than other predominant lactobacilli, suggesting that it has a greater capacity to produce acidic fermentation metabolites. Since fermentation of lactulose by *L. crispatus* dramatically lowers the pH (Collins et al., 2018) and BV organisms are known to be susceptible to acidity, it follows that this is responsible for their death in *L. crispatus* supernatants.

Though no studies have isolated or characterized the predicted bacteriocins present in the *L. crispatus* genome, this species possesses ones that have high nucleotide congruence with enterolysin A, helveticin J (66), and gassericin A (65). These bacteriocins were initially identified in *Enterococcus faecalis, Lactobacillus helveticus*, and *L. gasseri*, respectively, and have bactericidal activity against sensitive indicators within a broad pH range, including neutrality, and under anaerobic conditions (65, 243–245). Since no killing effects were observed in the present work at neutral pH, this suggests that bacteriocins were not responsible for antagonism against the BV-associated bacteria. Furthermore, bacteriocins are antimicrobials that typically target bacteria similar to the producer species; they occupy the same nutritional and physical niche. As the observed activity affects diverse species (*G. vaginalis, A. vaginae, and P. bivia*), which are quite distinct in their metabolic capacity, lineage, and physical structure from *L. crispatus*, it is unlikely that bacteriocins are
responsible. Lastly, bacteriocins are not abundantly produced unless bacteria are cultured on a solid surface such as agar, while broth medium was utilized here.

Considering the abundant factors involved in bacterial adhesion to host tissue, it is not necessarily surprising that lactulose had no effect on this property of *L. crispatus*. Despite some variation between strains, *L. crispatus* adhesion in the urogenital tract is predominantly receptor-mediated through adhesins and therefore not due to non-specific interactions such as hydrophobicity (45, 246). This binding exists only at low pH, which could not be obtained in the present assay while still maintaining epithelial cell viability, potentially explaining these results.

Currently, the adhesins responsible for *L. crispatus* ATCC 33820 attachment are unknown. In other lactobacilli, adhesins can bind to either vaginal epithelium receptors or to mucosal secretions. An example of the former in *L. crispatus* is the *Lactobacillus* epithelium adhesin (LEA) protein, which mediates binding to outermost epithelial cells (76), but this protein is not present in ATCC 33820. Though not yet established in *L. crispatus*, mucus-binding proteins (247) are found in other vaginal lactobacilli. Although the selection of VK2 (E6/E7) cells was based partially on their ability to produce mucin 5B (234), many other mucins found in cervical secretions could also be targets for *L. crispatus* adherence. Thus, an extension of this work should utilize primary cell lines and ultimately human subjects to evaluate adhesion.

Importantly, there was no negative impact of lactulose on *Lactobacillus* adhesion, as some prebiotics and other oligosaccharides have been observed to do this (248). Because most interactions between bacteria and the vaginal epithelium or mucous rely on attachment through glycoproteins or other carbohydrate-based moieties, free saccharides are able to bind these sites and therefore shield them from interaction, sometimes even actively co-aggregating bacteria (75, 249). In this case, lactulose averts these effects, possibly due to its dissimilar structure to mannose and other sugar components of adherence proteins, but also because it is actively degraded by *L. crispatus*.

The unexpected dose-dependent detriment of lactulose to VK2 cell viability emphasizes the need to carefully select active concentrations in developing treatments in humans,
particularly since the mechanism of action is currently unknown. Lactulose derivatives have also been shown to induce apoptosis in melanoma cells through inhibition of galectin signaling, but these compounds are significantly modified and contain additional lactose residues and amines (250). Nonetheless, certain pitfalls of cell culture cast doubt on the in vivo translation of this result. Although VK2 cells produce mucin 5A, the vaginal wall is typically coated with secretions from not only vaginal epithelial cells, but also originating from the Bartholin’s gland and the cervix (251). This protective coat would limit the sugar’s direct interaction with host tissue. In addition, VK2 cells lack the ultrastructure of the vaginal wall, a stratified squamous tissue, where the outermost layer consists of pyknotic cells with no metabolic activity, preparing to be shed (238). Thus, lactulose would not contact active epithelium unless damage were to occur. Given these limitations and the effectiveness of 0.5% (w/v) lactulose in stimulating lactobacilli, this low dose should be considered for vaginal administration.

The beneficial properties of lactulose on L. crispatus are shown here to be specifically mediated by increased lactic acid production and acidic pH, while bacterial adherence is not affected. This is not necessarily a limitation, as the sheer quantity of lactic acid profoundly improves L. crispatus antagonism against BV organisms both in culture and in vivo, and represents a significant aspect of vaginal health. Given the current limitations of cell culture, lactulose should still be considered for use in retaining a lactobacilli-dominated vaginal microbiota.

3.5 Materials and Methods

3.5.1 Time-kill assay

To obtain spent supernatants, L. crispatus ATCC 33820 was grown for 24 hours in 0.5% (w/v) lactulose-supplemented or control dextrose-free MRS, then centrifuged at 4500 × g for 5 min. In order to measure the effect of pH on antagonism, half of the supernatants from each condition were neutralized to pH 8.0 ± 0.1 with HCl and NaOH. Supernatants were then sterilized using 0.2 µm filters. BV organisms G. vaginalis ATCC 14018, A. vaginae ATCC BAA-55, P. bivia ATCC 29303, and M. curtisi ATCC 35241 were inoculated into
sterile *L. crispatus* supernatants at approximately $10^6$ CFU/mL and growth was monitored using spot plating method on Columbia Blood Agar (CBA) every 3 hours.

### 3.5.2 Effects of lactulose on *L. crispatus* adherence to vaginal epithelium and epithelial viability

The vaginal epithelium cell line VK2/E6E7 (ATCC CRL-2616) was cultured in keratinocyte serum-free media supplemented with 50 µg/mL of bovine pituitary extract, 0.1 ng/mL of epidermal growth factor and 0.4 mM CaCl$_2$ (Life Technologies, Gibco) at 37°C and 5% CO$_2$.

For the adherence assay, VK2/E6E7 cells were inoculated into a 24-well plate to a density of $10^5$ viable cells/well and allowed to fully differentiate. *L. crispatus* was grown in 1% (w/v) lactulose-supplemented, 2% (w/v) dextrose, or control dextrose-free MRS for 18 hours. Approximately $10^7$ CFU/mL *L. crispatus* from each pre-treatment were then seeded onto VK2/E6E7 cells for 1 hour. Cells were briefly rinsed with PBS to remove non-adherent bacteria. Remaining adherent bacteria were dislodged by treating cells with 0.05% trypsin for 10 min, then plated on MRS to determine viable counts.

In order to measure the viability of VK2/E6E7 in lactulose, KSFM solutions with increasing concentrations of lactulose (0.5%, 1%, and 5% w/v) were prepared. Once VK2/E6E7 cells reached approximately 80% confluence and were therefore still actively proliferating, cell media was replaced with lactulose-treated or control media. Cells were incubated for 1 hour, then viability was determined using the Vybrant® MTT cell proliferation assay kit (ATCC), according to the manufacturer’s instructions.
Chapter 4

Influence of glycogen metabolism on the vaginal microbiota
4.1 Abstract

Selective stimulation of bacteria by abundant glycogen in the vaginal ecosystem undoubtedly helps to shape the composition of the resident microbiota. Although it was previously thought that lactobacilli were the main beneficiaries of this carbon source, we observed here that *L. crispatus*, *L. gasseri*, and *L. jensenii* were not stimulated by glycogen, while *L. iners*, and bacterial vaginosis (BV)-associated *G. vaginalis*, *P. bivia*, *M. curtisii*, and *A. vaginae* were. To better understand the impact of this fermentation on bacterial interactions, supernatants were collected, replenished with glycogen, and *L. iners* or BV-associated organisms were re-inoculated. Growth in glycogen appeared self-limiting, as *L. iners* and *G. vaginalis* grown in their own supernatants were rapidly killed. Furthermore, metabolites from *L. iners* growth in glycogen were antagonistic toward these BV-associated organisms. Supernatant neutralization revealed that acidic pH was responsible for killing *P. bivia* and *M. curtisii*. In contrast, antagonism of *G. vaginalis* by *L. iners* could not be explained by pH, depletion of nutrients required for glycogen metabolism, or secretion of amylase-inhibiting lactic acid. Given the current lack of knowledge regarding glycogen utilization by vaginal microorganisms, further investigations into this interaction between host and bacteria should be considered before interventions using glycogen are introduced.

4.2 Introduction

Glycogen, a polysaccharide consisting of glucose monomers linked linearly by α-1,4-glycosidic bonds with branching α-1,6-glycosidic bonds, is the major carbon source available in the female genital tract (238). It is predominantly produced by the stratified epithelium lining the vaginal canal, then freed into surrounding cervicovaginal fluid when the outermost cells are sloughed off and broken apart by bacterial cytolsins (252, 253). Typical levels of free vaginal glycogen in cervicovaginal fluid are highly variable in healthy women, ranging anywhere from 0.1–32 μg/μL (188). This variation is generally attributed to differences in circulating estrogen, which triggers glycogen production in vaginal epithelial cells (34, 188). Since estrogen concentrations fluctuate throughout the menstrual cycle and are depleted following menopause, so too do vaginal glycogen levels vary (37, 38).
Like all abundant carbon sources in any given ecosystem, glycogen plays an essential role in selecting for and shaping the microbes that can survive in this niche, as those that express the genes necessary to utilize glycogen will have a metabolic advantage. Since glycogen commonly acts as a glucose storage vehicle in prokaryotes and eukaryotes alike, many bacteria already possess the machinery necessary to degrade intracellular glycogen (254). This typically includes a glycogen phosphorylase, a $\alpha$-1,4-glycosidase that cleaves off smaller sugars from ends of glycogen’s linear chains (255) and a debranching enzyme that targets the $\alpha$-1,6-glucose branching points (256). However, glycogen’s complex, highly branched structure prevents its transport across cell membranes; thus, these enzymes must be secreted outside the cell. Once the extracellular glycogen is broken into its glucose and maltose components, carbohydrate transporters such as phosphotransferases are required to actively import them into the cell (257, 258). Most of these enzymes have been characterized in *Escherichia coli* strains, and there has been surprisingly little research to date on their expression in common vaginal bacteria, despite the prevalence of this carbon source in the vagina.

Because of this gap in knowledge, there remains significant controversy over glycogen’s net impact on vaginal microbiota homeostasis. Initially, glycogen was thought to supplement vaginal lactobacilli because high glycogen levels were associated with a *Lactobacillus*-dominant microbiota and acidic vaginal pH (34, 188, 189). In particular, glycogen concentrations are elevated in microbiota colonized primarily by *L. crispatus* and *L. jensenii*, compared to *L. iners* (189). Nevertheless, despite few rare species of lactobacilli (*L. acidophilus, L. leichmannii*, and *L. salivarius*) having the ability to utilize glycogen (259), commonly dominant strains of *L. crispatus, L. jensenii*, and *L. gasseri*, as well as *L. plantarum*, have been observed to not utilize it at all (190). This paradigm was seemingly resolved with the discovery that human $\alpha$-amylases are also released by epithelial cells into vaginal fluid, where they metabolize glycogen into glucose and maltose (191). Thus, the host was believed to extracellularly degrade glycogen into counterparts fermentable by lactobacilli, where they increased in composition and released acidic lactic acid as a by-product. This non-specific mechanism is problematic though, because these simple sugars are metabolized almost ubiquitously by bacteria. Furthermore, these studies
did not include the most abundant *Lactobacillus* colonizer, *L. iners*, which has not yet been evaluated for glycogen fermentation ability.

In order to fully comprehend its impact on the vaginal microbiota, it is important to consider glycogen metabolism by microbes that are not beneficial to the host. A diversity of such organisms emerge in a common condition known as bacterial vaginosis (BV), where the commensal *Lactobacillus* population and acidic pH are depleted (82). Conversely, levels of strictly anaerobic bacteria increase, sometimes causing unpleasant odor and discharge (58, 260), as well as increased risk for infections (22). Upon comparison of the bacterial transcriptome during vaginal health and BV, Macklaim *et al.* (83) observed that genes responsible for glycogen metabolism were upregulated during BV and were mapped to several BV-associated organisms. The genome of *G. vaginalis*, a predominant organism involved in BV pathogenesis, contains conserved genes for glycogen catabolism (261). It is reasonable to postulate then, that glycogen may stimulate the growth of unwanted BV organisms rather than lactobacilli. We therefore aimed to measure the glycogen fermenting capacity of both common vaginal lactobacilli and BV-associated bacteria, and elucidate how this influences the interaction between these members of the vaginal microbiota.

## 4.3 Results

### 4.3.1 Glycogen fermentation by common vaginal lactobacilli and BV-associated organisms

Upon inoculation of preferred media with 0.5% glycogen, growth of *L. iners* was stimulated, while all other lactobacilli were not affected (*Figure 4.1A and B*). This directly coincided with significant media acidification in *L. iners* supplemented with glycogen compared to control (p<0.0001, *Figure 4.2*). As glycogen metabolism by *L. iners* has not yet been evaluated, we searched its transcriptome for genes potentially responsible for this phenotype. *L. iners* was found to increase its expression of pullulanase, a starch degrading enzyme, during BV onset (Macklaim *et al.*, unpublished). Common BV organisms *G. vaginalis* and *A. vaginae* increased in viability with glycogen as the sole carbon source, at early time points for the former (p<0.0001) and at 48 hours for the latter organism (p<0.05).
**Figure 4.1: Growth of common vaginal lactobacilli in 0.5% glycogen**

Growth of predominant vaginal *Lactobacillus* species in dextrose-free NYC III (*L. iners*) or MRS (all others), with or without supplementation with 0.5% (w/v) glycogen. (A) Viability (in CFU/mL) was tracked using spot plating, with significance determined at each time point using mixed ANOVA with Sidak’s multiple comparisons test (**p<0.001, ****p<0.0001). (B) Total bacteria (OD$_{600}$) after reaching plateau phase of growth, with significance determined using Student’s t-test (****p<0.0001).
Figure 4.2: Media pH following growth of common vaginal lactobacilli in 0.5% glycogen

Media acidity measured using pH test strips following plateau phase of *Lactobacillus* growth in dextrose-free NYC III (*L. iners*) or MRS (all others), with or without supplementation with 0.5% (w/v) glycogen. Significance determined using Student’s t-test (****p<0.0001).
(Figure 4.3A). This resulted in greater overall turbidity for both bacteria (p<0.0001, Figure 4.3B), as well as significant drops in media pH following growth (p<0.01 and p<0.05; Figure 4.4). Although *P. bivia* and *A. vaginae* had no increase in viable counts with glycogen administration at the selected time points, both experienced an overall increase in bacterial turbidity (p<0.0001 and p<0.05, respectively; Figure 4.3A and B), as well as lowered media pH (p<0.01 and p<0.05, respectively; Figure 4.4).

4.3.2 Bidirectional antagonism of BV-associated organisms and *L. iners* with glycogen stimulation

Since glycogen-inoculated BV bacteria and *L. iners* induced media acidity, and BV-organisms are known to be sensitive to low pH (53), we evaluated their viability in spent supernatants of *L. iners* and BV-organisms’ growth in glycogen. When inoculated in glycogen-spent *L. iners* supernatants, viability of *L. iners* (p<0.0001), *G. vaginalis* (p<0.0001), *M. curtisi* (p<0.0001), and *P. bivia* (p<0.05) fell below the limit of detection, significantly lowering them compared to control (Figure 4.5). Restoration of *M. curtisi* and *P. bivia* was achieved when these supernatants were neutralized to pH 7.0 (Figure 4.5). In contrast, *L. iners* and *G. vaginalis* viability was significantly improved upon supernatant neutralization (p<0.0001), but remained lower in abundance than in control *L. iners* supernatants (p<0.05 and p=0.05, respectively; Figure 4.5).

4.3.3 Lactate does not affect the growth of *G. vaginalis* or *L. iners* in glycogen

To determine whether products from *G. vaginalis* grown in glycogen exerted a similar self-killing, *L. iners*-antagonizing effect, *G. vaginalis* and *L. iners* were grown in *G. vaginalis* glycogen-spent supernatants. This was also to shed light on whether the previously observed killing of *G. vaginalis* and *L. iners* by *L. iners* supernatants was due to the depletion of nutrients required for glycogen metabolism. While glycogen-supplemented *G. vaginalis* supernatants significantly antagonized both *G. vaginalis* (p<0.0001) and *L. iners* (p<0.05) compared to control-grown *G. vaginalis* supernatants, neutralization completely restored their viability to levels comparable to control (p<0.001 and p<0.05, respectively; Figure 4.6).
Figure 4.3: Growth of BV-associated bacteria in 0.5% glycogen.
Growth of BV-associated bacteria in dextrose-free NYC III with or without supplementation with 0.5% (w/v) glycogen. (A) Viability (in CFU/mL) was tracked using spot plating, with significance determined at each time point using mixed ANOVA with Sidak’s multiple comparisons test (*p<0.05, ****p<0.0001). (B) Total bacteria (OD₆₀₀) after reaching plateau phase of growth, with significance determined using Student’s t-test (*p<0.05, ****p<0.0001).
Figure 4.4: Media pH following growth of BV-associated bacteria in 0.5% glycogen. Media acidity measured using pH test strips following plateau phase of BV-associated organism growth in dextrose-free NYC III with or without supplementation with 0.5% (w/v) glycogen. Significance determined using Student’s t-test (*p<0.05, **p<0.01).
Figure 4.5: Viability of BV organisms in spent glycogen-supplemented *L. iners* supernatants

*L. iners* supernatants from growth in dextrose-free NYC III broth with or without 0.5% (w/v) glycogen were collected. Subsets of glycogen-grown supernatants were neutralized to pH 7 using HCl and NaOH, then all were sterilized with 0.2 µm filters. Supernatants were replenished with 0.5% glycogen, then approximately $10^6$ viable bacteria were inoculated in supernatants and grown anaerobically for 48 hours. Viability was tracked using spot plating at designated time points. Dotted line indicates the limit of detection and values falling below were replaced with 2/3 the limit of detection. Significance was evaluated on log-transformed values using mixed ANOVA with Sidak’s multiple comparisons test (*p<0.05, **p<0.01, ****p<0.0001).
Figure 4.6: Viability of *G. vaginalis* and *L. iners* in spent glycogen-supplemented *G. vaginalis* supernatants

*G. vaginalis* supernatants from growth in dextrose-free NYC III broth with or without 0.5% (w/v) glycogen were collected. Subsets of glycogen-grown supernatants were neutralized to pH 7 using HCl and NaOH, then all were sterilized with 0.2 µm filters. Supernatants were replenished with 0.5% glycogen, then approximately 10^6 viable *G. vaginalis* or *L. iners* were inoculated in supernatants and grown anaerobically until control viability declined. Viability was tracked using spot plating at designated time points. Dotted line indicates the limit of detection and values falling below were replaced with 2/3 the limit of detection. Significance was evaluated on log-transformed values using mixed ANOVA with Sidak’s multiple comparisons test (*p<0.05, ***p<0.001, ****p<0.0001).
Lactic acid is a common by-product of anaerobic fermentation and has been shown to potently interfere with alpha-amylases responsible for glycogen metabolism in other microorganisms (262). Since *L. iners* fermentation produces abundant lactic acid, we evaluated if the concentrations produced by glycogen metabolism were responsible for limiting the proliferation of *L. iners* and *G. vaginalis* in glycogen. NYC III broth containing glycogen was acidified to pH 4.0 using lactic acid to simulate fermentation products of *L. iners*, then neutralized to the same pH as control media. Interestingly, *L. iners* and *G. vaginalis* inoculation in this lactate-treated media had no effect on their growth in glycogen (Figure 4.7).
Figure 4.7: *L. iners* and *G. vaginalis* viability in lactate-treated NYC III media.

Dextrose-free NYC III media with 0.5% (w/v) glycogen was prepared, with or without lactate. Lactate treatment was performed by bringing media to pH 4.0 with concentrated lactic acid, then neutralizing to pH 7.0 with NaOH. Both conditions were subsequently sterilized using 0.2 um filters. Significance between lactate and control was determined on log-transformed values using mixed ANOVA with Sidak’s multiple comparisons test.
4.4 Discussion

The present work reveals that, despite previous clarity on the subject, there is still much unknown about the role of glycogen in shaping the vaginal microbiome. We report for the first time that *Lactobacillus iners* directly metabolizes glycogen, unlike any other known predominant vaginal *Lactobacillus* spp. Furthermore, the broad glycogen-degrading capacity of several common BV-associated microbes is confirmed. We then show that by-products from this degradation affect the dynamic between glycogen-utilizing vaginal organisms, potentially resulting in a self-regulating system. Despite several attempts at determining the product(s) responsible for this antagonism, they remain elusive and will require further investigation.

*L. iners* is the most abundant vaginal *Lactobacillus* species, being present in instances of eubiosis and BV alike (88, 180). Although its cohabitation with dysbiotic organisms has led some to conclude that *L. iners* strains are less beneficial than other lactobacilli, there has been substantial debate over whether *L. iners* is simply better adapted to the vaginal niche (46, 47). Proponents of the former cite the observation that an *L. iners*-dominated microbiota tends to fluctuate more to a diverse, BV-like state than other predominant lactobacilli (39), tends to have less acidic vaginal pH (193). Furthermore, *L. iners* strains tend not to produce hydrogen peroxide (263, 264), previously thought to play a large role in defending against pathogens. While this may be viewed as an inability for *L. iners* to fight off microbes associated with dysbiosis, the latter hypothesis suggests that it may be better adapted to survive in and therefore tolerates the invasion of these microbes. This flexibility may be a result of its small genome that relies heavily on acquisition of nutrients from its environment, or its unique cell-anchoring proteins and carbohydrate utilization mechanisms that define its survival in the vagina (47, 48). Regardless, *L. iners* is considered an essential component of the vaginal microbiota in health, especially in women from African or Hispanic descent who are commonly dominated by this organism (33, 193).

Our finding that *L. iners* has the unique capacity for glycogen degradation largely supports this hypothesis, as glycogen is the most abundant carbohydrate source available in cervicovaginal fluid (34, 188). It was previously considered very rare for vaginal
lactobacilli to process glycogen (48, 191, 259), though no studies directly examined utilization by *L. iners*. However, previous work demonstrated that an *L. iners*-dominated microbiota has greater α-amylase activity than an *L. crispatus*-dominated vaginal microbiota (43). This capability may reflect adaptation mechanisms of *L. iners* to fluctuations in the vaginal environment. During BV, anaerobic organisms consume the ample free vaginal glycogen, leaving little for host mucosal α-amylases to digest into glucose and mannose (191), the primary carbon sources readily available for *L. crispatus, L. jensenii* and *L. gasseri*. While this loss is detrimental to other lactobacilli, *L. iners* increases its expression of the epithelium pore-forming protein inerolysin (83, 265), which lysed these cells to release glycogen. Given that *L. iners* upregulates four α-1,6-glucosidases (83) and a pullulanase during BV that potentially degrade glycogen, it is likely that this provides a mechanism for carbohydrate scavenging during low sugar availability. Thus, *L. iners* is able to effectively compete with BV organisms such as *Prevotella* spp. and *Gardnerella vaginalis*, which also produce cytolysins and glycogen-degrading enzymes (83, 252, 253), and which we have directly shown to metabolize glycogen here. Further characterization of these gene products in several *L. iners* strains and confirmation of their glycogen-degrading properties will shed light into which genes are truly responsible for this phenotype and whether it is broadly conserved across *L. iners* strains.

Due to the abundance of this polysaccharide in the vaginal niche, glycogen metabolism undoubtedly shapes interactions between inhabitants of the vaginal microbiome. We found that glycogen is broadly utilized by common BV organisms, supporting previous observations of their genetic capacity to do so (83). Surprisingly, utilization of glycogen by these microbes and *L. iners* produced acidic metabolites that lowered media pH, despite their known sensitivity to acid. This brought to question whether the glycogen metabolism process was self-limiting, which was indeed the case for both *L. iners* and *G. vaginalis*, whose supernatants had a self-killing effect. Therefore, glycogen metabolism might help to explain the spontaneous recovery from BV that some women experience (266, 267). Fluctuations in the vaginal microbiota occur frequently and from a number of known factors, including menstruation (37), sexual activity (112), and presence of external bacterial reservoirs (115); however, none of these explain the restoration of *Lactobacillus* dominance. We posit that newly burgeoning BV organisms capitalize on the largely
concentrated glycogen available in cervicovaginal fluid, rapidly producing acidic metabolites that slow their growth and lead to one of two outcomes: (i) Either the acid-tolerant lactobacilli that may have survived, including *L. iners*, are able to re-establish themselves, or (ii) BV organism metabolism slows to reach homeostasis in a dysbiotic state. Longitudinal studies following glycogen and the microbiota will be needed to further validate this conclusion. In addition, there is a need for vaginal models that support co-colonization of epithelial cells with the diversity of microbes present in the human vagina, which would better reveal how introduction of BV organisms or changing glycogen concentrations affect microbial balance and host symbiosis.

Another surprising discovery was that, despite similar degrees of acidity produced, *L. iners* supernatants were more detrimental to the viability of *L. iners* and *G. vaginalis* than products from *G. vaginalis*. Similarly to other lactobacilli, *L. iners* carbohydrate fermentation produces mainly lactic acid (268), while *G. vaginalis* secretes predominantly acetic acid and some butyric (19), lactic and succinic acids (260, 269). Different acid profiles may have varied effects on bacterial survival, with lactic acid being particularly toxic to BV-associated organisms (60). This, however, does not rule out additional secretions that may impart the increased killing effect of *L. iners*.

We used neutralized *L. iners* supernatants following glycogen stimulation to gain additional insight into the characteristics of antagonistic secretions produced. For *P. bivia* and *M. curtisii*, neutralization of *L. iners* supernatants completely restored their viability, indicating that acidic pH was responsible for this antagonism. In contrast, the remaining differences in *L. iners* and *G. vaginalis* survival between control and neutralized *L. iners* supernatants suggests an alternative explanation. The most probable being that the initial growth of *L. iners* with glycogen uses up additional nutrients necessary for glycogen metabolism. However, since *G. vaginalis* should utilize the same nutrients to purport this phenotype, and neutralized *G. vaginalis* supernatants had no effect on viability, this is unlikely. An alternative hypothesis is that *L. iners*-derived lactic acid specifically inhibits enzymes responsible for glycogen degradation, preventing *L. iners* and *G. vaginalis* from utilizing this carbon source. Others have shown that lactic acid is a potent bacterial α-amylase inhibitor (262), which would inhibit the breakdown of glycogen into maltose and
glucose by these organisms. We tested whether the concentration of lactic acid that was necessary to acidify the media to the same extent as \textit{L. iners} fermentation was sufficient to inhibit the growth of \textit{L. iners} and \textit{G. vaginalis} in glycogen at a neutral pH, but no change was observed. Perhaps since protonated lactic acid, present at lower pH, is more detrimental to bacterial survival, then neutralization abrogates lactic acid’s effect (53). Furthermore, the concentration necessary to inactivate alpha-amylases can be extremely high, equating to pH 2.93 (270). Thus, lactic acid does not fully explain the antagonistic properties of \textit{L. iners} following growth with glycogen as the primary carbon source.

With the currently limited research on glycogen’s role in shaping the vaginal microbiota, there is a serious need for controlled experiments. Although human studies have correlated free vaginal glycogen with a \textit{Lactobacillus}-dominated microbiota and low pH (188, 189), this remains insufficient to justify exogenous administration of glycogen. Certain products, such as the Luvena® vaginal moisturizer, have already been developed under the false notion that glycogen selectively stimulates commensal lactobacilli. Furthermore, glycogen broadly stimulates BV-associated bacteria, shown here, and the parasite \textit{Trichomonas vaginalis} (271). An improved understanding of glycogen metabolism by vaginal microbes will provide insight into temporal dynamics of the vaginal microbiota. The apparent self-limiting nature of glycogen metabolism by BV organisms may warrant fewer antibiotic prescriptions for BV, rather focusing on the reintroduction of lactobacilli, such as in a probiotic (130, 131). Lastly, future studies should determine the \textit{L. iners} metabolite responsible for the inhibition of glycogen utilization by BV-associated organisms, as the potential applicability of such a compound for modulating the vaginal microbiota could be profound. Given the clear importance of glycogen in the vaginal environment, this research provides a solid foundation for understanding its impact on resident microbes.

4.5 Materials and Methods

4.5.1 Bacteria and growth conditions

Representative strains of vaginal bacteria were selected for glycogen fermentation capacity and obtained from ATCC. Lactobacilli used were \textit{Lactobacillus iners} AB-1, \textit{L. gasseri} ATCC 33323, \textit{L. crispatus} ATCC 33820, and \textit{L. jensenii} ATCC 25258, and BV-associated
organisms were *Gardnerella vaginalis* ATCC 14018, *Atopobium vaginae* ATCC BAA-55, *Mobiluncus curtisii* ATCC 35241, and *Prevotella bivia* ATCC 29303. Lactobacilli, except *L. iners*, were cultured in modified de Mann, Rogosa and Sharpe (MRS) media containing no dextrose in anaerobic conditions at 37°C. BV-associated organisms and *L. iners* were grown in dextrose-free New York City (NYC) III medium in an anaerobic chamber (80% N₂, 10% CO₂, and 10% H₂) at 37°C.

4.5.2 Measurement of glycogen fermentation

Bacterial growth was measured upon addition of 0.5% (w/v) glycogen (type II, from oyster, Sigma) using both spot plating and optical density measurements. *L. gasseri, L. crispatus,* and *L. jensenii* were diluted and plated on MRS agar, while *L. iners* and BV-associated organisms grew on Columbia blood agar (CBA). To determine viable counts, spot plating was performed at designated time points until plateau phase was reached. Optical density reading at 600 nm was then used to approximate total bacteria following growth. Glycogen fermentation was determined to occur by measuring the media pH after reaching plateau phase.

4.5.3 Isolation of and growth in bacterial supernatants

Supernatants were extracted from *L. iners* and BV-associated bacteria grown in NYC III media with or without 0.5% (w/v) glycogen by centrifuging at 4500 × g for 10 min and decanting into fresh tubes. Spent glycogen-inoculated supernatants were then either untreated or neutralized to pH 7.0 ± 0.2 using HCl and NaOH. Approximately 10⁶ CFU/mL bacteria were seeded into supernatants and spot plating was used to measure viability over time.

To determine the effect of lactate in the media on the ability of *L. iners* and *G. vaginalis* to proliferate in glycogen, dextrose-free NYC III broth with 0.5% glycogen was brought to pH 4.0 ± 0.2 with lactic acid, then both this and control glycogen-supplemented NYC III broth were neutralized to pH 7.0 ± 0.5 using HCl and NaOH. *L. iners* and *G. vaginalis* were then inoculated in both media at a density of approximately 10⁶ CFU/mL.
Chapter 5

Multi-mycotoxin biomarker detection in a cohort of Rwandan women using LC-MS/MS and association with women’s health outcomes
5.1 Abstract

Mycotoxins are a widespread problem for food safety and women’s health due to their carcinogenic and teratogenic effects and worldwide prevalence. Despite extensive reporting of aflatoxins (AF), fumonisins (F), ochratoxin A (OTA) and zearalenone (ZEN) in popular Rwandan foods, the exposure levels of these fungal toxins have not been explored. In this chapter, I use a state-of-the-art metabolomics approach to quantify a panel of mycotoxins in a cohort of 149 Rwandan women that participated in a study of the BV microbiota and metabolome to further understand the intersection of women’s health and environmental toxins. Plasma AF-albumin was detected in 81% of women at a mean concentration of 2.26 pg/mg albumin, representing long-term exposure to AFB₁. Urinary biomarkers AFM₁, FB₁, FB₂, OTA and ZEN were detected in 49%, 30%, 15%, 71% and 51% of individuals in this population, with mean quantity of positives being 170, 59, 64, 684 and 307 pg/mg creatinine, respectively. AFM₁, a urinary biomarker for AFB₁ ingestion, was associated with increased intake of vegetables, potentially relating to the common contamination of cassava in Rwanda with Aspergillus spp. In addition, elevated levels of the plasma AFB₁ biomarker AF-albumin were observed in pregnant women. Additional studies are needed to delineate the source of contamination in Rwanda, but given the known negative consequences of mycotoxin exposure, systematic improvements to food processing and storage are drastically needed to reduce exposure in this high-risk population.

5.2 Introduction

Mycotoxins are a serious concern for human health, especially in the developing world where an estimated 4.5 billion people are chronically exposed (192). These compounds are secondary metabolites released into the environment mainly by species of three major fungi: Aspergillus, Fusarium, and Penicillium. Various crops predominant in developing nations in Africa including maize, millet, cassava, peanuts, and products formed from these crops, are commonly contaminated by these organisms. This is in large part due to environmental conditions, but also because of weaker regulations on food cultivation and storage. Typically, fungi require a warm, humid climate and a nutritional host to proliferate and produce toxic compounds, which largely resembles the tropical temperament of
African countries (272, 273). Therefore, without proper dehumidification, cold storage, antifungal application, and frequent chemical testing, fungal contamination can occur during several stages of crop preparation, including plant growth, storage, transportation, or at the final location of sale (274). Given the food scarcity in impoverished nations and the fact that cooking practices do not reduce these compounds’ stability, contaminated foods are often still ingested, causing serious problems to human health (275).

The main mycotoxins of interest and significance to humans are aflatoxins (AF), fumonisins (F), zearalenone (ZEN), and ochratoxin A (OTA). Aflatoxins B₁, B₂, G₁ and G₂ are common contaminants of maize and peanuts, mostly produced by *Aspergillus flavus* and *A. parasiticus* (276) among few other *Aspergillus* spp. (277). Aflatoxin B₁ is considered a class I carcinogen and is tightly regulated under Health Canada at a maximum level of 15 ppb in food. Metabolism of this compound produces a highly carcinogenic epoxide, which intercalates DNA to cause mutations and greatly increases the risk for cancer development (278–280, 144). This may be partially responsible for the high rates of liver cancer in African countries, especially in Rwanda where it is the leading cancer (281, 282). High acute exposure also results in aflatoxicosis, a severe form of hepatitis, that can lead to jaundice, and potentially death (283). Frequent aflatoxin outbreaks have occurred throughout Africa in recent years, perhaps the most notable occurring in 2004 in Kenya, resulting in 317 cases of aflatoxicosis and 125 deaths between January and June alone (284). Fumonisins B₁ and B₂, produced by *Fusarium* spp., almost ubiquitously contaminate maize in Africa (158, 285). Along with liver cancer (162), fumonisin intake has been associated with esophageal cancer (161) and neural tube defects in development (163). Certain *Fusarium* spp. also produce ZEN, a compound that structurally and functionally imitates estrogen, thereby dysregulating endocrine signalling (173), potentially stimulating breast cancer progression (174), and causing fetal abnormalities (27). Recent work has also observed that ZEN disrupts the immune system, attenuating the production of antibodies and macrophage-derived peroxide (286). Similarly to fumonisins, ZEN is found in several types of grains and cereals worldwide, including maize and corn (287). In contrast, ochratoxin A is not believed to broadly contaminate foods in Africa, though its widespread prevalence in European and American beverages, such as coffee and
wine, warrants investigation. OTA can be produced by several *Aspergillus* spp. and *Penicillium verrucosum*, and is mainly associated with nephropathy in humans (177).

From a women’s health perspective, mycotoxin ingestion is a serious issue in developing nations. Not only do the broad carcinogenic and toxic effects apply, but also pathologies specifically related to females. For example, exposure to mycotoxins increases risks of developing tumours of the endometrial lining, ovaries, and breasts. In addition, pregnant women ingesting mycotoxins are at risk for late-term abortion and fetal abnormalities because of their toxic effects in development (288). Furthermore, breastfeeding women can pass AFM1, FB1, and OTA to newborns, an occurrence reported in women from African countries (289). These risks are specifically targeted to women, who contribute 60-80% of the agricultural workforce in African countries and are therefore highly exposed to such toxins (183). Therefore, women of reproductive age are important in determining the effects of environmental toxins on population health, as it directly affects incoming generations.

Though studies in Africa have reported human contact with mycotoxins, the substantial differences in climate and eating habits between individual countries warrants investigation into the Rwandan population. Our unique focus on Rwandan women is important and well-founded, since their common food sources have been shown to be significantly contaminated with these compounds. Approximately 5.4%, 0.9%, and 46.8% of Rwandan cassava crops are reportedly contaminated with deoxynivalenol, ochratoxin A, and zearalenone, respectively (157). This crop is the second most popularly consumed crop in Rwanda, only proceeded by maize. Though not directly studied in Rwanda, maize is also frequently colonized by toxigenic *Fusarium* and *Aspergillus* spp. in other populations. ELISA measurements of FB1 and ZEN in banana beers made in Kigali, Rwanda also quantified mean concentrations of 32.65 and 1.43 µg/kg, respectively (171). Despite prevalent contamination of popular Rwandan staple foods with mycotoxins, human exposure levels have been under-reported. Since this better reflects clinically significant outcomes, we sought to characterize a broad profile of fungal toxins that may be present in Rwandan women of reproductive age.
5.3 Results

5.3.1 Multi-mycotoxin detection in plasma and urine of Rwandan women

Using liquid chromatography-mass spectrometry, plasma AFB$_1$ and urine aflatoxins, fumonisins, OTA, and ZEN were quantified in Rwandan women. The largest proportion of samples were contaminated with plasma AFB$_1$ at 81%, followed by OTA at 71%, ZEN at 51%, AFG$_1$ at 50%, and AFM$_1$ at 49% (Table 5.1). Of values above the limit of quantification, the highest mean concentrations occurred for OTA at 684 pg/mg creatinine, ZEN at 307 pg/mg creatinine, and AFM$_1$ at 170 pg/mg creatinine (Table 5.1 and Figure 5.2). Plasma albumin-conjugated AFB$_1$ was present at a mean level of 2.22 pg/mg albumin, with measurements ranging from 0.36 to 9.34 pg/mg albumin (Figure 5.1A). While urinary AFM$_1$ is a validated biomarker for short-term exposure to dietary aflatoxin B$_1$ (290), AFB$_1$-albumin reflects exposure over several weeks (291). Therefore, to observe consistency in measurements and whether exposure was changing over time, we analyzed samples for AFB$_1$-albumin across a 30-day interval. There was some variation within individuals in aflatoxin B$_1$ plasma concentrations, but no overall difference was observed (p>0.05, Figure 5.1B).

Table 5.1: Summary of detected plasma and urinary mycotoxin levels

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>&lt;LOD n</th>
<th>%</th>
<th># detectable n</th>
<th>%</th>
<th>Total n</th>
<th>Mean of positives</th>
</tr>
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<tr>
<td>AFB$_1$-lysine</td>
<td>21</td>
<td>19%</td>
<td>91</td>
<td>81%</td>
<td>112</td>
<td>2.22*</td>
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<td>68</td>
<td>51%</td>
<td>66</td>
<td>49%</td>
<td>134</td>
<td>169.91#</td>
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<tr>
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<td>124</td>
<td>89%</td>
<td>15</td>
<td>11%</td>
<td>139</td>
<td>6.24#</td>
</tr>
<tr>
<td>AFB$_2$</td>
<td>130</td>
<td>94%</td>
<td>9</td>
<td>6%</td>
<td>139</td>
<td>2.25#</td>
</tr>
<tr>
<td>AFG$_1$</td>
<td>70</td>
<td>50%</td>
<td>69</td>
<td>50%</td>
<td>139</td>
<td>14.06#</td>
</tr>
<tr>
<td>AFG$_2$</td>
<td>127</td>
<td>91%</td>
<td>12</td>
<td>9%</td>
<td>139</td>
<td>1.51#</td>
</tr>
<tr>
<td>FB$_1$</td>
<td>135</td>
<td>98%</td>
<td>3</td>
<td>2%</td>
<td>138</td>
<td>59.37#</td>
</tr>
<tr>
<td>FB$_2$</td>
<td>118</td>
<td>85%</td>
<td>21</td>
<td>15%</td>
<td>139</td>
<td>64.41#</td>
</tr>
<tr>
<td>OTA</td>
<td>41</td>
<td>29%</td>
<td>98</td>
<td>71%</td>
<td>139</td>
<td>684.39#</td>
</tr>
<tr>
<td>ZEN</td>
<td>61</td>
<td>49%</td>
<td>64</td>
<td>51%</td>
<td>125</td>
<td>306.98#</td>
</tr>
</tbody>
</table>

LOD = limit of detection; * pg/mg albumin, # pg/mg creatinine
Figure 5.1: Concentration of AFB$_1$-albumin adduct in plasma of Rwandan women
Plasma aflatoxin B$_1$ in Rwandan women, expressed in pg/mg albumin. Plasma AFB$_1$-albumin levels were quantified using targeted LC-MS (A) in 112 individuals, and (B) before and after a trial consuming probiotic *L. rhamnosus* GR-1 and *L. reuteri* RC-14 daily for 30 days. Concentrations were determined by comparison to 13C-labelled AFB$_1$-lysine standard directly injected into samples. No statistical differences were observed between day 0 and day 30, according to paired t-test (p>0.05).
Figure 5.2: Concentration of various mycotoxins in urine of Rwandan women
Levels of urinary aflatoxins M₁, B₁₂, and G₁₂, fumonisins B₁ and B₂, ochratoxin A, and zearalenone in Rwandan women, expressed in ng/mg creatinine.
5.3.2 Association of dietary habits, location, and pregnancy outcomes to mycotoxin levels

Upon stratification of mycotoxin levels by metadata, including dietary habits (vegetable and fruit consumption), pregnancy status, history of preterm labour and infertility, and rural vs. urban residence, several trends were identified. AFM$_1$ levels were elevated in women reporting daily vegetable consumption, compared to those reporting only a few times a week (*p<0.05, Figure 5.3). Additionally, plasma AFB$_1$ levels were elevated in pregnant women compared to non-pregnant women in this population (***p<0.01, Figure 5.4).
Figure 5.3: Urinary aflatoxin M₁ concentrations are positively correlated with vegetable consumption

Levels of urinary aflatoxin M₁ in Rwandan women, expressed in ng/mg creatinine. Vegetable consumption was self-reported in clinical visits upon patient recruitment. Significance was determined on log-transformed values using Welch’s two-sample t-test with Bonferroni’s multiple test correction (*p<0.05).
Figure 5.4: Pregnant Rwandan women have elevated plasma aflatoxin B$_1$ levels
Levels of plasma albumin-conjugated aflatoxin B$_1$ in Rwandan women, expressed in pg/mg albumin. Pregnancy status was reported in clinical visits upon patient recruitment. Significance was determined on log-transformed values using Welch’s two-sample t-test with Bonferroni’s multiple test correction (**p<0.01).
5.4 Discussion

We report here, using highly reliable and state-of-the-art metabolomics techniques, levels of a panel of important fungal toxins in biological samples from Rwandan women. The techniques we have used, namely isotopically-labelled spiked standards with solid phase extraction, produce accurate results while reducing the effect of matrices on sensitivity (292, 293). Signal suppression is typically high for plasma (294) and urine (295) samples. Therefore, despite the popularity of dilute-and-shoot methods for urine mycotoxin analysis, our methodology was also highly effective, as observed by the large detection percentage in our study compared to others (168, 169) and high accuracy and precision (Data not shown). Additionally, we have adopted creatinine normalization to adjust for urine dilution; however, recent work brings this method into question considering the inter-individual variation in creatinine excretion (296). To account for this, we also utilized the validated 8-colour urine chart for measurement of urine concentration, and found that the two were positively related (Figure 5.5). Given this, we are confident in the reliability of our measurements.

Despite numerous studies on mycotoxin contamination in food products and in humans from other nations in Africa, no studies have attempted to quantify human exposure to mycotoxins in Rwanda. However, the mean of 170 pg AFM1/mg creatinine in this study is much in line with that found in cohorts from Ghana and Zimbabwe using similar methodology, which ranged from 162–831 pg/mg creatinine (151, 297, 298). Much lower concentrations were observed in adults from Cameroon and Kenya, at 40 and 5 pg/mg creatinine, respectively (169, 299), despite the latter population having historically been highly exposed to AFB1. In contrast to German and Italian populations, for example, where exposure risks are much lower, urinary AFM1 was only detectable in 0–6% of the population (300, 301). Although urine biomarkers reflect short-term exposure to AFB1, chronic exposure can be delineated from plasma AFB1-lysine adducts. We observed widespread contamination of AFB1-albumin (mean of 2.2 pg AFB1/mg albumin) in 81% of the Rwandan women, demonstrating that reproductive-age women are chronically exposed to aflatoxin. In further support of this, there was consistency in the overall level of this blood biomarker in a subset of individuals measured again after 30 days. This is
consistent with a cohort of Egyptian women, whose serum AFB1-albumin levels were 4.9 pg/mg albumin (150). One source of AFB1 in this population may be cassava plants, due to the observed correlation between vegetable intake and urinary AFM1. Cassava was a popularly reported component of their vegetable intake, and it is reported as the most highly cultivated crop in Rwanda, with 3,159,551 tonnes being produced in 2014 alone (302). Other potential sources that were not indicated in this study include maize and peanuts (154, 155), which are frequently colonized by toxigenic Aspergillus spp.

Interestingly, pregnant women in this cohort had elevated levels of circulating aflatoxin B1 compared to their non-pregnant counterparts. This could be explained by increased food intake because of higher energy demands, since an estimated additional 350 kcal/day and 500 kcal/day are needed for the second and third trimesters, respectively (303). However, since only limited self-reported dietary information was obtained, and pregnancy status was not associated with differences in vegetable, fruit, or fish consumption (Data not shown), we can only speculate on this association. Furthermore, there is little to no data on actual energy intake in pregnant Rwandan women. For example, in an Indian cohort, the cumulative increase in calorie intake during the second and third trimesters plateaued at approximately 380 kcal/day (304). Regardless, this finding suggests there is an increased risk for harm to pregnant women and their developing fetus.

Fumonisin B2 was present in 21/139 (15%) of samples in this population, much more prevalent than FB1 at 3/138 (2%). This is in direct opposition to the consistent trend in the literature of FB1 being more abundant than FB2 in food and humans (169, 170). However, since toxicological studies in rats and cell culture have concluded that FB1 and FB2 have similarly detrimental effects (305, 306), this may not affect clinical outcomes. Regardless, research into crops containing fumonisin contamination in Rwanda have either focused entirely on FB1 (171), shown consistently higher levels of FB1 than FB2 (156), or not detected either (157), leaving many questions unanswered about the source of FB2 in this study. Without fully understanding the impact of elevated FB2 in human studies, the presently observed concentrations will require further investigation (169, 170, 300).
Of the urinary mycotoxins measured, OTA was most commonly detected (71%), and was highly elevated at a mean concentration of 684 pg/mg creatinine. This is approximately 10-fold higher than most other studies measuring OTA in African cohorts (169, 170). However, the prevalence of Rwandan staple foods exceeding acceptable values of OTA, including peanuts, maize, and cassava (156, 157), are also quite high. OTA is generally considered a problem of developed nations due to its prevalence in coffee-based products imported from tropical regions and necessity for strict regulations on OTA in stored wheat. Nevertheless, OTA-producing *Aspergillus* spp. have been isolated from Rwandan green coffee beans in multiple studies (176, 307). As OTA is known to have nephrotoxic effects, it may therefore contribute to the alarming rates of chronic kidney disease in Sub-Saharan Africa (308). Given this new information, more research must be done on this toxic compound and its biological impact in Rwanda.

Significant ZEN contamination is prevalent in cassava from Rwanda, with 11.7% of these crops containing levels over the regulatory limit (157, 171). It is not surprising, then, that ZEN exposure in this population is widespread, contaminating urine at a mean level of 307 pg/mg creatinine. Similar assessments in Cameroon and South Africa observed comparable levels of 210 and 529 pg/mg creatinine, respectively (168, 169). To counteract the significant exposure of this population, several studies have focused on utilizing probiotic organisms to sequester and detoxify zearalenone. Probiotic *Lactobacillus reuteri* with cloned genes for zearalenone degradation and *Bacillus amyloliquefaciens* have recently been developed with notable success, as they sequester ZEN from absorption and can convert it to non-toxic metabolites (309, 310).

In this study, we have quantified mycotoxins of importance in a novel cohort of 139 Rwandan women. Aflatoxin, ochratoxin A and zearalenone were widely prevalent in this population, with plasma AFB1-albumin disparately affecting pregnant women. Given limited dietary information, only aflatoxin B1 could be linked to vegetable consumption. However, additional studies will be needed to elucidate the exact source of AFB1 and other prevalent toxins, especially OTA. Although no associations were observed between mycotoxin concentrations and adverse fertility outcomes, the impact of prolonged exposure to these concentrations in reproductive aged women will be important for future
work. Given their potential negative consequences on this vulnerable population, therapeutics such as probiotics to mitigate exposure to mycotoxins and systematic improvements to food safety measures are of utmost importance (311).

5.5 Materials and Methods

5.5.1 Human sample collection and ethics

Recruitment and sample collection of blood and urine from 149 pre-menopausal Rwandan women (ages 18–55) was performed at the Central University Hospital of Kigali. Ethics approval was obtained under the University of Western Ontario Heath Sciences Research Ethics Board (HSREB#102886, Appendix A.2) and the Ethics Committee of Kigali University Teaching Hospital (Ref#EC/CHUK/035/12, Appendix A.3). A subset of 30 women were recruited for a clinical trial of probiotic lactobacilli [urex-cap-5 (RePhresh Pro-B), 10⁹ L. rhamnosus GR-1 and L. reuteri RC-14] ingested daily for 30 days, and additional samples were collected for these individuals at the end of the trial. First, informed consent was read and signed by patients, and a health history questionnaire (containing information about fertility, diet, residence, past/present infections, etc.) was filled out. Blood collection, including malaria test, was performed by Dr. Stephen Rulisa or a registered nurse. Two tubes of blood (3 mL each) were collected from each patient into vacutainer tubes. Patients were then instructed to self-collect a urine sample into a sterile collection tube. Samples were stored at -80°C, where possible, until processing.

5.5.2 AFB₁-lysine quantification in plasma samples

Plasma was processed for quantification of albumin-conjugated AFB₁ as per the method by McMillan et al. (312). Briefly, plasma samples were thawed on ice for 30 min before 35 μL was aliquoted into a fresh vial for albumin quantification. To precipitate proteins, 805 μL of 8:2 methanol:water was added to 250 μL of plasma and samples were vortexed for 15 sec (313). Following centrifugation at 15 000 rpm for 15 min, supernatant was transferred to a fresh vial and stored at -80°C. The protein pellet was resuspended in 230 μL PBS with agitation using a Teflon pestle. Upon reconstitution, samples were spiked with 75 μL of 1.0 ng/mL 13C, 15N-labelled AFB₁-lysine standard. To release plasma aflatoxin from conjugated albumin, 230 μL of 6.5 mg/mL Pronase® (nuclease-free, Sigma,
Oakville, CA) was added to each sample, vortexed for 10 sec, and incubated overnight at 37°C with agitation at 1300 rpm. Following incubation, 460 μL of MilliQ water was added to samples, and subjected to Solid Phase Extraction (SPE) using the Oasis® MAX 1cc 30 mg extraction cartridge, according to the following established procedure (314).

Cartridges were activated and equilibrated with two 1 mL aliquots of methanol and MilliQ water, respectively. Samples were then loaded at a flow rate of 0.5 mL/min prior to washing with 1.0 mL MilliQ water. Impurities were removed using washes with 1.0 mL of 70% (v/v) methanol, 1.0 mL of 1% NH₄OH in methanol, and 0.5 mL of methanol. Cartridges were then dried for 5 min on high, before final elution with 800 μL of 2% formic acid (FA) in methanol. Samples were dried using nitrogen gas, then reconstituted in 150 μL of 25% methanol and transferred to vial inserts for LC-MS.

LC-MS analysis was performed using an Easy-nLC 1000 nano-flow system coupled with a Q-Exactive Orbitrap mass spectrometer. 10 μL of each sample was injected onto a 100 μm × 2 cm Acclaim C18 PepMap™ trap column and washed with 20 μL of 0.1% FA. Bound compounds were then eluted onto a 75 μm × 15 cm Acclaim C18 PepMap™ analytical column (Thermo Scientific, Waltham, US). A flow rate of 300 nL/min was used throughout the entirety of the run. The gradient started at 5% B (acetonitrile + 0.1% FA) and increased to 35% over 20 min, then increased to 90% B over 2 min and held for 5 min. The trap and analytical columns were equilibrated with 6 μL of 0.1% FA in water between each run. The injection needle and loop system were washed during analysis with isopropanol, acetonitrile:water (50:50) and finally with 0.1% FA in water. The nanospray voltage was set at 1.90 kV, capillary temperature 275°C, and S-lens RF level 60. Each sample was analyzed by a combination of full MS and parallel reaction monitoring (PRM). Full MS was acquired between mass range 150–900 m/z at 35,000 resolution, automatic gain control (AGC) target of 5 × 10⁶ and maximum injection time (IT) of 256 ms. PRM analysis monitoring the AFB₁-Lys and AFB₁⁻¹³C₆⁻¹⁵N₂⁻⁵⁷⁻⁵⁵⁻⁵⁴⁻⁵₁⁻⁴⁰⁻⁴⁹⁻⁴⁸⁻⁴⁷⁻⁴⁶⁻⁴⁵⁻⁴⁴⁻⁴³⁻⁴²⁻⁴¹⁻⁴⁰⁻³⁹⁻³⁸⁻³⁷⁻³⁶⁻³⁵⁻³⁴⁻³³⁻³²⁻³¹⁻³⁰⁻²⁹⁻²⁸⁻²⁷⁻²⁶⁻²⁵⁻²⁴⁻²³⁻²²⁻²¹⁻²⁰⁻¹⁹⁻¹⁸⁻¹⁷⁻¹⁶⁻¹⁵⁻¹⁴⁻¹³⁻¹²⁻¹¹⁻¹⁰⁻⁹⁻⁸⁻⁷⁻⁶⁻⁵⁻⁴⁻³⁻²⁻¹⁻⁰⁺⁺⁺⁺ m/z isolation window, 17,500 resolution, AGC target 5 × 10⁶ and 25 NCE. Peak areas were integrated in the Xcalibur software package at 5 ppm mass accuracy. A calibration curve consisting of seven
concentrations of unlabelled AFB\textsubscript{1}-Lys: 30, 200, 400, 1000, 2000, 3000 and 4000 pg/mL each with 500 pg/mL AFB\textsubscript{1}-^{13}\text{C}\text{6}^{15}\text{N}\text{2}-\text{Lys} was constructed with a linear fit.

5.5.2.1 Bromocresol purple albumin quantification assay

Plasma albumin levels were determined using the modified method by Hill & Wells (315). To prepare the bromocresol purple (BCP) reagent, 60 µM BCP was dissolved in 0.1 M acetate buffer containing 0.15 M sodium chloride and pH brought to 5.5. In a 96-well plate, 180 µL of BCP reagent was added to 20 µL of 11-fold saline-diluted plasma. Samples were processed for 2 min before measuring absorbance at 603 nm. Comparison to a standard curve of human albumin at concentrations ranging from 10–60 mg/mL was utilized to quantify albumin in each sample.

5.5.3 Multi-mycotoxin biomarker quantification in urine

Urine samples were thawed on ice for 30 min, then 200 µL were transferred to a fresh vial for creatinine quantification. 5 mL of each urine sample was diluted 1:1 with deionized water and centrifuged at 4500 × g for 10 min to remove solid matter. Supernatants were then transferred to fresh containers and set to pH 7.0 with ammonium hydroxide and formic acid. Samples were then spiked with 10 µL of a working solution containing ^{13}\text{C}, ^{15}\text{N}-labelled internal standards at the following concentrations: 60 ng/mL aflatoxin M\textsubscript{1}, 12 ng/mL fumonisin B\textsubscript{1}, 30 ng/mL fumonisin B\textsubscript{2}, and 30 ng/mL ochratoxin A. Quantification of aflatoxins B\textsubscript{1}, B\textsubscript{2}, G\textsubscript{1}, G\textsubscript{2}, and zearalenone were also performed by comparison to standard curves run with each batch. Diluted urine was run through SPE using the Oasis Max 6cc 150 mg extraction cartridge, according to the following procedure.

Cartridges were activated and equilibrated with two 5 mL aliquots each of methanol and deionized water, in that order. Samples were then loaded at a flow rate of 0.5 mL/min and rinsed with 5.0 mL deionized water. Each column was eluted with 3 mL of 100% methanol and collected for analysis of aflatoxins and zearalenone. Columns were then washed with 2 mL each of 5% (v/v) NH\textsubscript{4}OH in methanol, 100% methanol, and 2% formic acid in 25% methanol. Cartridges were then dried for 5 min on high, before final elution with 5× 750 µL of 2% formic acid in methanol. Both collected fractions were dried using nitrogen gas,
then reconstituted in 250 μL of 25% methanol with agitation at 4500 x g and transferred to LC-MS vials.

Reconstituted urine samples were analyzed using an Agilent 1290 Infinity HPLC coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo) with a HESI (heated electrospray ionization) source. For HPLC, 10 μL of each sample was injected into a ZORBAX Eclipse plus RRHD C18 (2.1 × 50 mm × 1.8 μm) column, maintained at 35°C using a flow rate of 0.3 mL/min. Mobile phase (A) consisted of 0.1% formic acid in LC-MS grade water and mobile phase (B) consisted of 0.1% formic acid in acetonitrile. The initial composition of 0% (B) was held constant for 0.5 min, then increased to 100% over 3.0 min. Mobile phase B was then held at 100% for 2.0 min, then returned to 0% over 0.5 min for a total run time of 6.0 min.

The HESI source was operated under the following conditions: nitrogen flow of 17 and 8 arbitrary units for the sheath and auxiliary gas respectively, probe temperature and capillary temperature of 450°C and 400°C respectively, S-Lens RF level of 45, and capillary voltage of 3.9 kV. Targeted analytes and internal standards were monitored by LC-MS/MS acquisition mode using the following settings: 1.2 m/z isolation window, normalized collision energy of 35, resolution of 17 500, AGC target 5 x 10⁶, and maximum injection time of 110 ms. Standard curves were run with every batch to account for sample variation, or absolute quantification was performed by isotope dilution method. The target analytes were monitored by MS/MS using the conditions shown in Table 5.2. Aflatoxins and ZEN were measured in SPE fractions collected from the initial 100% methanol elution, while fumonisins and OTA were measured in SPE fractions collected from the final 2% FA in methanol elution.

<table>
<thead>
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<th>Name</th>
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<th>Product ion m/z</th>
<th>NCE</th>
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<td>43</td>
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For absolute and relative quantification of mycotoxins of interest, seven-dilution calibration curves were created consisting of: Unlabelled AFM$_1$ (0.023, 0.046, 0.23, 0.46, 2.3, 4.6, and 23 ng/mL) each with 1.2 ng/mL labelled AFM$_1$; unlabelled AFB$_1$ and AFG$_1$ (0.046, 0.092, 0.46, 0.92, 4.6, 9.2, and 46 ng/mL); unlabelled AFB$_2$ and AFG$_2$ (0.0115, 0.023, 0.115, 0.23, 1.15, 2.3, and 11.5 ng/mL); unlabelled FB$_1$ (1.15, 2.3, 11.5, 23, 115, 230, and 1150 ng/mL) each with 6 ng/mL labelled FB$_1$; and unlabelled FB$_2$, OTA, and ZEN (0.46, 0.92, 4.6, 9.2, 46, 92, and 460 ng/mL) each with 6 ng/mL labelled FB$_2$ and labelled OTA. Following data acquisition, peak areas were quantified using the Xcalibur software package. Peaks were identified within a retention time window and width of 0.5 and 0.75 min respectively, at 50 ppm mass accuracy. Peak detection and integration using the Genesis method was performed, with a signal-to-noise threshold of 0.5 and signal-to-noise minimum peak height of 3.0 respectively. Automatic processing was supplemented with manual confirmation of peaks.

5.5.3.1 Measurement of additional urine characteristics

Urine creatinine levels were measured using the Creatinine (urinary) Colorimetric Assay Kit (Cayman Chemical Co., Ann Arbor, MI, USA), according to the manufacturer’s instructions. Urine hydration status was graded according to the validated 8-scale urine colour chart originally developed by Armstrong et al. (316, 317). Creatinine concentrations were plotted against urine colour in Figure 5.5. pH was measured following 1:1 dilution of urine with milli-Q water, and additional characteristics such as cloudiness and presence of blood were recorded.
Figure 5.5: Urine colour and creatinine concentration are positively related
Interquartile ranges of creatinine concentration stratified by urine colour, with outliers individually plotted. Colour was determined according to the 8-scale chart and creatinine was quantified using a creatinine (urinary) colorimetric assay kit. Line indicates mean creatinine level for each group.
5.5.4 Data processing and statistical analysis

Following peak detection, concentrations (in ng/mL) were calculated based on calibration curves for each mycotoxin. Values were excluded from additional analyses if below the limit of quantification. Plasma AFB$_1$ levels were normalized to serum albumin, while all urine mycotoxins were normalized to creatinine to account for variation in dilution of these fluids. All concentrations were log-transformed prior to statistical analysis to obtain normal distribution. Associations between mycotoxin levels and patient metadata were performed in R (r-project.org), accounting for multiple test correction. All graphing was done using GraphPad Prism 6 (La Jolla, CA).
Chapter 6

General Discussion and Conclusions
6.1 Discussion

This thesis has provided a series of studies that have increased our understanding of microbes, particularly their influence on women’s health. Lactobacilli in the vaginal microbiota are known to play a large part in preventing dysbiosis and infection by sexually transmitted diseases. Interventions to increase their abundance and production of beneficial lactate have been sparse until now. Furthermore, surprisingly little is still known about the role of the most predominant carbon source in the vagina—glycogen—on the vaginal microbiota. While much of this research has focused on issues predominantly affecting women in North America and Europe, there is a current lack of research on the effects of infectious microbes on women’s health in the developing world. An ongoing problem is chronic exposure to fungal toxins, which are found ubiquitously in crops from countries in Africa, and which have a range of effects on infertility, pregnancy outcomes, and women’s health, in general. Therefore, this thesis has expanded knowledge of exogenous and endogenous carbohydrates on the composition and activity of vaginal microbes, and the extent and impact of mycotoxin exposure in a group of high-risk women in Rwanda.

6.1.1 Lactulose supports vaginal acidity and maintenance of a Lactobacillus-dominated microbiota

In Chapter 2, I evaluated the potential of lactulose, against three other common prebiotics, to selectively stimulate vaginal lactobacilli without also propagating BV organisms or C. albicans. Lactulose was fermented by a broad array of common vaginal lactobacilli, namely L. iners and 16 L. crispatus isolates, while not influencing the growth of G. vaginalis, A. vaginae, P. bivia, or M. curtisii (Figure 2.1–2.3). However, the use of single cultures does not represent the diversity present in the vagina. As the current animal models for BV also only utilize single organism inoculation (182), I developed a batch culturing method using swabs taken directly from healthy or BV-affected women. Using this polymicrobial model, challenging with lactulose restored acidic pH and increased lactic acid production in both healthy and BV communities, and attenuated odor-associated amine secretion in the dysbiotic model (Figures 2.7, 2.11, and 2.12). Although lactulose treatment helped to maintain a Lactobacillus-dominated community in the healthy system,
the few remaining lactobacilli could not be stimulated in the BV model to restore a healthy profile (Figures 2.4, 2.5, 2.9, and 2.10).

In Chapter 3, I demonstrated direct antagonism of lactulose-stimulated \( L. \) \textit{crispatus} products against BV organisms, which was dependent on acidic pH (Figure 3.1). Unlike other carbohydrate-based interventions, lactulose supplementation does not interfere with \( L. \) \textit{crispatus} adherence (Figure 3.2), an important commensal property of this bacterium (76). This supports a mechanism whereby this beneficial organism ferments lactulose into abundant lactic acid, creating a hostile environment for acid-labile microbes. However, caution must be taken should future extensions of this research arise, as lactulose detrimentally affects the viability of vaginal epithelial cells (Figure 3.3).

As an extension to these promising findings, lactulose must be evaluated further in a randomized controlled clinical trial for the maintenance of a healthy vaginal microbiota. Given the high incidence of periodic fluctuations from homeostasis to dysbiosis during the menstrual cycle and in pregnancy (39, 318), a therapeutic that supports microbial eubiosis would be clinically useful. Furthermore, there is promise for lactulose in conjunction with treatments known to stimulate \( Lactobacillus \) spp., such as probiotic GR-1 and RC-14 (131), in a two-pronged synbiotic approach that takes advantage of the metabolic benefits of lactulose. This research has direct application for use in women, and is worth continuing to potentially reduce the burden of antibiotic use in BV. Additionally, the polymicrobial batch culture model developed in this thesis will be valuable for future research evaluating vaginal prebiotics or other microbiota interventions, as it more closely resembles the diversity present \textit{in vivo}.

6.1.2 Glycogen utilization by BV organisms and \textit{Lactobacillus iners} is self-limiting

Much of the previous work evaluating the role of glycogen in shaping the vaginal microbiota relied on retrospective correlations to healthy women. With the work done in Chapter 4 of this thesis, glycogen metabolism was directly observed by BV-associated \( G. \) \textit{vaginalis}, \( A. \) \textit{vaginae}, \( P. \) \textit{bivia} and \( M. \) \textit{curtisii}, as well as the first report of its utilization by vaginal lactobacilli—\( L. \) \textit{iners} (Figures 4.1–4.4). Interestingly, acidic metabolites produced
in this degradation process by *L. iners* and *G. vaginalis* had a self-killing effect, but also antagonized other dysbiotic members of the vaginal microbiota (Figures 4.5–4.6). Secretions from *L. iners* treated with glycogen remained antagonistic to *G. vaginalis* upon neutralization (Figure 4.5), suggesting a metabolite was produced that either prevented glycogen utilization by *G. vaginalis* or was bacteriostatic. These findings emphasize that the widely stated reason that lactobacilli propagate in the vagina because of glycogen, is misleading at best.

Others have reported lactic acid to be inhibitory to α-amylases (262), enzymes known to catabolize glycogen. However, lactic acid at the same concentration as was present in *L. iners* supernatants had no effect on *G. vaginalis* or *L. iners* growth in glycogen (Figure 4.7). Furthermore, the remaining inhibitory effect was not due to nutrient depletion because neutralized, spent supernatants from *G. vaginalis* did not similarly prevent *L. iners* growth in glycogen (Figure 4.6). Therefore, despite an attempt to characterize the nature of this substance, it remains elusive.

A rational next step will be to perform metabolomic analysis on these supernatants to examine compounds secreted by *L. iners* upon glycogen metabolism for their inhibition of other organisms. This could be combined with an examination of the *L. iners* genome for candidate molecules, albeit none have been found so far (47). Future work should also aim to characterize genes for glycogen metabolism in a broader profile of vaginal organisms, not just in *L. iners* isolates, to determine how conserved this phenotype is. With this information, targeted inhibition of these enzymes may be possible as an intervention to limit the nutrient availability of organisms associated with dysbiosis. As there are feminine health products already integrating glycogen under the pretense that it will beneficially modify the vaginal microbiota, it is essential that research be done to understand its true impact in this system. The work in this thesis provides support against its exogenous administration due to the potential to stimulate BV organisms.
6.1.3 Rwandan women are significantly exposed to aflatoxins, fumonisins, ochratoxin A, and zearalenone

In Chapter 5 of this thesis, I developed a method and measured the biological exposure of 139 Rwandan women to a panel of fungal toxins with worldwide importance: Aflatoxin B\textsubscript{1}, fumonisin B\textsubscript{1} and B\textsubscript{2}, ochratoxin A and zearalenone. Aflatoxins were found to be widely present in this population. Indeed, 81% and 49% of women had detectable levels of AFB\textsubscript{1}-albumin and AFM\textsubscript{1}, respectively, the two major biomarkers for AFB\textsubscript{1} exposure in blood and urine (290, 291). Since AFM\textsubscript{1} in urine was positively correlated to vegetable intake, I posited that widely popular crops known to be contaminated with aflatoxins, such as cassava (154, 157), could be the source. Increased long-term aflatoxin exposure was observed in pregnant compared to non-pregnant Rwandan women, which raises serious concern over the health of mothers and their developing fetuses.

Urinary levels of ochratoxin A and zearalenone were also surprisingly high, with mean concentrations of 684 and 307 pg/mg creatinine in the 71% and 51% of women that had detectable levels, respectively. Fortunately, higher levels of these mycotoxins were not associated with adverse fertility outcomes, as determined by comparison to patient metadata. However, without knowing the source and extent of this contamination in the entire population, the impact of these elevated levels remains unknown. Regardless, reporting of ochratoxin A challenges the notion that it is not a frequent contaminant in African nations, and provides rationale to continue studying its influence in the developing world. Fumonisins B\textsubscript{1} and B\textsubscript{2} were less extensively detected, with only 2% and 15% of women having quantifiable levels.

Extending this research to larger populations in Rwanda with carefully collected dietary information would be beneficial to understanding the widespread impact and food source of the contamination. Given the known negative consequences of mycotoxin exposure, therapeutics such as probiotics that might mitigate exposure to, and absorption of, mycotoxins (309, 310) and systematic improvements to food safety measures are of utmost importance. Women in Africa are disparately affected by crop-related toxins due to their dominance in the farming workforce and in meal preparation (183), so studies focused on the impact on women are essential. This thesis provides a quantification of mycotoxin
levels in reproductive-age Rwandan women, which were previously unreported, and provides insight into some possible sources of aflatoxin B₁ and the elevated risk for pregnant women.
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Appendix A: Human Ethics Approval

Appendix A.1: University of Western Ontario Health Sciences Research Ethics Board (HSREB) approval for acquisition of vaginal swabs from healthy women and women with BV

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the amendment to the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guidelines for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number EB 00000940.

[Signature]

Ethics Officer, on behalf of the HSREB Chair

[Signature]

Ethics Officer, [Name]
Appendix A.2: University of Western Ontario HSREB approval for Rwanda study

Principal Investigator: Dr. Gregor Reid  
File Number: 102896  
Review Level: Full Board  
Approved Local Adult Participants: 0  
Approved Local Minor Participants: 0  
Protocol Title: Pilot study investigating bacteria (microbiota), their by-products (metabolites) and environmental toxins in relation to reproductive health in Rwandan women  
Department & Institution: Schulich School of Medicine and Dentistry/Microbiology & Immunology, Western University  
Sponsor: UWO

Ethics Approval Date: December 11, 2012  
Ethics Expiry Date: September 30, 2013

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This is to notify you that the University of Western Ontario Health Sciences Research Ethics Board (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada CH Good Clinical Practice Practices: Consolidated Guidelines, and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this HSREB also complies with the membership requirements for HSREB's as defined in Division 6 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request form.

Members of the HSREB that are named as investigators in research studies, or declare a conflict of interest, do not participate in discussions related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

[Signature]

Ethics Officer to Contact for Further Information

[Contact Information]

This is an official document. Please retain the original in your files.

Western University, Schulich School of Medicine and Dentistry, London, ON, Canada N6A 5C6  
1.800.668.3036 1.519.854.2800 905.525.9111

[Contact Information]
Appendix A.3: Ethics Committee of Kigali University Teaching Hospital approval for Rwanda study

CENTRE HOSPITALIER UNIVERSTAIRE
UNIVERSITY TEACHING HOSPITAL
Centre Hospitalier Universitaire de Kigali
Ethics Committee / Comité d’éthique

July 13th 2012

Ref.: EC/CHUK/035/12

Review Approval Notice

Dear Amy McMillan,

Your research project: “Pilot Study Investigating Bacteria (Microbiota), their by-products (Metabolome) and Environmental Toxins in Relation to Reproductive Health in Rwandan Women”

During the meeting of the Ethics Committee of Kigali University Teaching Hospital (KUTH) that was held on 13/07/2012 to evaluate your protocol of the above mentioned research project, we are pleased to inform you that the Ethics Committee/CHUK has approved your protocol. You are required to present the results of your study to KUTH Ethics Committee before publication.

PS: Please note that the present approval is valid for 12 months.

Yours sincerely,

Dr George NTAKIYIRUTA,
The Vice President, Ethics Committee,
Kigali University Teaching Hospital

<<University teaching hospital of Kigali Ethics committee operates according to standard operating procedures (Sops) which are updated on an annual basis and in compliance with GCP and Ethics guidelines and regulations>>

B.P. 655 Kigali- RWANDA Tel. Fax : 00 (250) 576638 E-mail : chuk.hospitala@chukigali.org>>
Curriculum Vitae

STEPHANIE LYNN COLLINS

Department of Microbiology & Immunology, Schulich School of Medicine & Dentistry, Western University, London, Ontario, Canada.

EDUCATION

2015–Present M.Sc. in Microbiology & Immunology
Western University, London, Canada
Supervisor: Dr. Gregor Reid

2010–2015 B.M.Sc. in Biochemistry & Cell Biology
Western University, London, Canada

PUBLICATIONS


SCHOLARSHIPS AND AWARDS

2017 International Scientific Association for Probiotics and Prebiotics (ISAPP) Students and Fellows Association (SFA) Travel Grant

2016–2017 Dr. RGE Murray Graduate Scholarship in Microbiology & Immunology

2015–2017 Western Graduate Research Scholarship

2016 ISAPP Best Poster Award

2016 ISAPP SFA Travel Grant

2016 Dr. FW Luney Graduate Travel Award in Microbiology & Immunology
2015  Dr. FW Luney Graduate Entrance Scholarship in Microbiology & Immunology

Summer 2015  Dean’s Undergraduate Research Opportunities Program

2011–2012  The Western Scholarship of Excellence

2010–2015  Western University Dean’s Honour List

PRESENTATIONS


WORK AND VOLUNTEER EXPERIENCE

2016–2017  Talks on Fridays Organizer
Lawson Health Research Institute, London, CA.

2016–2017  Secretary for the ISAPP SFA Executive Committee
Chicago, US.

Sep–Dec 2016  Teaching Assistant  
Microbiology & Immunology 3610F: Microbiology laboratory  
Western University, London, CA

Jan–Apr 2016  Teaching Assistant  
Microbiology & Immunology 2500B: The Biology of Infection and Immunity  
Western University, London, CA

2015–2016  Microbiology & Immunology Social Committee Member  
Western University, London, CA