Electronic Thesis and Dissertation Repository

12-14-2017 1:30 PM

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

Stephanie L. Collins, *The University of Western Ontario*

Supervisor: Reid, Gregor, *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology
© Stephanie L. Collins 2017

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

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:

Chapter 2: (Figures 2.1-2.8 were included in) Collins SL, McMillan A, Seney S, van der Veer C, Kort R, Sumarah M, and Reid G. 2018. Promising prebiotic candidate established by evaluation of lactitol, lactulose, raffinose, and oligofructose for maintenance of a *Lactobacillus*-dominated vaginal microbiota. Appl Environ Microbiol 84.

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.

Acknowledgments

First and foremost, I would like to thank my supervisor Dr. Gregor Reid for taking a chance on a student who knew nothing about bacteria. His passion for clinically-oriented research and openness to multi-disciplinary training allowed me to discover the area of research that I now find both exciting and rewarding, for which I am extremely grateful.

I would also like to thank Mark Sumarah, Justin Renaud, and all other members of the Sumarah lab for taking a chance on a student who knew nothing about analytical chemistry, and for the endless intellectual guidance and material support. I especially thank Mark Sumarah for providing feedback on this egregiously long thesis.

Thank you to my other advisory committee members, Drs. Carole Creuzenet and Jo-Anne Hammond for their guidance in the past few years, whose unique perspectives gave valuable insight into this project.

I am very grateful to current and past members of the Reid lab, without whom I would have surely lost my marbles. A special thanks to Amy McMillan for her mentorship early in my studies, and Shannon Seney for her patience and friendship in teaching me the absolute basics of research.

Lastly, I would like to thank my friends and family, especially mom, dad, and Peter, for their constant support and all the laughs throughout my time here.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

2-HV	2-hydroxyisovalerate
ACN	acetonitrile
AF	aflatoxin
AGC	automatic gain control
BCP	bromocresol purple
BV	bacterial vaginosis
CBA	Columbia blood agar
CFU	colony forming unit
CLR	centered log-ratio
CYP	cytochrome P450
EC	European Commission
F	fumonisin
FA	formic acid
FDA	Food and Drug Administration
FOS	fructo-oligosaccharides
GC-MS	gas chromatography-mass spectrometry
GHB	γ-hydroxybutyrate
GOS	gluco-oligosaccharides
H_2O_2	hydrogen peroxide
HESI	heated electrospray ionization

HIV	human immunodeficiency virus
HSV-2	herpes simplex virus 2
ISU	individual sequence unit
KSFM	keratinocyte serum-free medium
LEA	*Lactobacillus* epithelium adhesin
LC-MS	liquid chromatography-mass spectrometry
MOX	methoxyamine-HCl
MRS	de Mann, Rogosa, and Sharpe
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
NMR	nuclear magnetic resonance
NYC III	New York City III media
OD	optical density
OTA	ochratoxin A
OTU	operational taxonomic unit
PCA	principal component analysis
PBS	phosphate-buffered saline
PRM	parallel reaction monitoring
rRNA	ribosomal ribonucleic acid
RT-qPCR	real-time quantitative polymerase chain reaction
SCFA	short-chain fatty acid

SPE   solid phase extraction

TSB   tryptic soy broth

VDMP   vaginally defined media + proteose peptone

VK2   vaginal keratinocyte 2

ZEN   zearalenone

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

(30, 31). 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_2O_2), lactic acid, and bacteriocins (49). Early *in vitro* studies emphasized H_2O_2 as a major defense mechanism of lactobacilli due to its ability to kill *Gardnerella vaginalis* and sequester HIV-1 through the H_2O_2-peroxidase-halide pathway (50, 51), leading to the screening of vaginal probiotics on the basis of H_2O_2 production (52). However, the validity of this has come into question since H_2O_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_2O_2 is sequestered by the proteinaceous environment imparted by male ejaculate and vaginal secretions (55). Given

this, the biological impact of H_2O_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

(145). Recently, aflatoxin B_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_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_1 was found at a median level of 162 and 19.7 pg/mg creatinine in Zimbabwean and Egyptian women (150, 151). AFM_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_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_1 toxic effects and further work must address this.

1.3.2 Fumonisins (F)

Fumonisins B_1 and B_2 are mycotoxins produced by *Fusarium* spp., namely *Fusarium verticillioides*, previously known as *F. moniliforme* (158). *F. verticillioides* has the highest expression of FB_1 and FB_2 in humid conditions and suboptimal growth temperatures of 20°C and 20–30°C, respectively (159). Under these conditions, FB_1 and FB_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 FB_1 and FB_2 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 FB_1 and FB_2 represent validated biomarkers for exposure (167). Urinary FB_1 is typically found in significantly greater abundance than FB_2, reflecting similar proportions in contaminated crops. For example, in adult cohorts from South Africa, Cameroon, and Nigeria, reported urinary FB_1 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 FB_2 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 FB_1 and some FB_2 have been found in numerous food sources. Average detectable levels of FB_1 in Rwandan maize flour and cassava are similar, and range from 21.9–31.1 µg/kg, and for FB_2 are slightly lower at 10.1–17.0 µg/kg (156, 157). FB_1 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

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,

104). 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

L. crispatus 33820
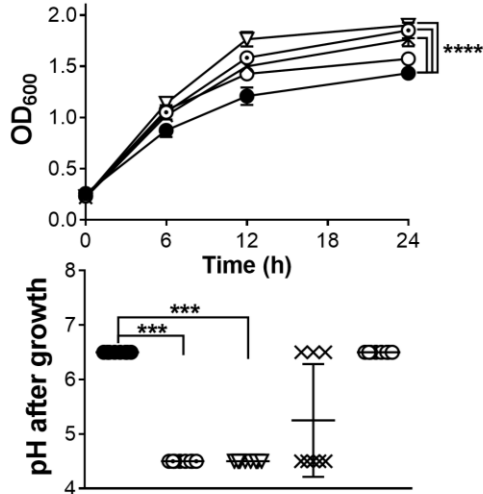
L. iners AB-1

L. vaginalis 49540
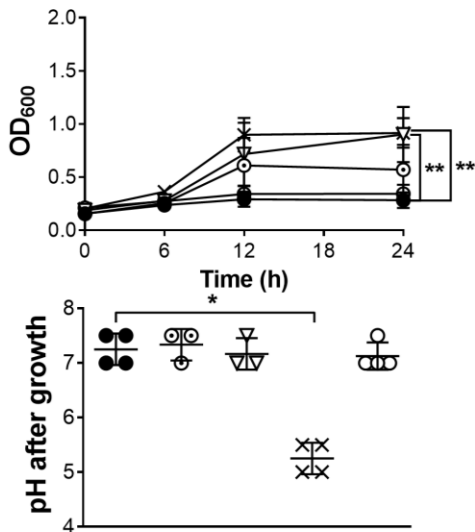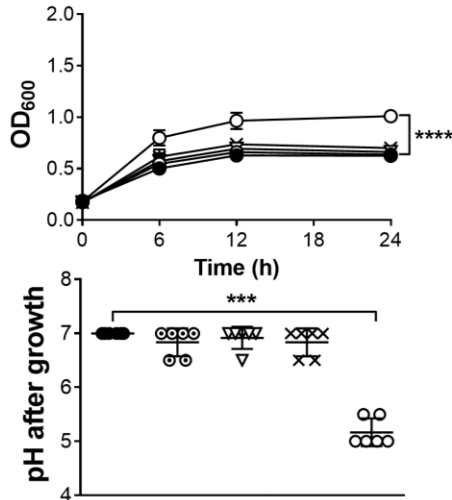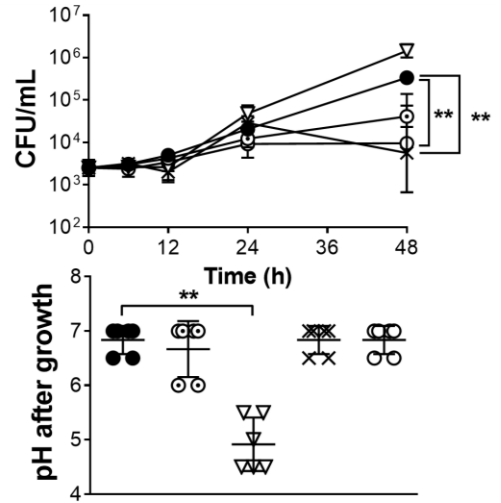
L. gasseri 33323

L. jensenii 25258
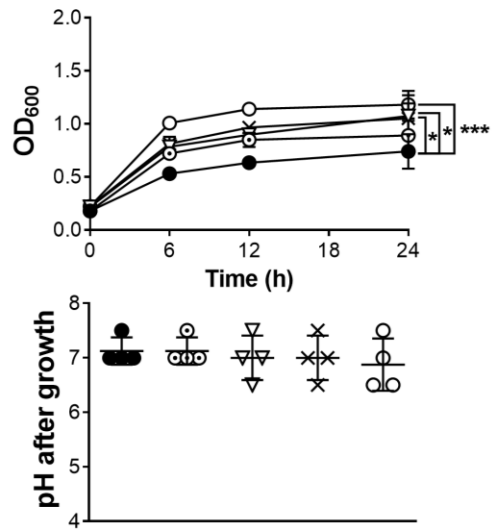
L. jensenii RC-28
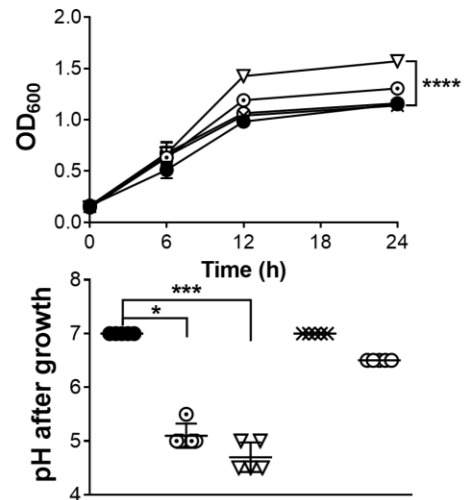
● No prebiotic ⊙ Lactitol ▽ Lactulose ✳ Raffinose ○ Oligofructose

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 $\pm$ SEM, with differences determined using 2-way ANOVA with Dunnett's multiple comparisons test. For pH measurements, bar heights are mean pH $\pm$ SD, with differences to control determined using Kruskall-Wallis' with Dunn's multiple comparisons tests.

Healthy   **BV**
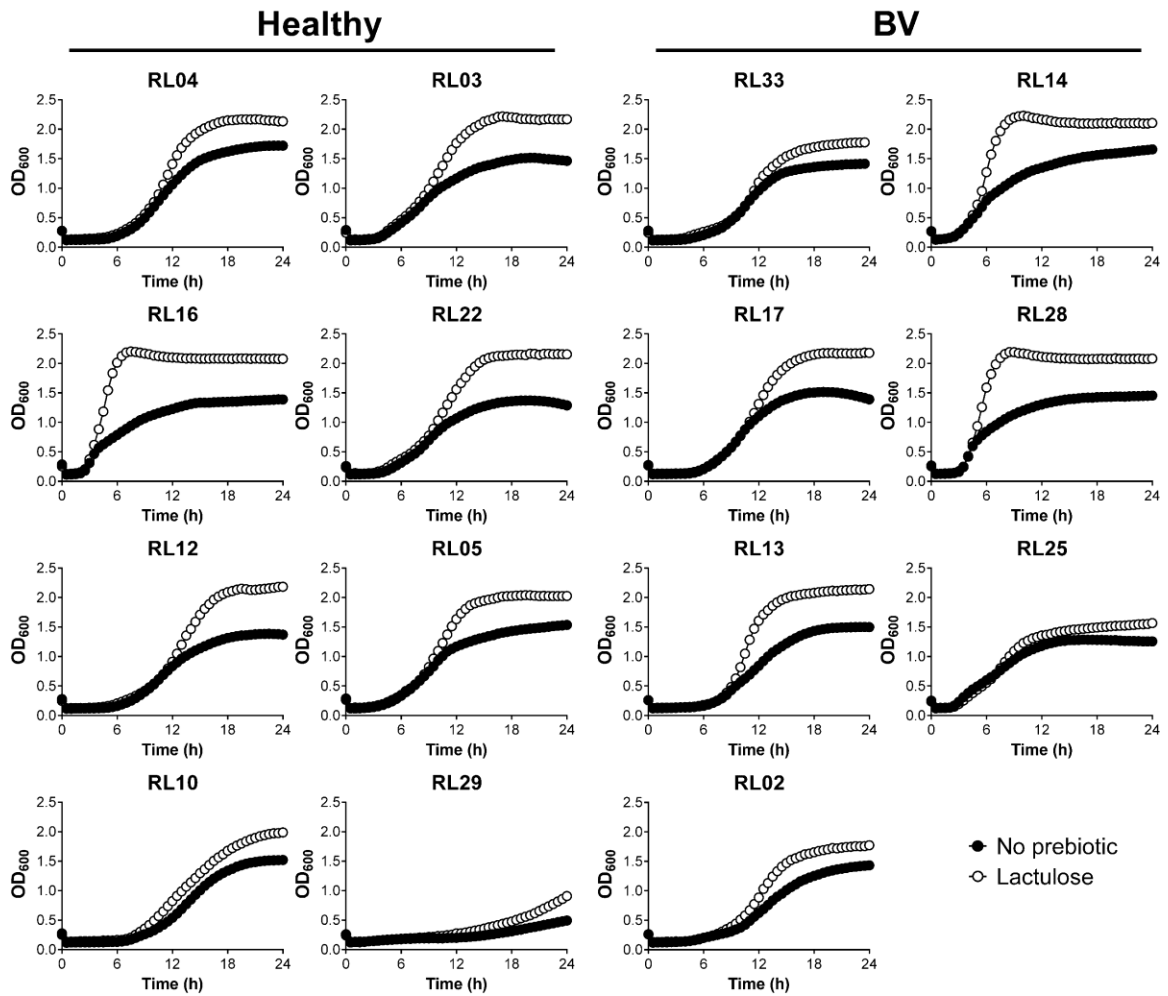
Figure 2.2: Lactulose is ubiquitously utilized by vaginal *L. crispatus* clinical isolates
Individual cultures of vaginal *L. crispatus* isolates obtained from women with or without BV were inoculated in dextrose-free MRS supplemented with 0.5% (w/v) lactulose. Points represent the mean $OD_{600} \pm SD$.
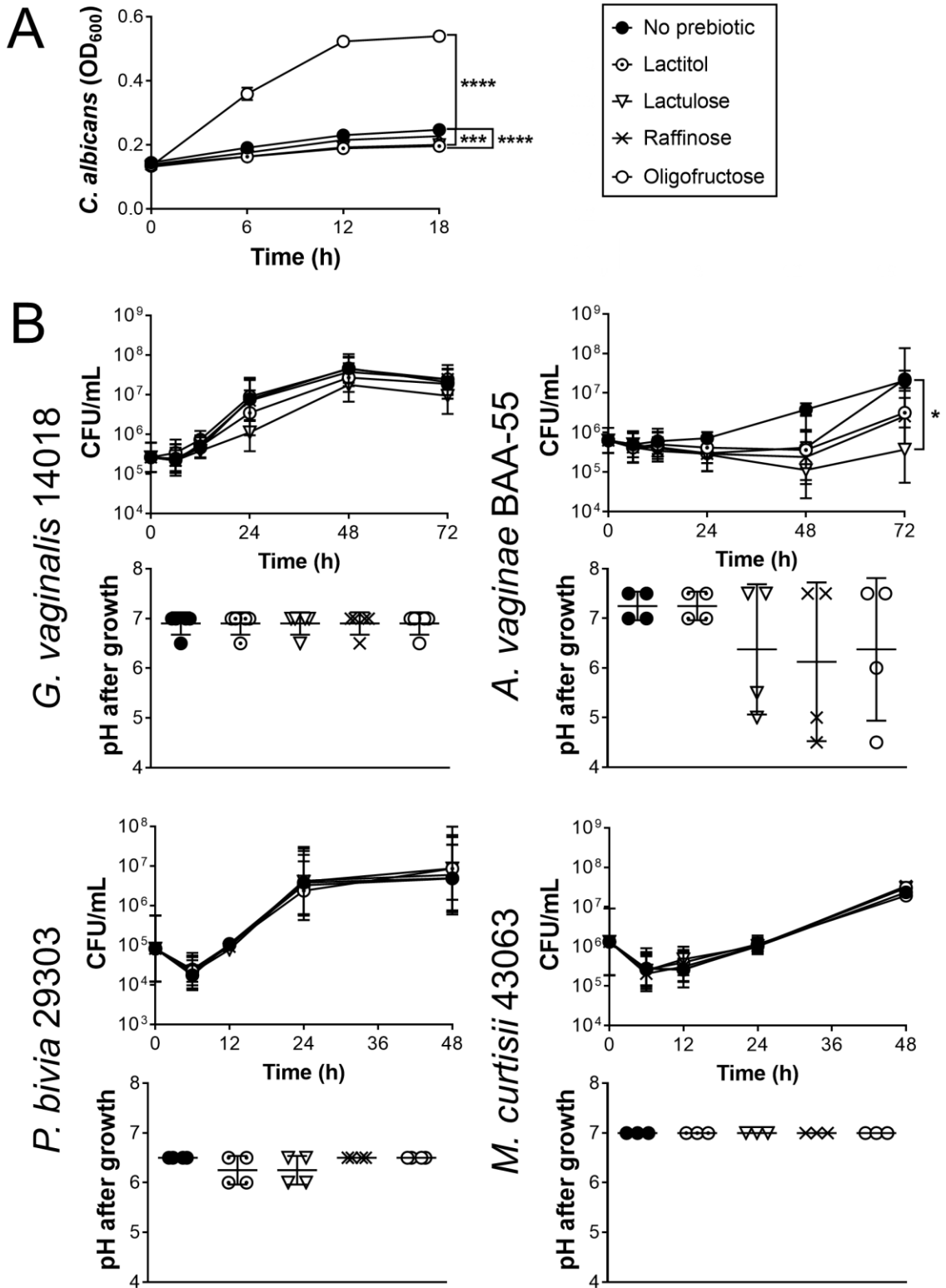
Figure 2.3: Growth and pH of *C. albicans* and BV organisms cultured in prebiotics
(A) *C. albicans* supplemented with 5% prebiotic. (B) BV-associated bacteria inoculated in 0.5% prebiotic media prior to pH testing. For growth curves, points represent mean (OD_{600}) or geometric mean (CFU/mL) of 3-7 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
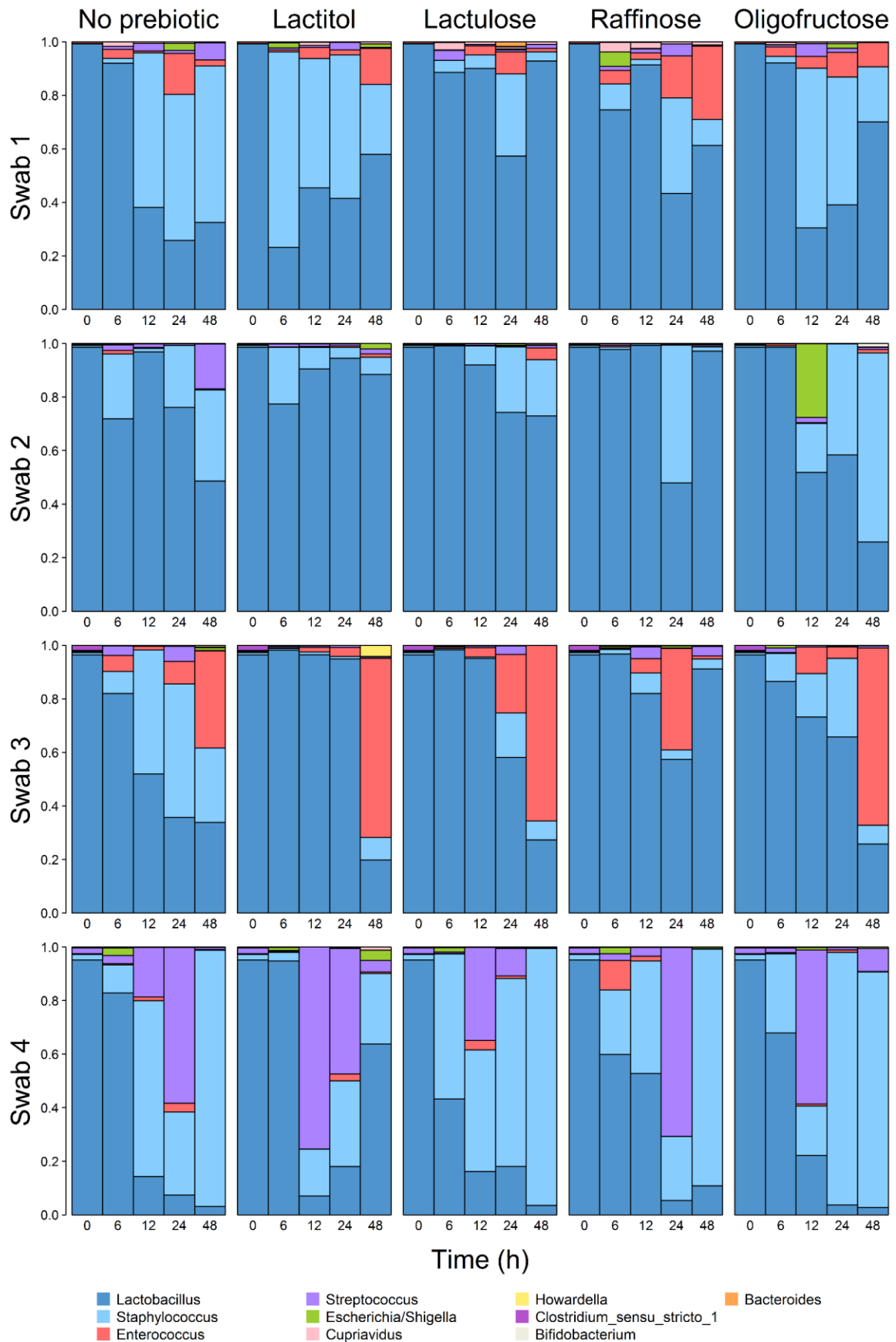
Figure 2.4: Swab microbiota dynamics throughout inoculation in prebiotics
Vaginal swab microbiota from four separate donors were cultured in dextrose-free vaginally-defined medium with 0.5% (w/v) prebiotic. Bar heights represent the fraction of the microbiota taken up by each genus at the designated sampling point, as determined by 16S rRNA sequencing of the V4 region.

A Total *Lactobacillus*
(16S rDNA copies/mL)

B *M. curtisii*
(cells/mL)

Swab 1 — Time (h)

Swab 2 — Time (h)

Swab 3 — Time (h)

Swab 4 — Time (h)

Average — Time (h)

0.23

● No prebiotic   ◉ Lactitol   ▽ Lactulose   ✳ Raffinose   ○ Oligofructose

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.
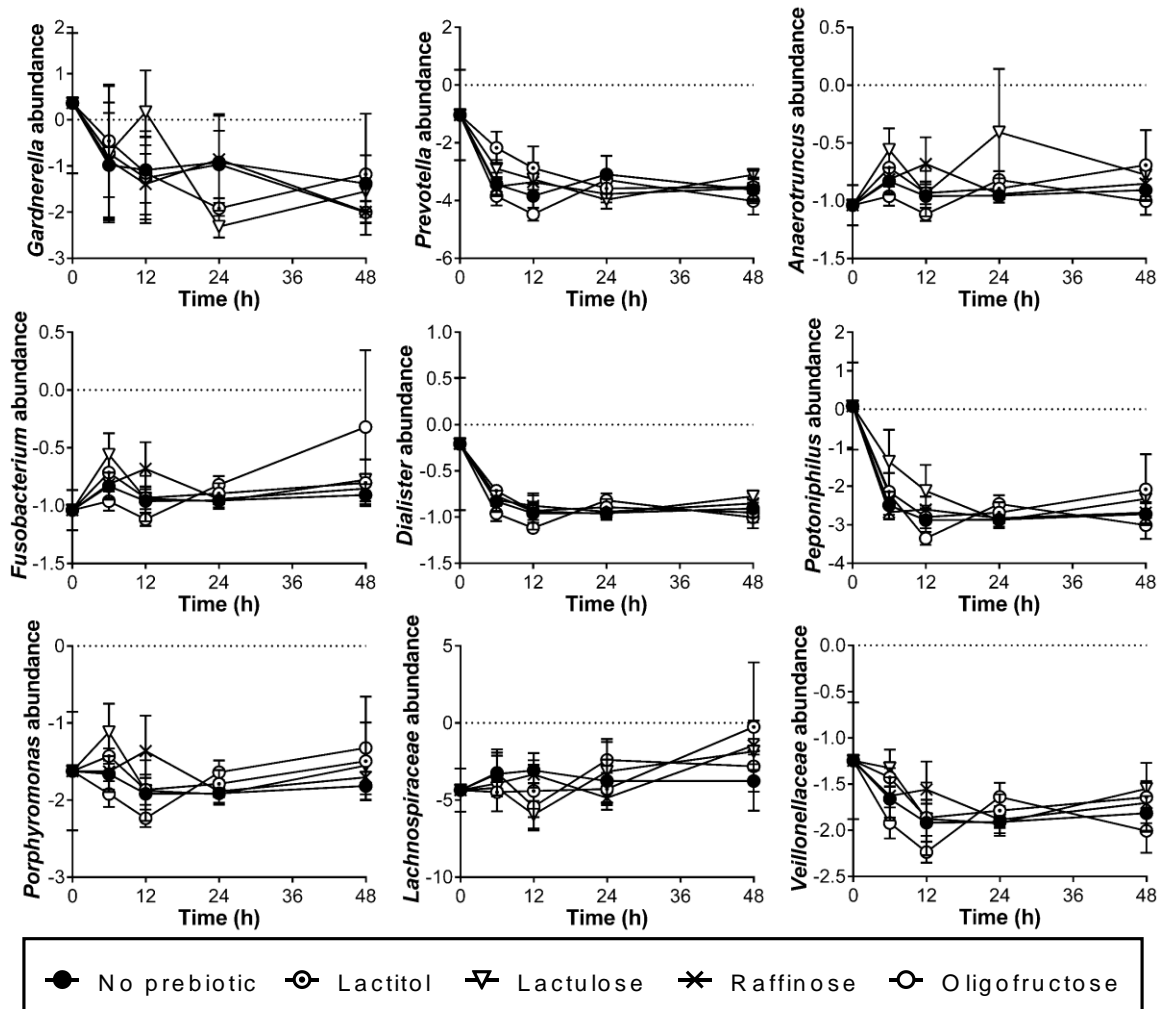
Figure 2.6: Abundance of various BV-associated organisms from healthy vaginal swab consortia grown in prebiotics

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).
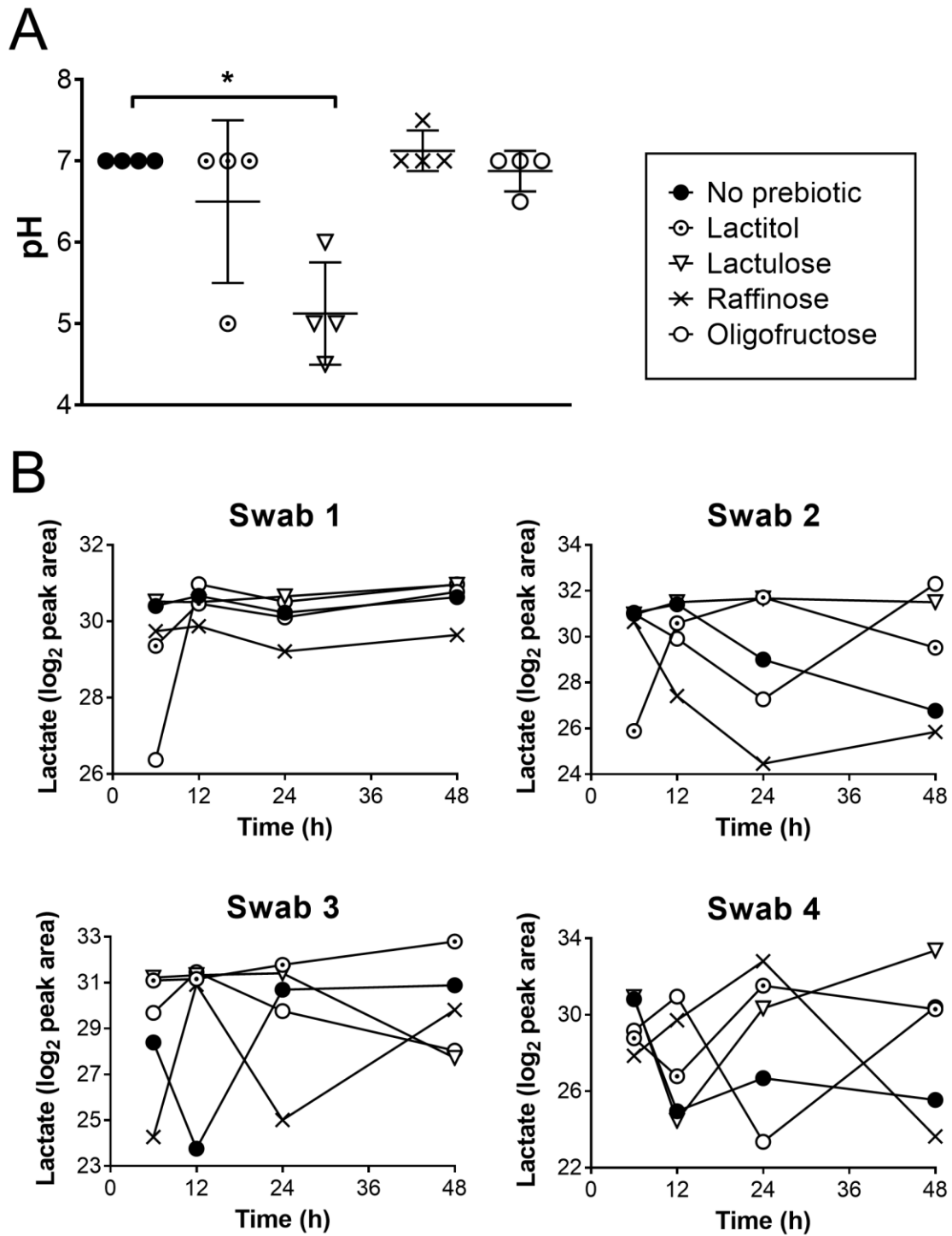
Figure 2.7: Metabolic analysis of healthy vaginal swab consortia in prebiotics
(A) Mean pH ± SD of swab communities following 48 hours of growth in prebiotics. Statistical significance determined according to the Kruskall-Wallis test with Dunn's multiple comparisons (*p<0.05). (B) Peak area of LC-MS-detected lactate, log2 corrected, in swab bacterial supernatant. Dots are individual measures of growth over time.
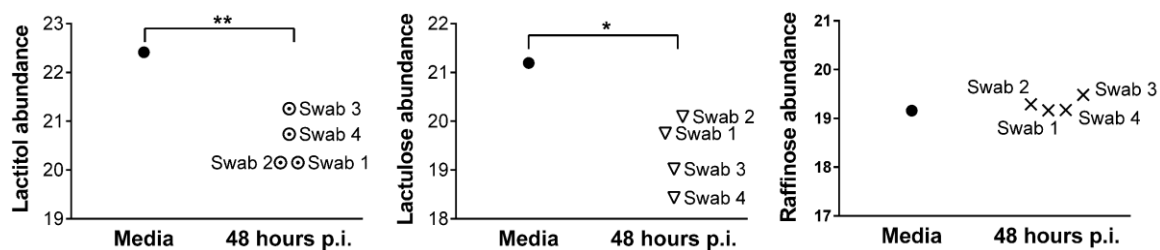
Figure 2.8: Abundance of lactitol, lactulose, and raffinose following healthy vaginal swab consortia growth

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, CLRs 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
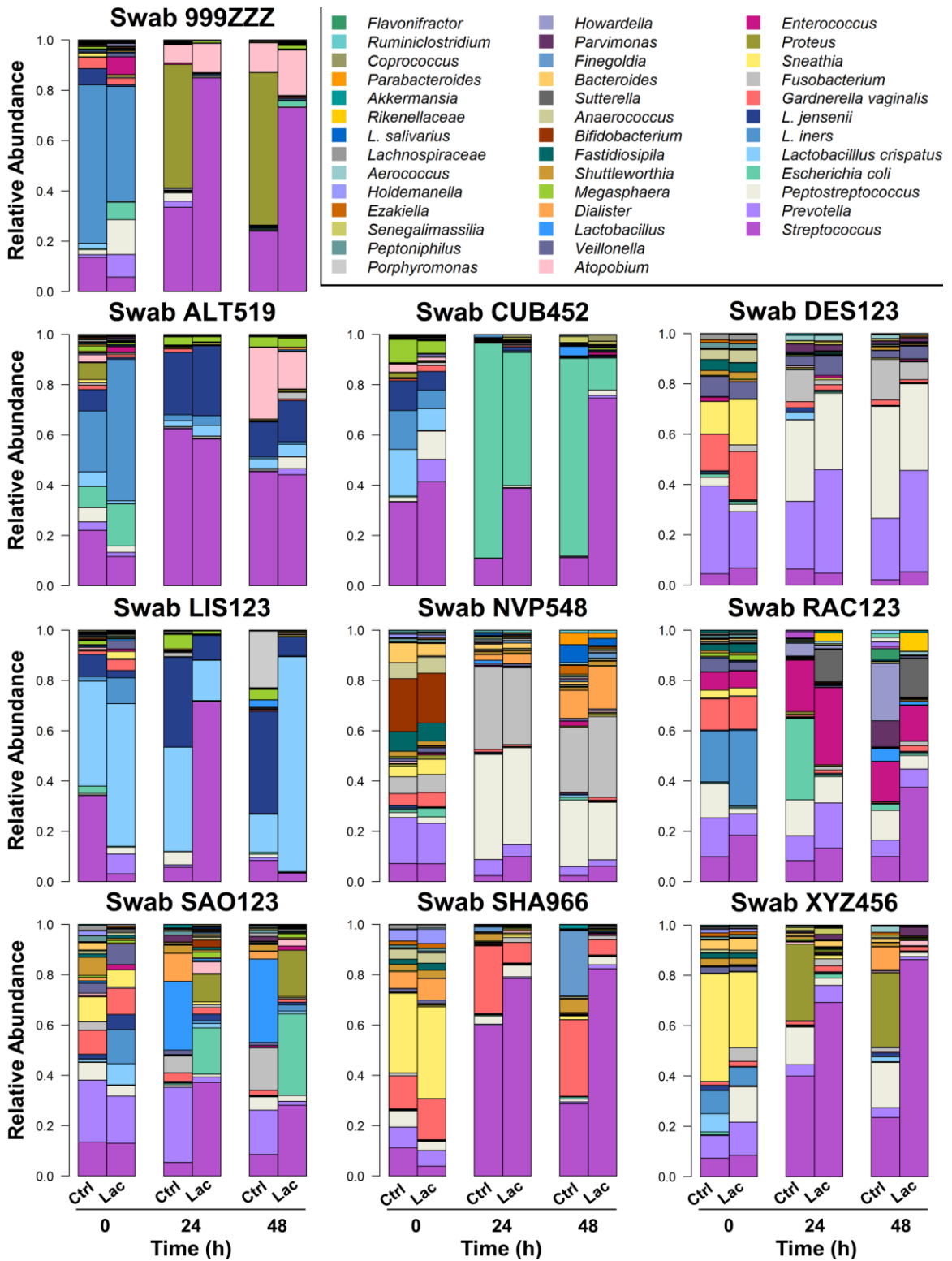
Figure 2.9: 16S rRNA microbiota composition of aberrant vaginal consortia inoculated with 1% (w/v) lactulose

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.
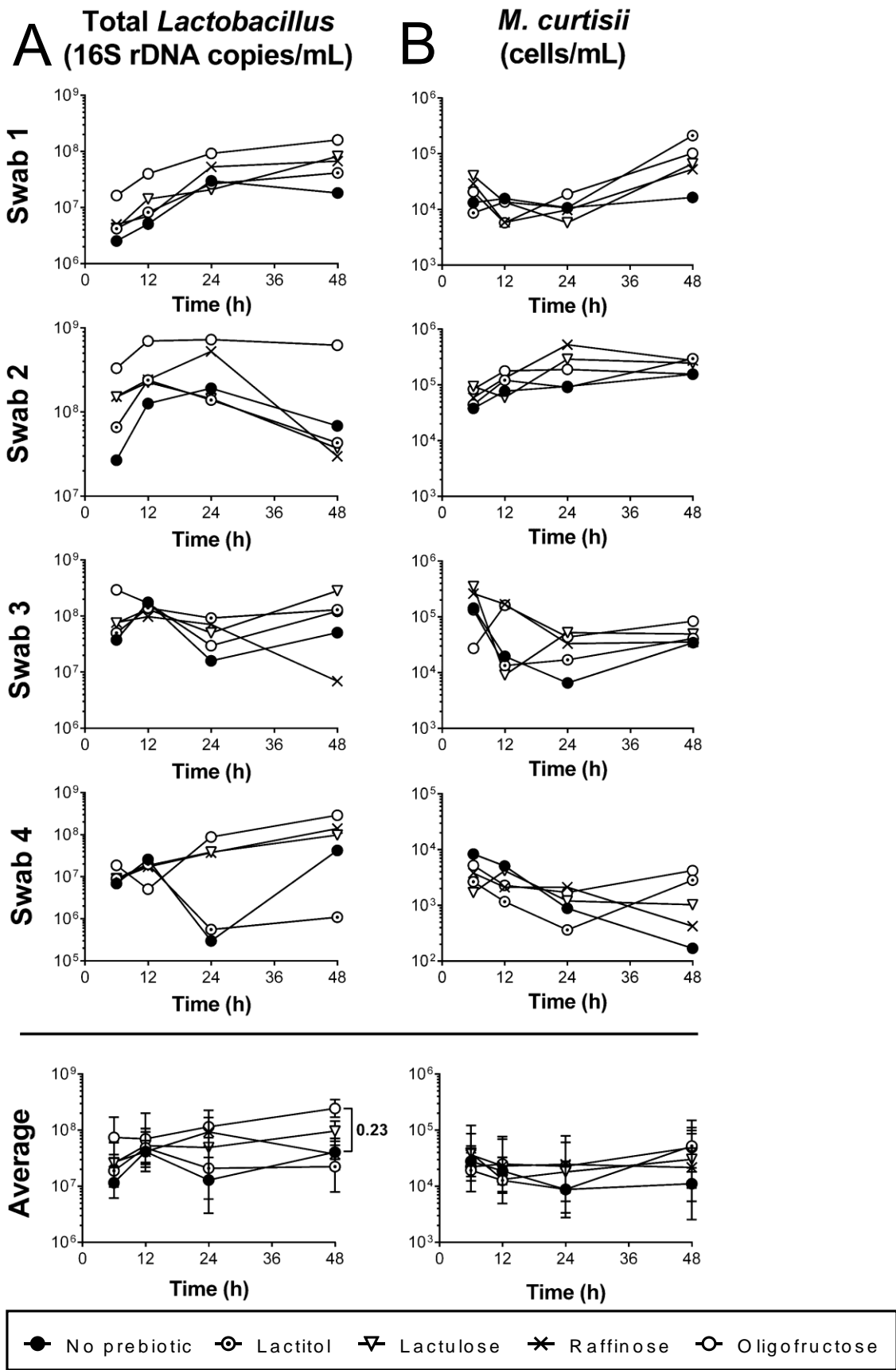
Lactobacillus was observed (**Figure 2.10**). Multivariate analyses of the aberrant microbiotas in lactulose and control media did not detect any overarching community differences in the two treatment groups (Data not shown).

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**).
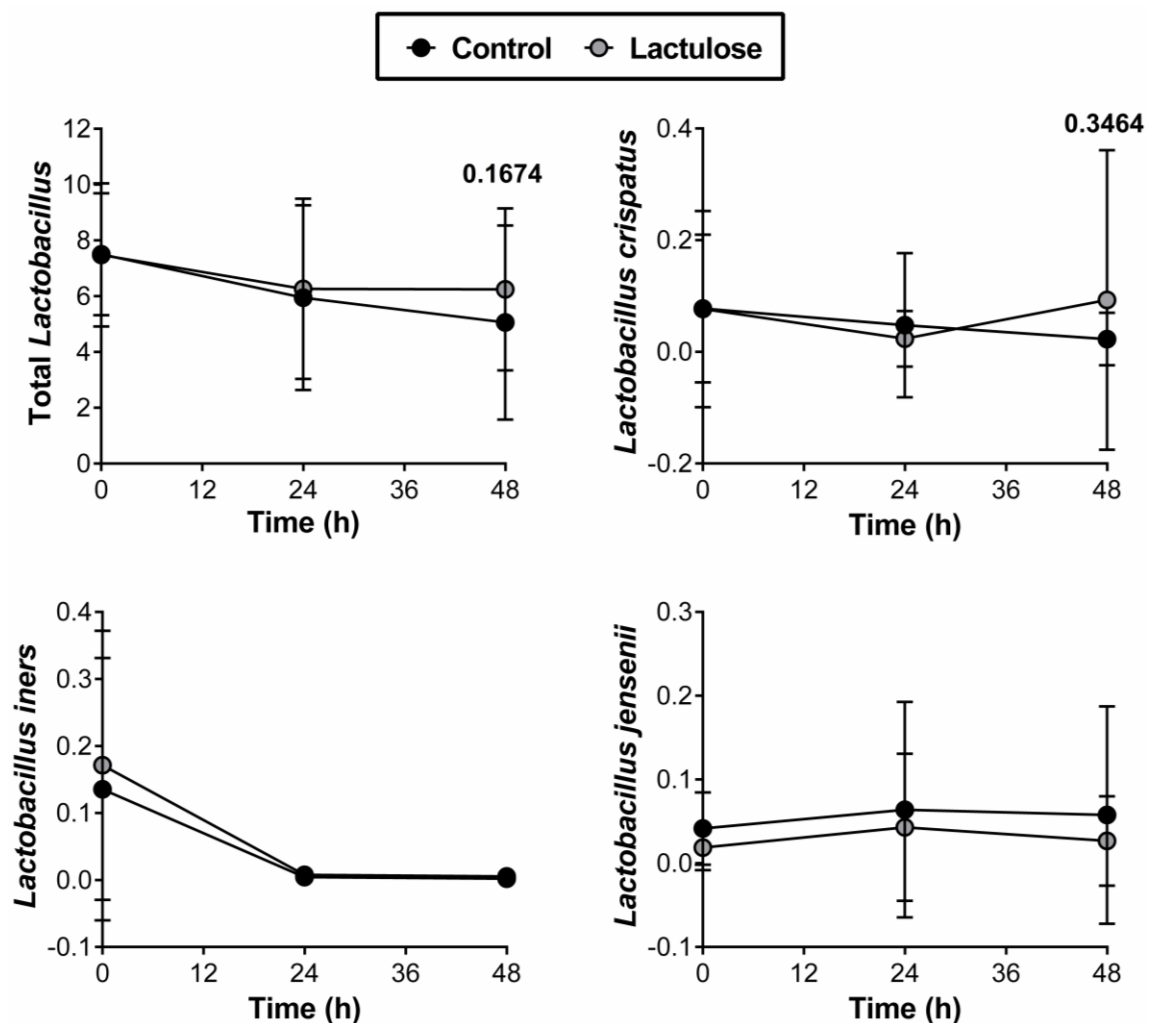
Figure 2.10: Mean abundance of *Lactobacillus* spp. in aberrant swab microbiotas treated with lactulose

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).
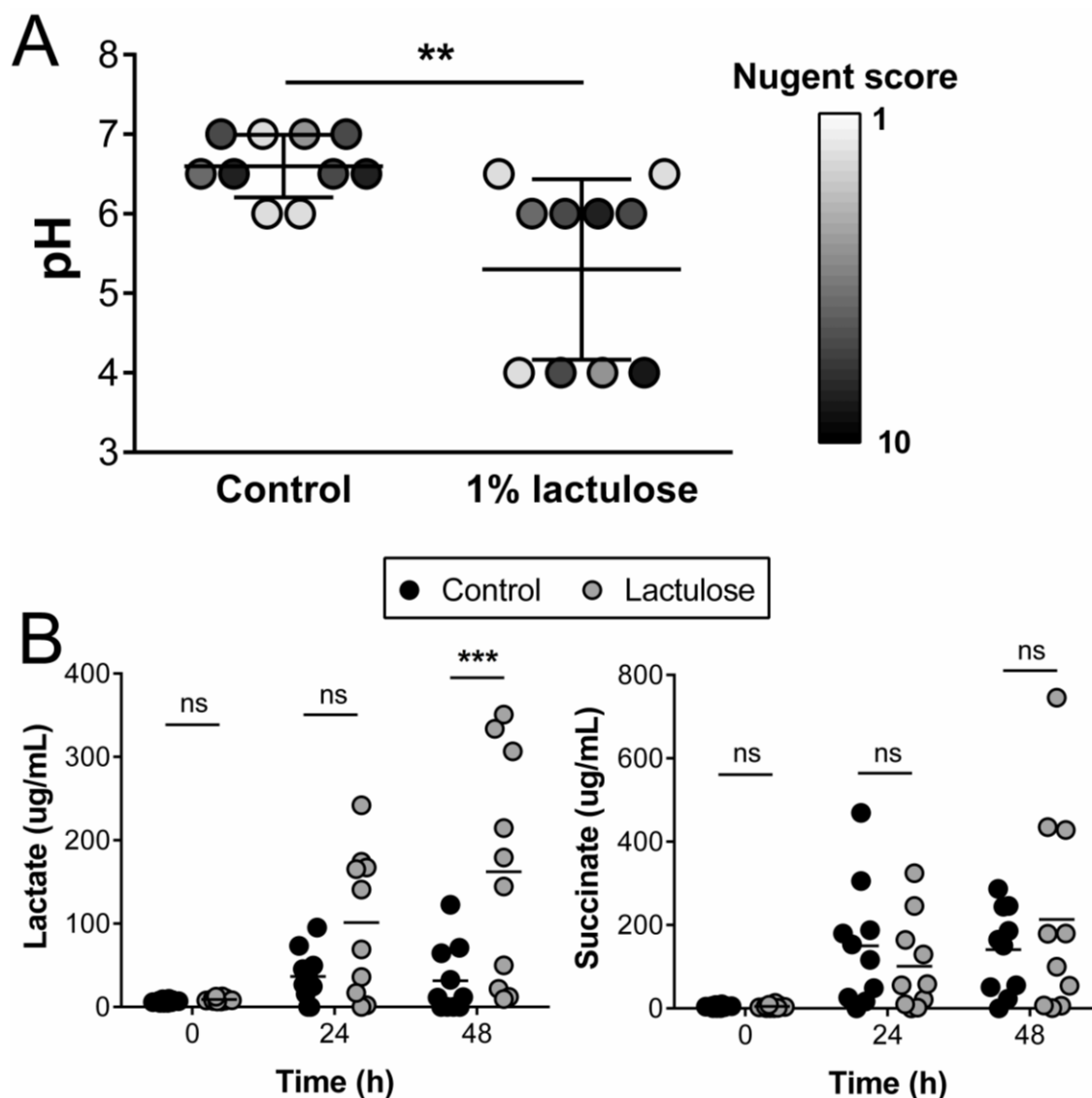
Figure 2.11: Treatment with lactulose triggers acid production by aberrant vaginal swab microbiotas *in vitro*

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).
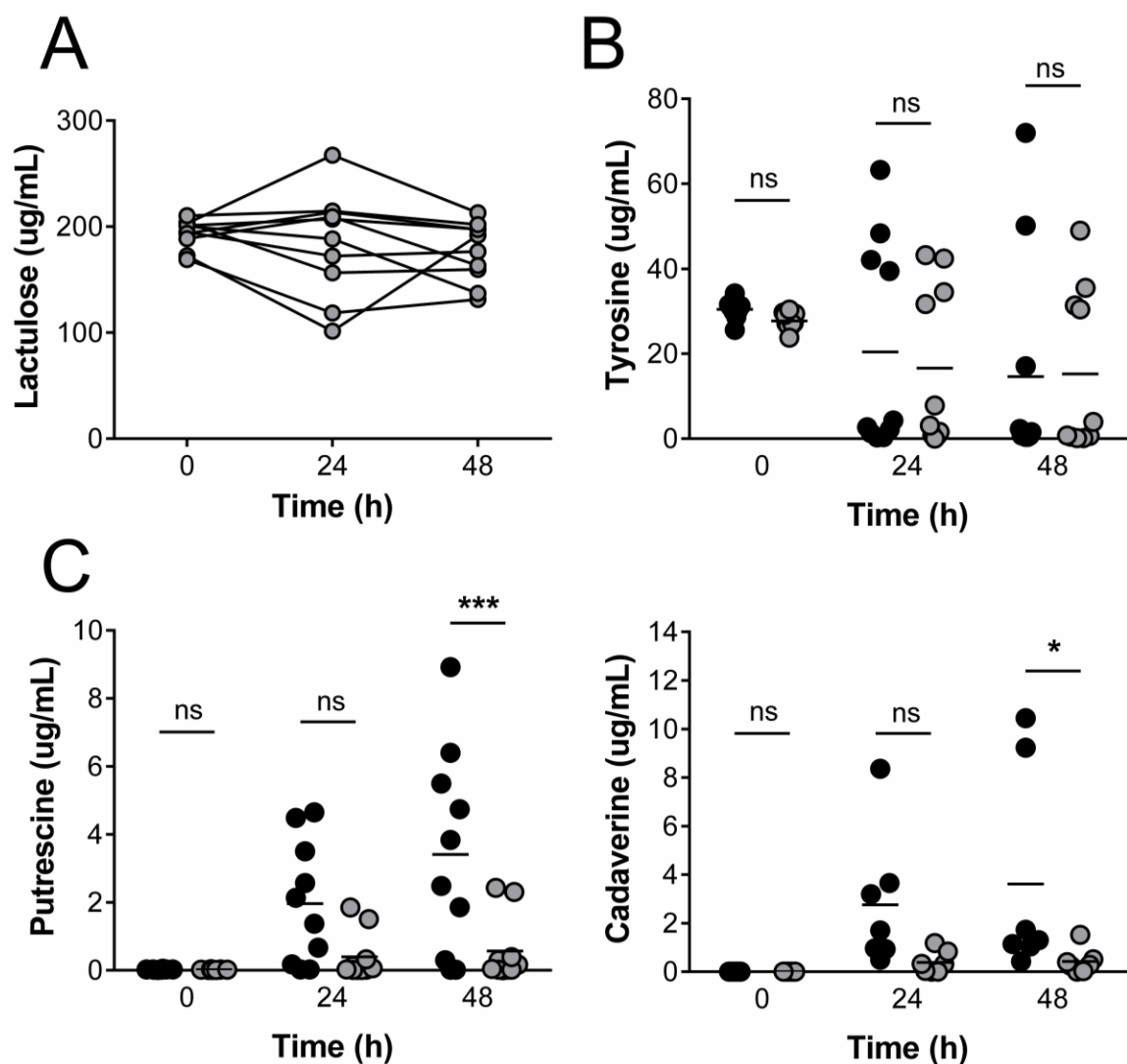
Figure 2.12: Levels of lactulose, tyrosine, and production of odor-associated BV metabolites by aberrant vaginal swab microbes

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 33820^T, *L. vaginalis* NCFB 2810, *L. gasseri* ATCC 33323^T, *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% N_2, 10% CO_2, 10% H_2). *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).

OD_{600} 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% N_2, 10% CO_2, 10% H_2) 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% O_2) 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% N_2, 10% H_2, 10% CO_2) 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/uL, 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-desulfatasing 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

Target	Primer sequence (5' to 3')	Thermal cycling parameters		
Total *Lactobacillus*	**LBF**: ATGGAAGAACACCAGTGGCG **LBR**: CAGCACTGAGAGGCGGAAAC	15 min 95 °C	37x	15s 95 °C, 45s 50 °C, 45s 72 °C
L. iners	**InersFw**: GTCTGCCTTGAAGATCGG **InersRev**: ACAGTTGATAGGCATCATC	15 min 95 °C	35x	15s 95 °C, 55s 60 °C, 60s 65 °C
L. crispatus	**LcrisF**: AGCGAGCGGAACTAACAGATTTAC **LcrisR**: AGCTGATCATGCGATCTGCTT	15 min 95 °C	40x	15s 95 °C, 60s 60 °C, 20s 72 °C
L. gasseri	**LgassF**: AGCGAGCTTGCCTAGATGAATTTG **LgassR**: TCTTTTAAACTCTAGACATGCGTC	15 min 95 °C	40x	15s 95 °C, 60s 57 °C, 60s 65 °C

L. jensenii	**LjensF**: AAGTCGAGCGAGCTTGCCTATAGA	15 min 95 °C	40x	15s 95 °C, 55s 60 °C, 60s 72 °C
	LjensR: CTTCTTTCATGCGAAAGTAGC			
L. vaginalis	**LV16s_23s_F**: GCCTAACCATTTGGAGGG	15 min 95 °C	37x	15s 95 °C, 30s 56 °C, 30s 72 °C
	LV16s_23s_R3: CGATGTGTAGGTTTCCG			
A. vaginae	**AV-F**: CCCTATCCGCTCCTGATACC	10 min 95 °C	40x	15s 95 °C, 20s 64 °C, 25s 72 °C
	AV-R: CCAAATATCTGCGCATTTCA			
G. vaginalis	**F-GV1**: TTACTGGTGTATCACTGTAAGG	10 min 95 °C	40x	45s 95 °C, 45s 55 °C, 45s 72 °C
	R-GV3: CCGTCACAGGCTGAACAGT			
M. curtisii	**F**: GCGATGGTTCCAGAGATGGGCCAGCCTT	2 min 95°C	40x	60s 95°C, 60s 65°C, 60s 72°C
	R: CACGAGTCCCCGGCCGAA			
P. bivia	**PBsulF**: ACGTTTGGGCAAAGCTCCTTGTCT	1 min 95 °C	40x	15s 94 °C, 40s 58 °C, 30s 72 °C
	PBsulR: GCGTGTACGCCAGTTGCAAGA			

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:H_2O, 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 3×10^6 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 8.3×10^4, 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**).
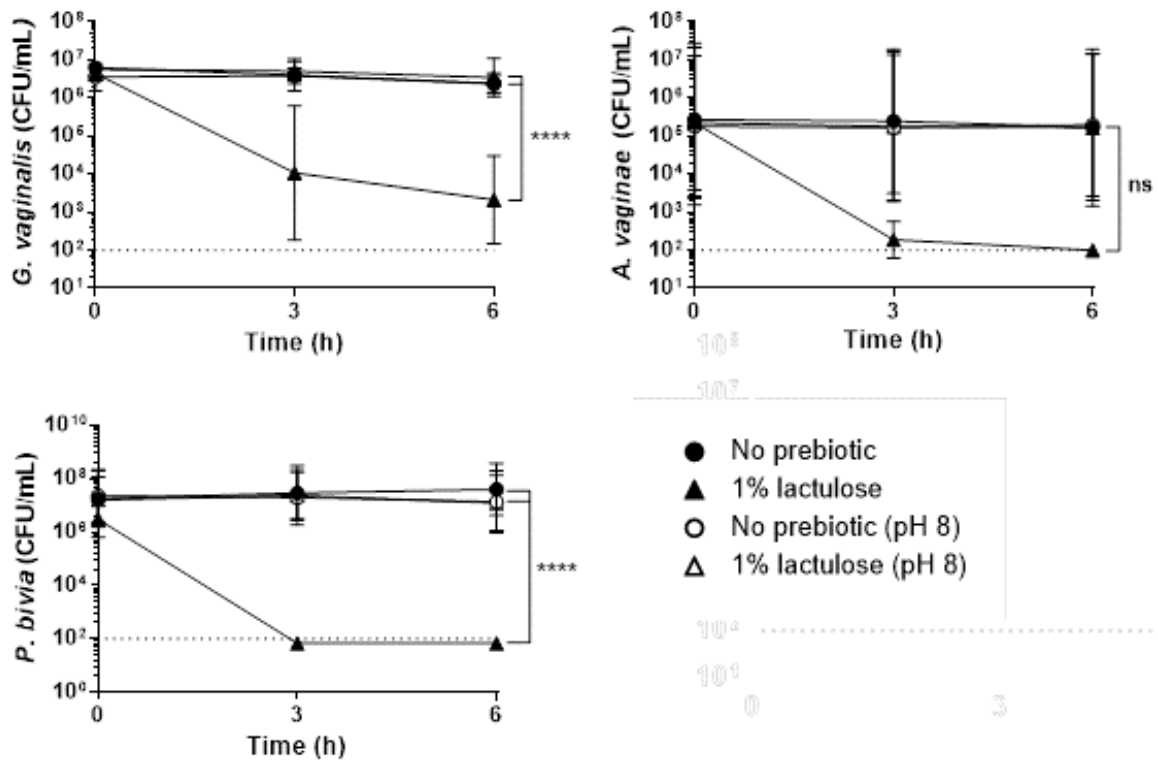
Figure 3.1: Lactulose-treated *L. crispatus* has pH-dependent antagonism against BV organisms

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).
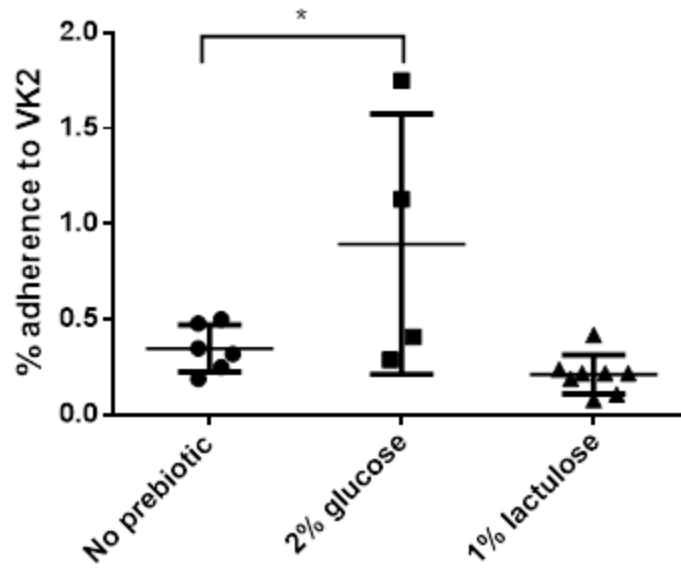
Figure 3.2: Lactulose does not affect *L. crispatus* attachment to vaginal epithelium
Approximately 10^6 *L. crispatus* cells were incubated for 1 hour on fully differentiated VK2/E6E7 vaginal cells. Adherence expressed as a percentage of *L. crispatus* bacteria seeded onto VK2/E6E7 cells. Statistical significance was determined according to one-way ANOVA and Dunnett's multiple comparison's test (*p<0.05).
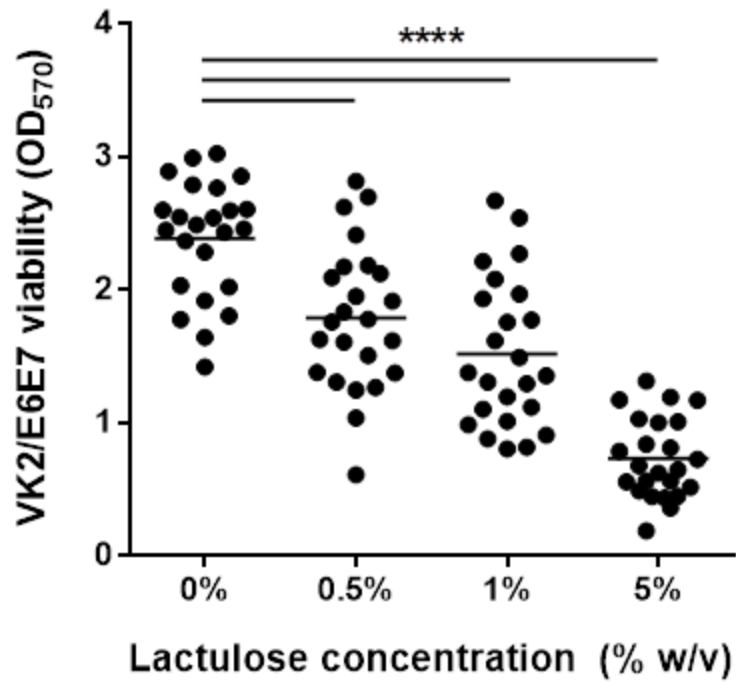
Figure 3.3. Lactulose is inhibitory to VK2/E6E7 cell viability in a dose-dependent manner

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. curtisii* 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 cytolysins (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 α-1,4-glycosidase that cleaves off smaller sugars from ends of glycogen's linear chains (255) and a debranching enzyme that targets the α-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 α-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)

A

Control   0.5% glycogen

B
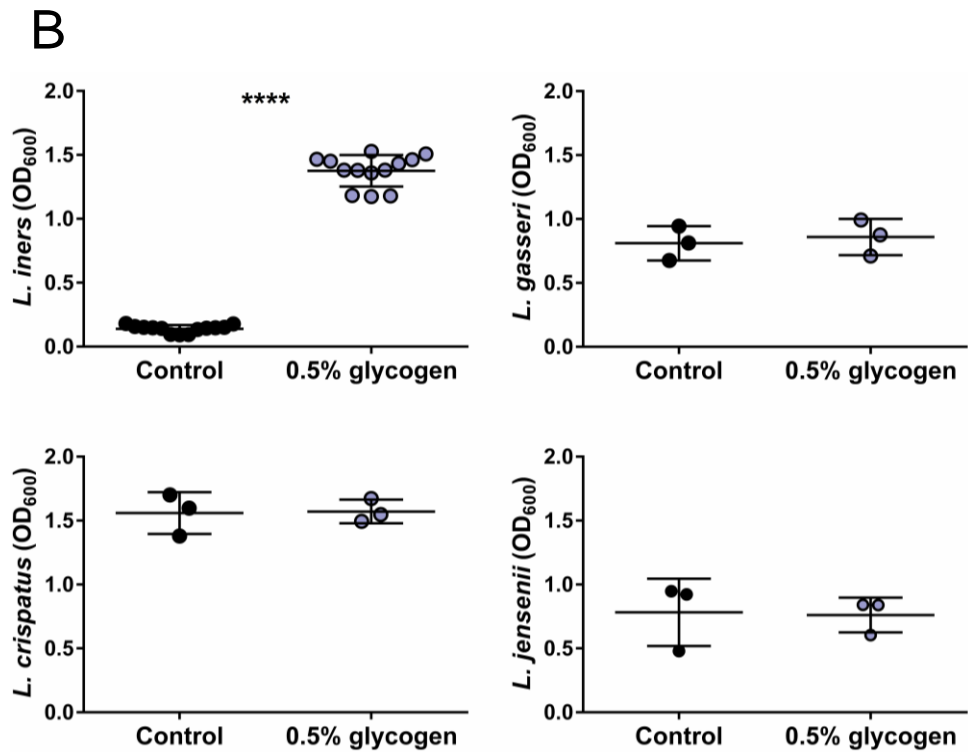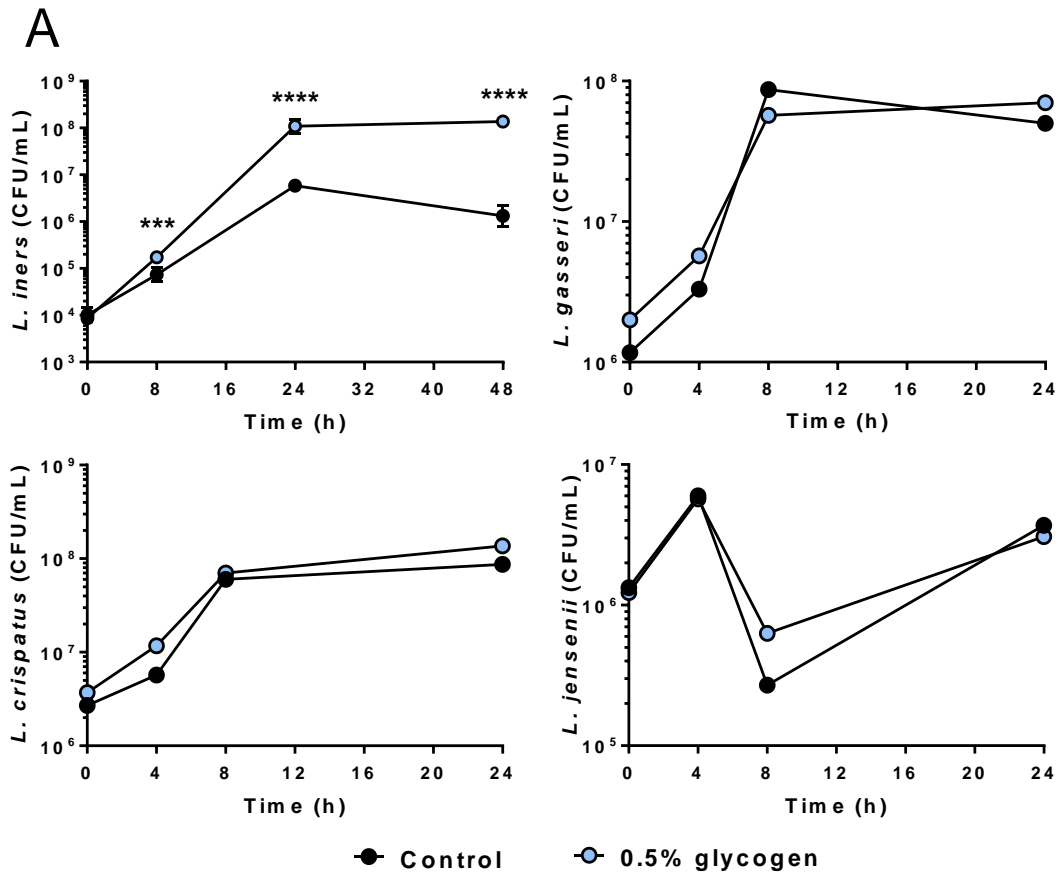
Figure 4.1: Growth of common vaginal lactobacilli in 0.5% glycogen

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 (****$p<00001$).
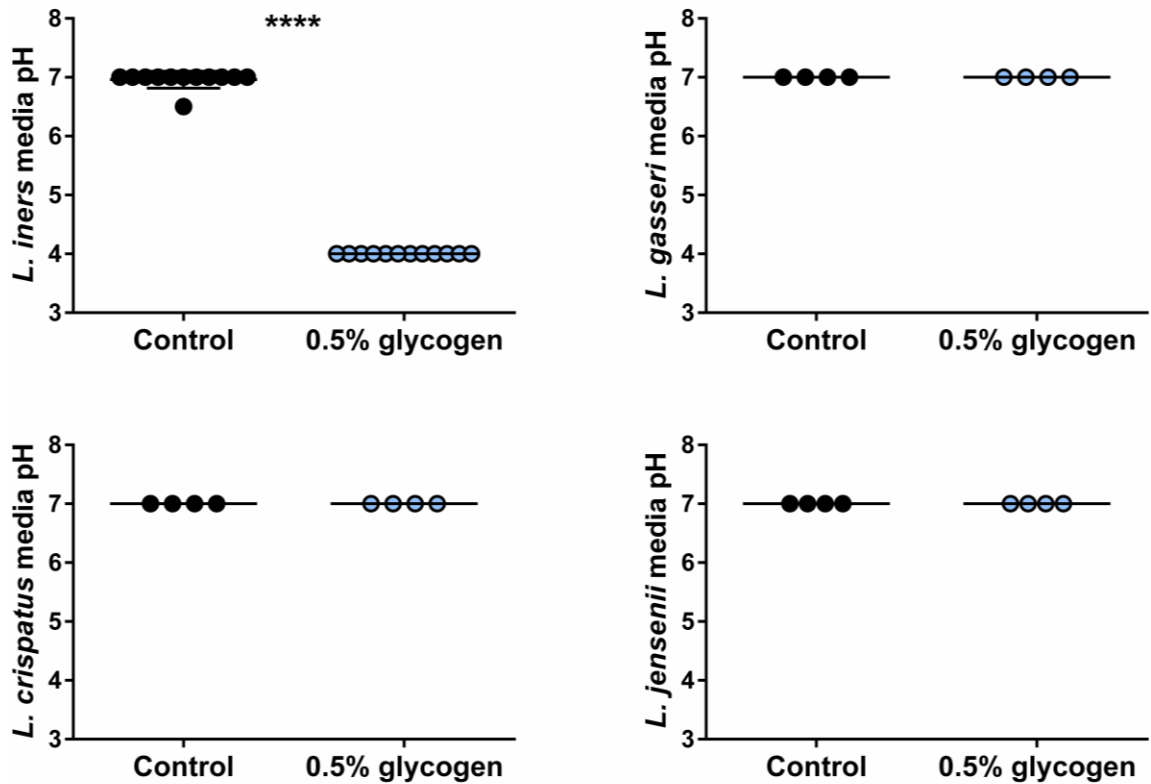
Figure 4.2: Media pH following growth of common vaginal lactobacilli in 0.5% glycogen

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. curtisii* ($p < 0.0001$), and *P. bivia* ($p < 0.05$) fell below the limit of detection, significantly lowering them compared to control (**Figure 4.5**). Restoration of *M. curtisii* 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**).
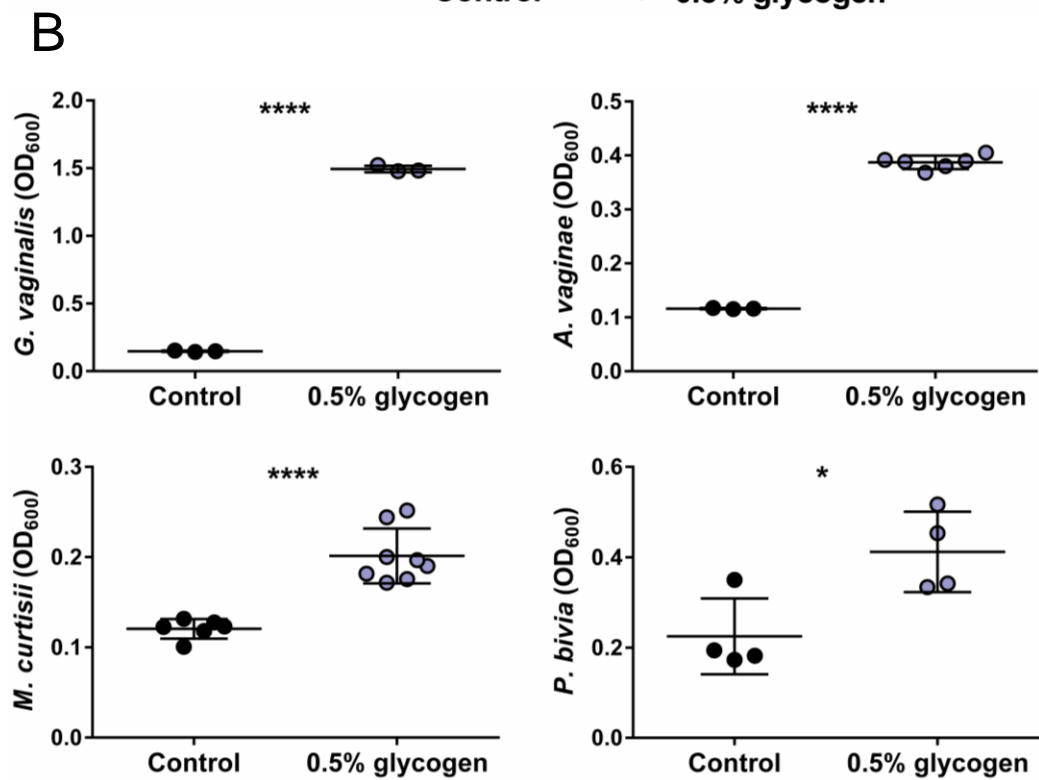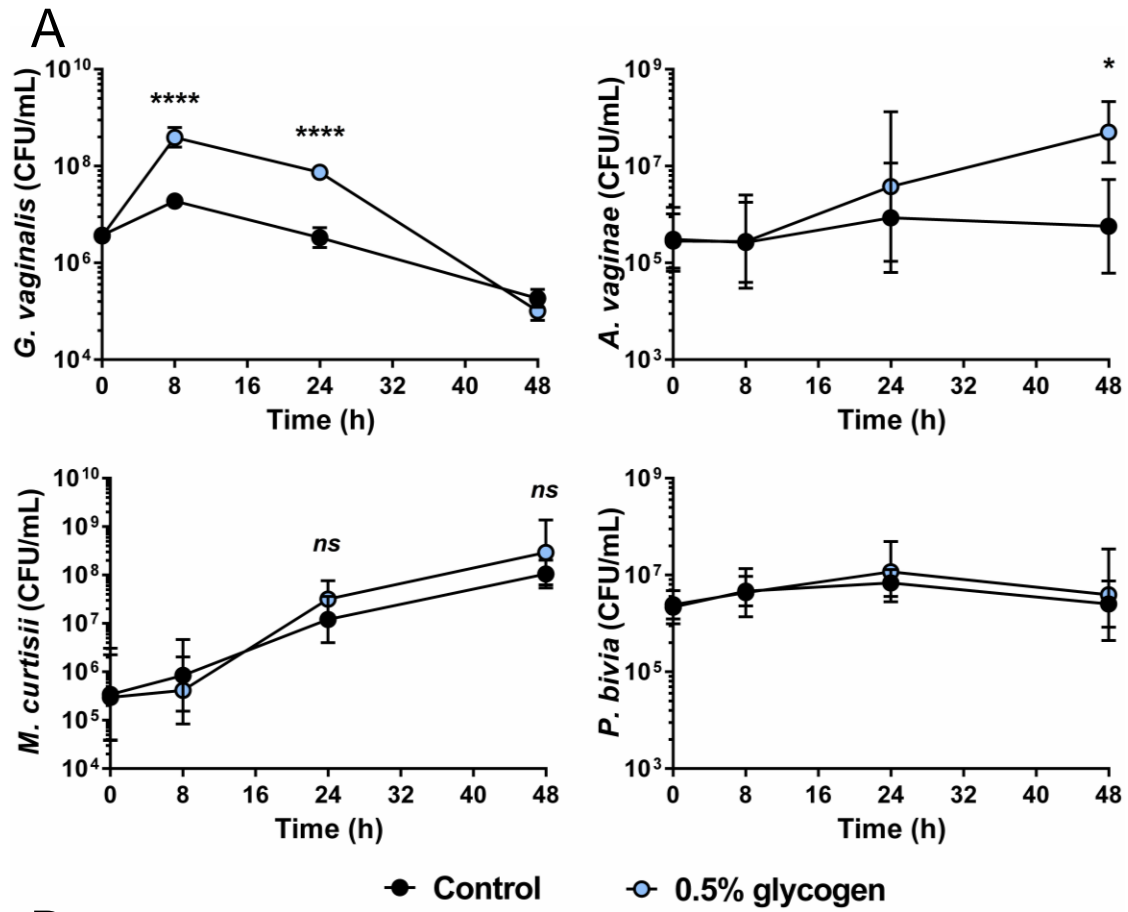
Figure 4.3: Growth of BV-associated bacteria in 0.5% glycogen.
Growth of BV-associated bacteria in dextrose-free NYC III with or without supplementation with 0.5% (w/v) glycogen. (A) Viability (in CFU/mL) was tracked using spot plating, with significance determined at each time point using mixed ANOVA with Sidak's multiple comparisons test (*p<0.05, ****p<0.0001). (B) Total bacteria (OD_{600}) after reaching plateau phase of growth, with significance determined using Student's t-test (*p<0.05, ****p<00001).
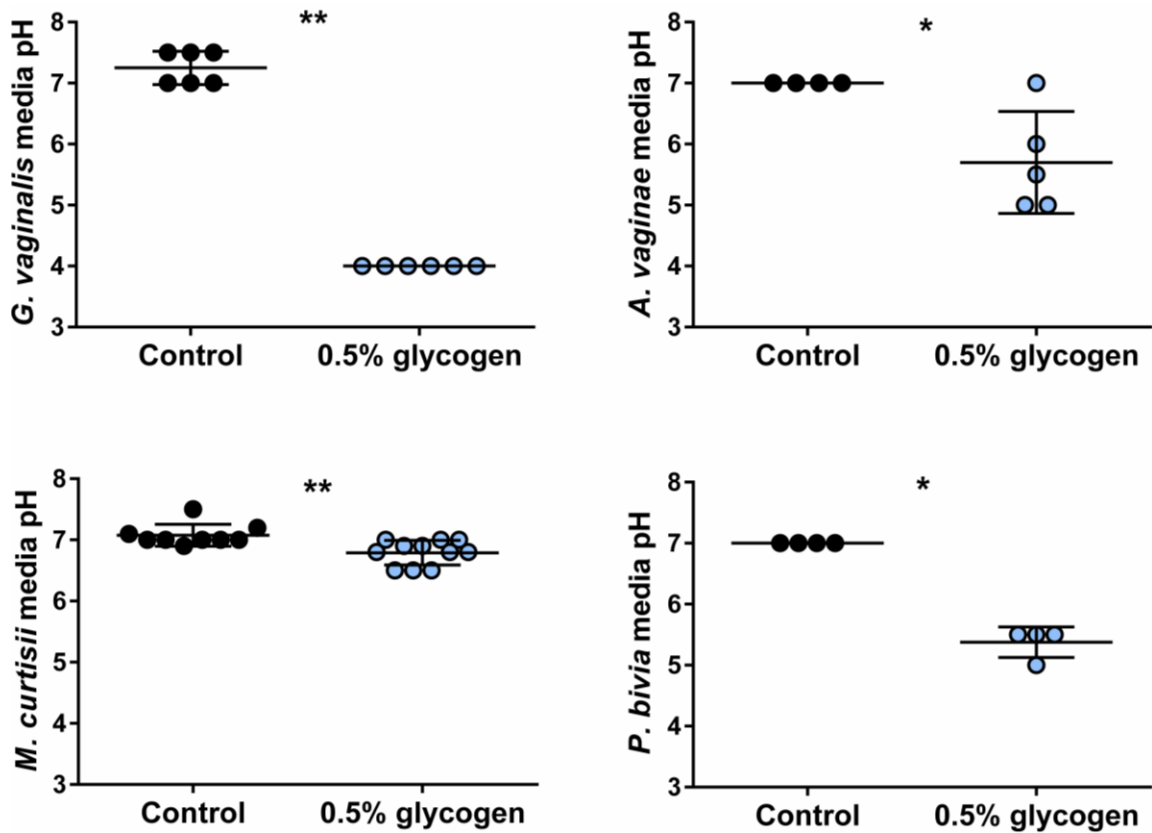
Figure 4.4: Media pH following growth of BV-associated bacteria in 0.5% glycogen. Media acidity measured using pH test strips following plateau phase of BV-associated organism growth in dextrose-free NYC III with or without supplementation with 0.5% (w/v) glycogen. Significance determined using Student's t-test (*$p < 0.05$, **$p < 0.01$).
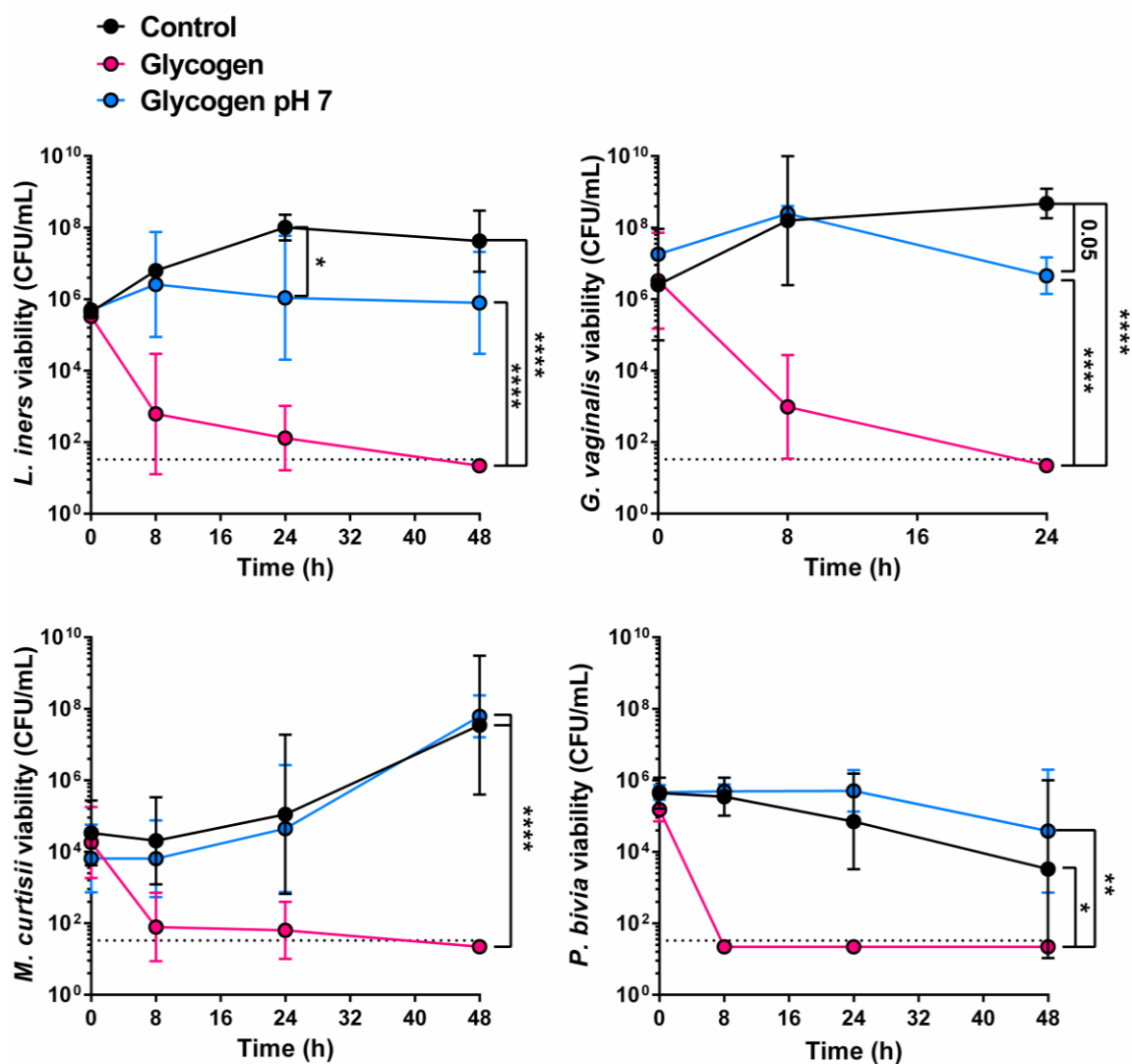
Figure 4.5: Viability of BV organisms in spent glycogen-supplemented *L. iners* supernatants

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).
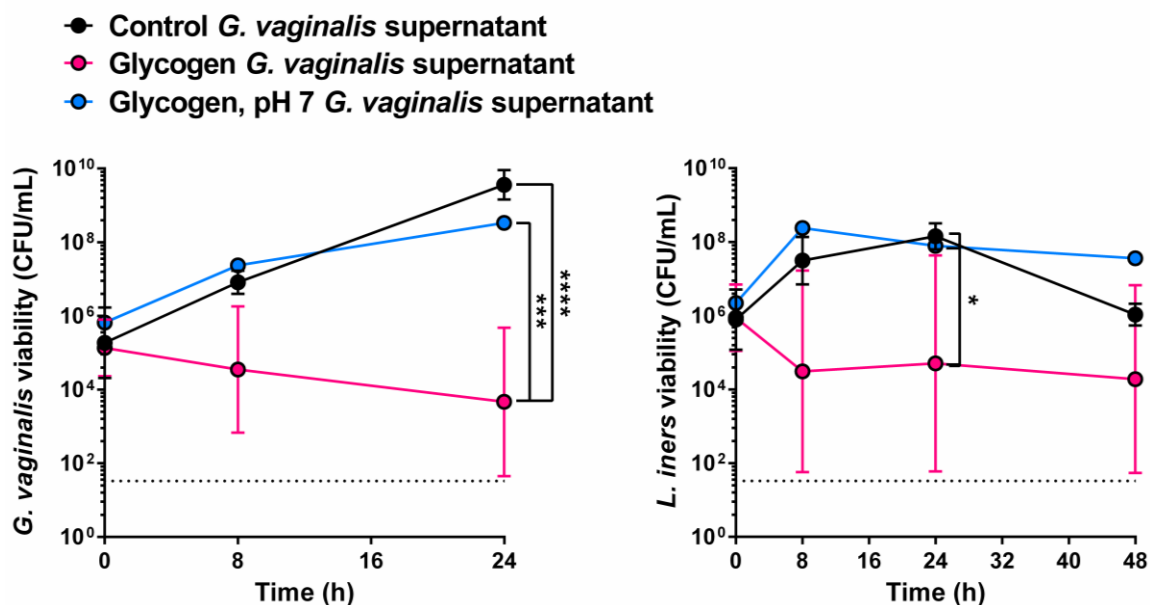
Figure 4.6: Viability of *G. vaginalis* and *L. iners* in spent glycogen-supplemented *G. vaginalis* supernatants

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**).
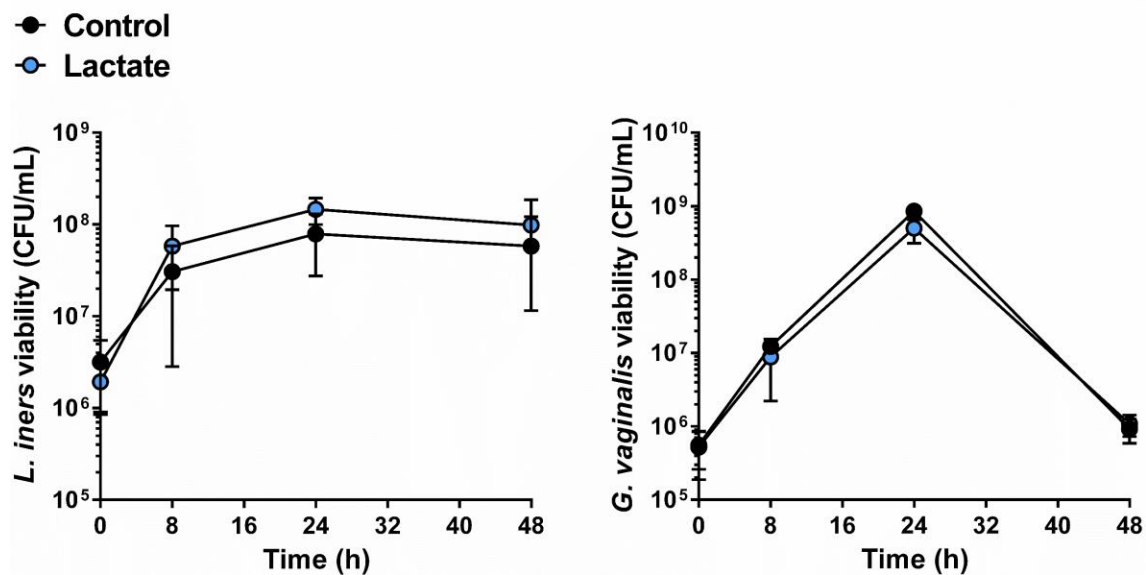
Figure 4.7: *L. iners* **and** *G. vaginalis* **viability in lactate-treated NYC III media.**
Dextrose-free NYC III media with 0.5% (w/v) glycogen was prepared, with or without lactate. Lactate treatment was performed by bringing media to pH 4.0 with concentrated lactic acid, then neutralizing to pH 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 lyses 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 *L. iners* fermentation was sufficient to inhibit the growth of *L. iners* and *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 *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 *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 *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 *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 *Lactobacillus iners* AB-1, *L. gasseri* ATCC 33323, *L. crispatus* ATCC 33820, and *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% N_2, 10% CO_2, and 10% H_2) 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 $4500 \times$ 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 10^6 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 10^6 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 AFB_1. Urinary biomarkers AFM_1, FB_1, FB_2, 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. AFM_1, a urinary biomarker for AFB_1 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 AFB_1 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 B_1, B_2, G_1 and G_2 are common contaminants of maize and peanuts, mostly produced by *Aspergillus flavus* and *A. parasiticus* (276) among few other *Aspergillus* spp. (277). Aflatoxin B_1 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 B_1 and B_2, 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 FB_1 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

Mycotoxin	<LOD		# detectable		Total	Mean of
	n	%	n	%	n	positives
AFB_1-lysine	21	19%	91	81%	112	2.22*
AFM_1	68	51%	66	49%	134	169.91[#]
AFB_1	124	89%	15	11%	139	6.24[#]
AFB_2	130	94%	9	6%	139	2.25[#]
AFG_1	70	50%	69	50%	139	14.06[#]
AFG_2	127	91%	12	9%	139	1.51[#]
FB_1	135	98%	3	2%	138	59.37[#]
FB_2	118	85%	21	15%	139	64.41[#]
OTA	41	29%	98	71%	139	684.39[#]
ZEN	61	49%	64	51%	125	306.98[#]

LOD = limit of detection; * pg/mg albumin; [#] pg/mg creatinine
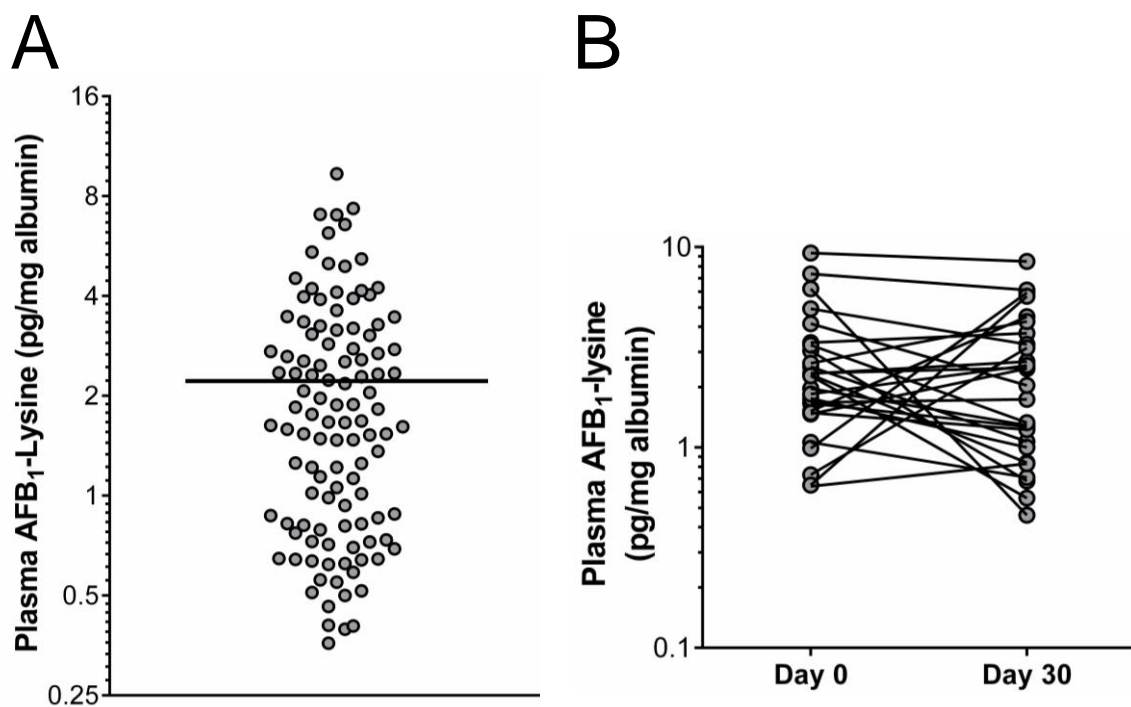
Figure 5.1: Concentration of AFB$_1$-albumin adduct in plasma of Rwandan women

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).
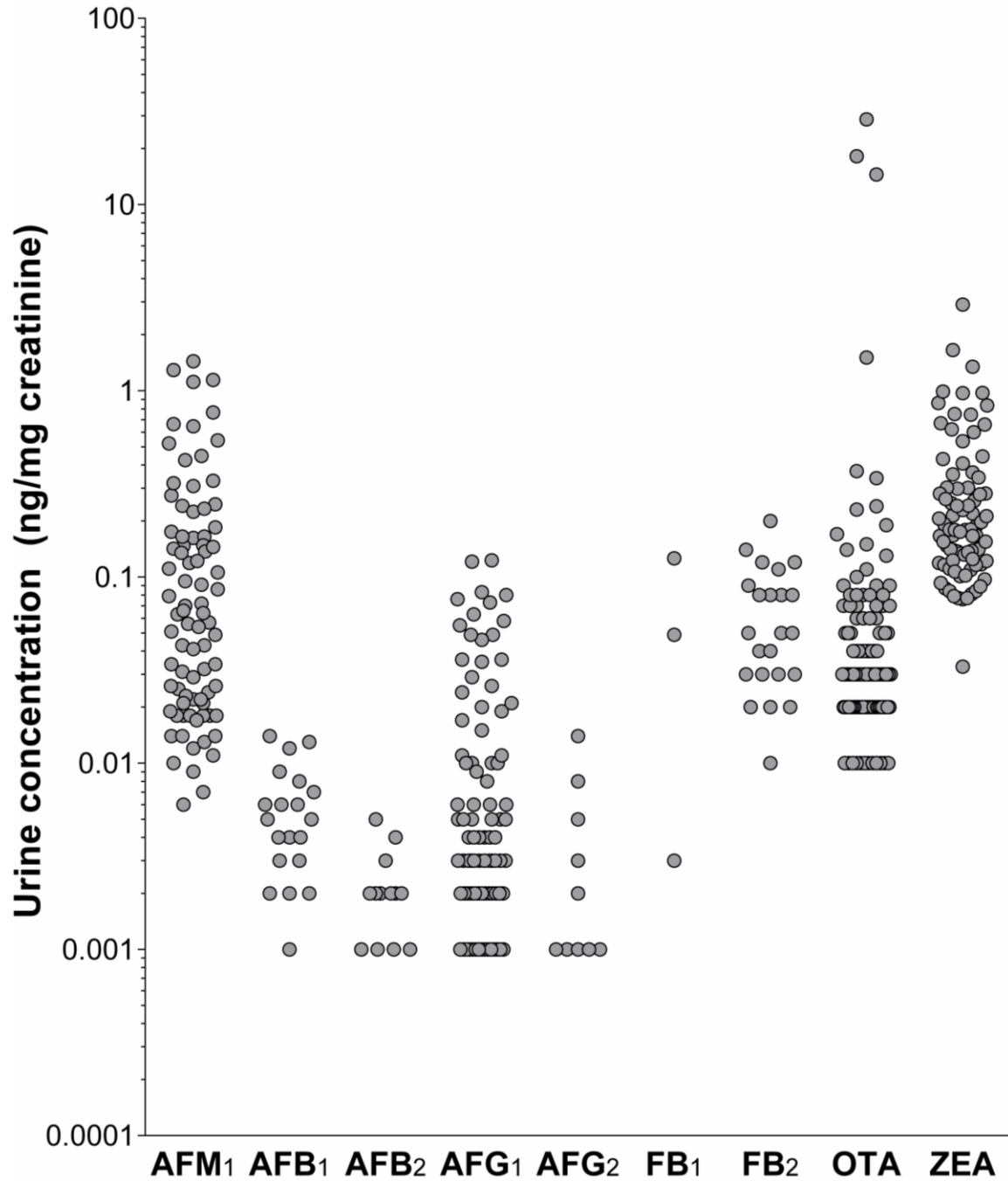
Figure 5.2: Concentration of various mycotoxins in urine of Rwandan women
Levels of urinary aflatoxins M_1, $B_{1/2}$, and $G_{1/2}$, fumonisins B_1 and B_2, ochratoxin A, and zearalenone in Rwandan women, expressed in ng/mg creatinine.

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**).
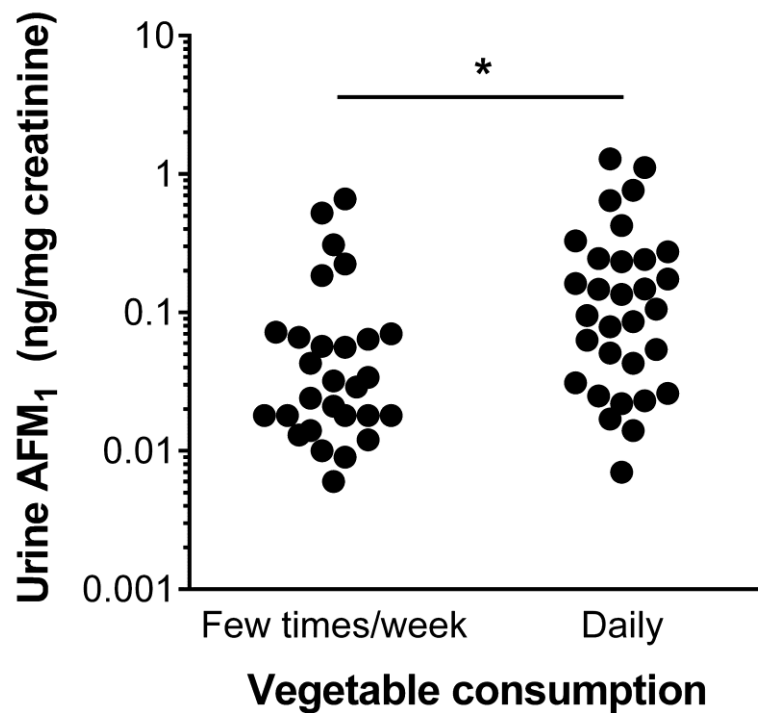
Figure 5.3: Urinary aflatoxin M₁ concentrations are positively correlated with vegetable consumption

Levels of urinary aflatoxin M_1 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 (*$p<0.05$).
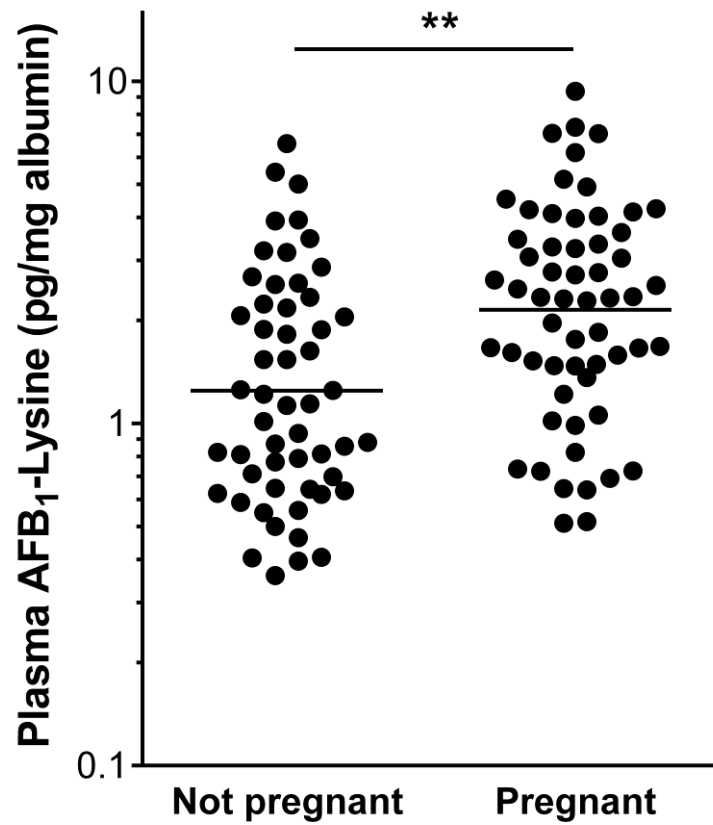
Figure 5.4: Pregnant Rwandan women have elevated plasma aflatoxin B1 levels
Levels of plasma albumin-conjugated aflatoxin B_1 in Rwandan women, expressed in pg/mg albumin. Pregnancy status was reported in clinical visits upon patient recruitment. Significance was determined on log-transformed values 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 AFM_1/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 AFB_1. In contrast to German and Italian populations, for example, where exposure risks are much lower, urinary AFM_1 was only detectable in 0–6% of the population (300, 301). Although urine biomarkers reflect short-term exposure to AFB_1, chronic exposure can be delineated from plasma AFB_1-lysine adducts. We observed widespread contamination of AFB_1-albumin (mean of 2.2 pg AFB_1/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 AFB_1-albumin levels were 4.9 pg/mg albumin (150). One source of AFB_1 in this population may be cassava plants, due to the observed correlation between vegetable intake and urinary AFM_1. 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 B_1 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 B_2 was present in 21/139 (15%) of samples in this population, much more prevalent than FB_1 at 3/138 (2%). This is in direct opposition to the consistent trend in the literature of FB_1 being more abundant than FB_2 in food and humans (169, 170). However, since toxicological studies in rats and cell culture have concluded that FB_1 and FB_2 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 FB_1 (171), shown consistently higher levels of FB_1 than FB_2 (156), or not detected either (157), leaving many questions unanswered about the source of FB_2 in this study. Without fully understanding the impact of elevated FB_2 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 AFB_1-albumin disparately affecting pregnant women. Given limited dietary information, only aflatoxin B_1 could be linked to vegetable consumption. However, additional studies will be needed to elucidate the exact source of AFB_1 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), 10^9 *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 AFB$_1$-lysine quantification in plasma samples

Plasma was processed for quantification of albumin-conjugated AFB$_1$ 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 AFB$_1$-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% NH_4OH 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 5×10^6 and maximum injection time (IT) of 256 ms. PRM analysis monitoring the AFB_1-Lys and AFB_1-$^{13}C_6^{15}N_2$-L-Lys transitions to m/z 394.1268 and 400.1413 respectively was accomplished using a 1.0 m/z isolation window, 17,500 resolution, AGC target 5×10^6 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$_1$-Lys: 30, 200, 400, 1000, 2000, 3000 and 4000 pg/mL each with 500 pg/mL AFB$_1$-^{13}C$_6$^{15}N$_2$-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}C, ^{15}N-labelled internal standards at the following concentrations: 60 ng/mL aflatoxin M$_1$, 12 ng/mL fumonisin B$_1$, 30 ng/mL fumonisin B$_2$, and 30 ng/mL ochratoxin A. Quantification of aflatoxins B$_1$, B$_2$, G$_1$, G$_2$, 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$_4$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 × 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 5×10^6, 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 5.2: Tandem-MS conditions for urine mycotoxin analytes

Name	Formula	Precursor *m/z*	Product ion *m/z*	NCE
AFM$_1$-labelled	C$_{17}$H$_{12}$O$_7$	346.12	288.12561	40
AFM$_1$	C$_{17}$H$_{12}$O$_7$	329.07	273.07523	43
AFB$_1$	C$_{17}$H$_{12}$O$_6$	313.07	241.04924	57
AFB$_2$	C$_{17}$H$_{14}$O$_6$	315.09	259.05991	59
AFG$_1$	C$_{17}$H$_{12}$O$_7$	329.07	243.05945	43

AFG$_2$	C$_{17}$H$_{14}$O$_7$	331.08	245.08093	60
FB$_1$-labelled	C$_{34}$H$_{59}$NO$_{15}$	756.51	356.38370	29
FB$_1$	C$_{34}$H$_{59}$NO$_{15}$	722.40	352.32092	29
FB$_2$-labelled	C$_{34}$H$_{59}$NO$_{14}$	740.51	358.39935	27
FB$_2$	C$_{34}$H$_{59}$NO$_{14}$	706.40	336.32574	27
OTA-labelled	C$_{20}$H$_{18}$ClNO$_6$	424.16	268.05771	22
OTA	C$_{20}$H$_{18}$ClNO$_6$	404.09	257.02087	22
ZEN	C$_{18}$H$_{22}$O$_5$	319.15	187.07556	32

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.
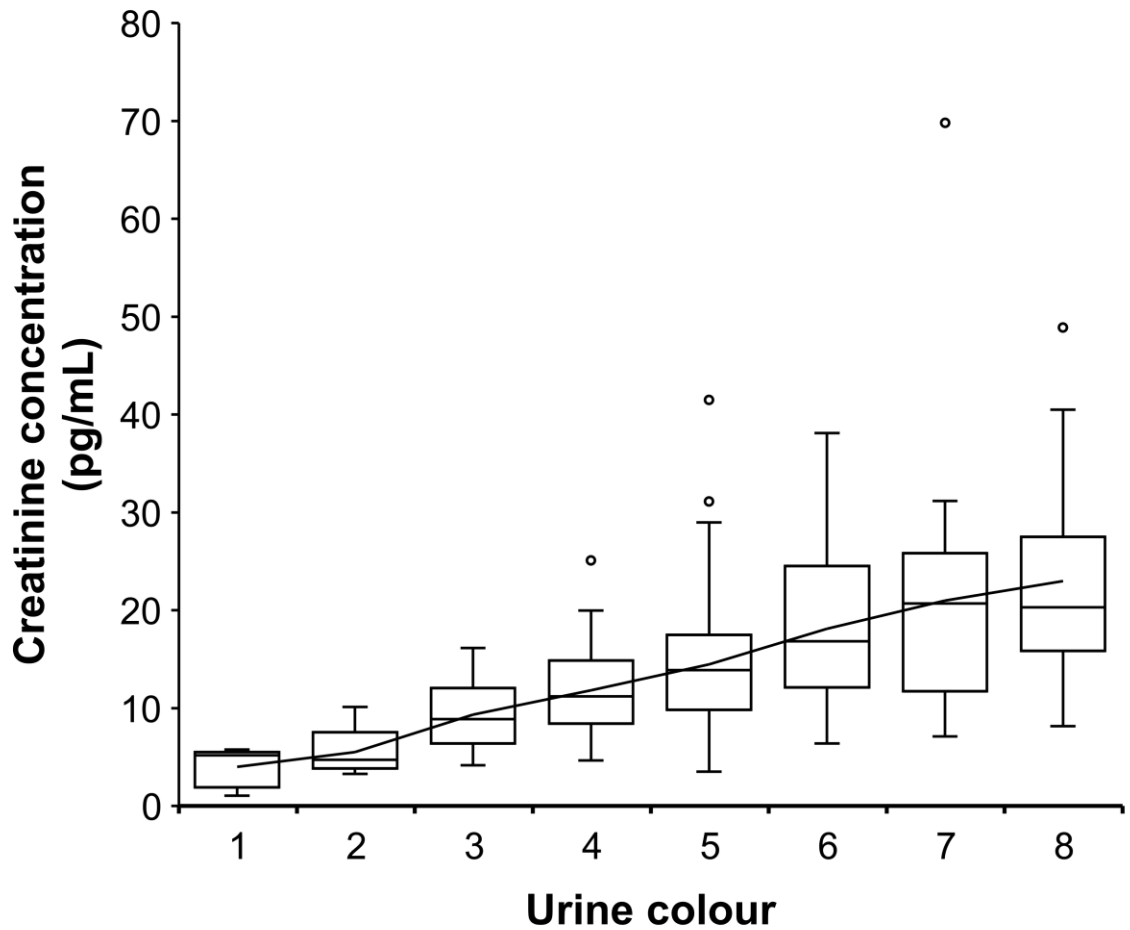
Figure 5.5: Urine colour and creatinine concentration are positively related
Interquartile ranges of creatinine concentration stratified by urine colour, with outliers individually plotted. Colour was determined according to the 8-scale chart and creatinine was quantified using a creatinine (urinary) 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. 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. 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 *in vivo*.

6.1.2 Glycogen utilization by BV organisms and *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. vaginalis*, *A. vaginae, P. bivia* and *M. curtisii*, as well as the first report of its utilization by vaginal lactobacilli—*L. 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_1, fumonisin B_1 and B_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_1-albumin and AFM_1, respectively, the two major biomarkers for AFB_1 exposure in blood and urine (290, 291). Since AFM_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_1 and B_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 B_1 and the elevated risk for pregnant women.

References

1. Urbaniak C, Cummins J, Brackstone M, Macklaim JM, Gloor GB, Baban CK, Scott L, O'Hanlon DM, Burton JP, Francis KP, Tangney M, Reid G. 2014. Microbiota of human breast tissue. Appl Environ Microbiol 80:3007–3014.

2. Lewis DA, Brown R, Williams J, White P, Jacobson SK, Marchesi JR, Drake MJ. 2013. The human urinary microbiome; bacterial DNA in voided urine of asymptomatic adults. Front Cell Infect Microbiol 3:41.

3. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto J-M, Hansen T, Paslier DL, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Consortium M, Antolin M, Artiguenave F, Blottiere H, Borruel N, Bruls T, Casellas F, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Forte M, Friss C, van de Guchte M, Guedon E, Haimet F, Jamet A, Juste C, Kaci G, Kleerebezem M, Knol J, Kristensen M, Layec S, Roux KL, Leclerc M, Maguin E, Minardi RM, Oozeer R, Rescigno M, Sanchez N, Tims S, Torrejon T, Varela E, de Vos WM, Winogradsky Y, Zoetendal E, Bork P, Ehrlich SD, Wang J. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65.

4. Whiteside SA, Razvi H, Dave S, Reid G, Burton JP. 2015. The microbiome of the urinary tract—a role beyond infection. Nat Rev Urol 12:81–90.

5. Bearfield C, Davenport ES, Sivapathasundaram V, Allaker RP. 2002. Possible association between amniotic fluid micro-organism infection and microflora in the mouth. BJOG 109:527–533.

6. DiGiulio DB. 2012. Diversity of microbes in amniotic fluid. Semin Fetal Neonatal Med 17:2–11.

7. DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, Kim CJ, Erez O, Edwin S, Relman DA. 2008. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: A molecular and culture-based investigation. PLoS One 3:e3056.

8. Jiménez E, Fernández L, Marín ML, Martín R, Odriozola JM, Nueno-Palop C, Narbad A, Olivares M, Xaus J, Rodríguez JM. 2005. Isolation of commensal bacteria

from umbilical cord blood of healthy neonates born by cesarean section. Curr Microbiol 51:270–274.

9. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A 107:11971–11975.

10. Grönlund M-M, Gueimonde M, Laitinen K, Kociubinski G, Grönroos T, Salminen S, Isolauri E. 2007. Maternal breast-milk and intestinal bifidobacteria guide the compositional development of the Bifidobacterium microbiota in infants at risk of allergic disease. Clin Exp Allergy 37:1764–1772.

11. Martin V, Maldonado-Barragán A, Moles L, Rodriguez-Baños M, del Campo R, Fernández L, Rodríguez JM, Jiménez E. 2011. Sharing of bacterial strains between breast milk and infant feces. J Hum Lact 28:36–44.

12. Zoetendal EG, Akkermans ADL, Akkermans-van Vliet WM, de Visser JAGM, de Vos WM. 2001. The host genotype affects the bacterial community in the human gastrointestinal tract. Microb Ecol Health Dis 13:129–134.

13. Blekhman R, Goodrich JK, Huang K, Sun Q, Bukowski R, Bell JT, Spector TD, Keinan A, Ley RE, Gevers D, Clark AG. 2015. Host genetic variation impacts microbiome composition across human body sites. Genome Biol 16:191.

14. Swann JR, Want EJ, Geier FM, Spagou K, Wilson ID, Sidaway JE, Nicholson JK, Holmes E. 2011. Systemic gut microbial modulation of bile acid metabolism in host tissue compartments. Proc Natl Acad Sci U S A 108 Suppl 1:4523–4530.

15. Strachan DP. 2000. Family size, infection and atopy: the first decade of the "hygiene hypothesis." Thorax 55:S2–S10.

16. Yamini D, Pimentel M. 2010. Irritable bowel syndrome and small intestinal bacterial overgrowth. J Clin Gastroenterol 44:672–675.

17. Barnett C, Nazzal L, Goldfarb DS, Blaser MJ. 2015. The presence of *Oxalobacter formigenes* in the microbiome of healthy young adults. J Urol.

18. Savignac HM, Couch Y, Stratford M, Bannerman DM, Tzortzis G, Anthony DC, Burnet PW. 2015. Prebiotic administration normalizes lipopolysaccharide (LPS)-induced anxiety and cortical 5-HT2A receptor and IL-1β levels in male mice. Brain Behav Immun.

19. McMillan A, Rulisa S, Sumarah M, Macklaim JM, Renaud J, Bisanz JE, Gloor GB, Reid G. 2015. A multi-platform metabolomics approach identifies highly specific biomarkers of bacterial diversity in the vagina of pregnant and non-pregnant women. Sci Rep 5:14174.

20. Pryde SE, Duncan SH, Hold GL, Stewart CS, Flint HJ. 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiol Lett 217:133–139.

21. Urbaniak C, Gloor GB, Brackstone M, Scott L, Tangney M, Reid G. 2016. The microbiota of breast tissue and its association with breast cancer. Appl Environ Microbiol 82:5039–5048.

22. Wiesenfeld HC, Hillier SL, Krohn MA, Landers DV, Sweet RL. 2003. Bacterial vaginosis is a strong predictor of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infection. Clin Infect Dis 36:663–668.

23. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. 1986. Independent associations of bacterial vaginosis and *Chlamydia trachomatis* infection with adverse pregnancy outcome. JAMA 256:1899–1903.

24. Ralph SG, Rutherford AJ, Wilson JD. 1999. Influence of bacterial vaginosis on conception and miscarriage in the first trimester: cohort study. BMJ 319:220–223.

25. Cicinelli E, Matteo M, Tinelli R, Pinto V, Marinaccio M, Indraccolo U, de Ziegler D, Resta L. 2014. Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage as confirmed by improved pregnancy outcome after antibiotic treatment. Reprod Sci 21:640–647.

26. Zhang Y, Jia Z, Yin S, Shan A, Gao R, Qu Z, Liu M, Nie S. 2014. Toxic effects of maternal zearalenone exposure on uterine capacity and fetal development in gestation rats. Reprod Sci 21:743–753.

27. Collins TFX, Sprando RL, Black TN, Olejnik N, Eppley RM, Alam HZ, Rorie J, Ruggles DI. 2006. Effects of zearalenone on in utero development in rats. Food Chem Toxicol 44:1455–1465.

28. McHardy IH, Goudarzi M, Tong M, Ruegger PM, Schwager E, Weger JR, Graeber TG, Sonnenburg JL, Horvath S, Huttenhower C, McGovern DP, Fornace AJ, Borneman J, Braun J. 2013. Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface reveals exquisite inter-relationships. Microbiome 1:17.

29. Fredricks DN, Fiedler TL, Marrazzo JM. 2005. Molecular identification of bacteria associated with bacterial vaginosis. N Engl J Med 353:1899–1911.

30. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. 2005. Diversity of the human intestinal microbial flora. Science 308:1635–1638.

31. Hayashi H, Sakamoto M, Benno Y. 2002. Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol Immunol 46:535–548.

32. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. 2011. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A 108:4680–4687.

33. Zhou X, Brown CJ, Abdo Z, Davis CC, Hansmann MA, Joyce P, Foster JA, Forney LJ. 2007. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. ISME J 1:121–133.

34. Mirmonsef P, Modur S, Burgad D, Gilbert D, Golub ET, French AL, McCotter K, Landay AL, Spear GT. 2015. Exploratory comparison of vaginal glycogen and *Lactobacillus* levels in premenopausal and postmenopausal women. Menopause 22:702–709.

35. Hillier SL, Lau RJ. 1997. Vaginal microflora in postmenopausal women who have not received estrogen replacement therapy. Clin Infect Dis 25:S123–S126.

36. Heinemann C, Reid G. 2005. Vaginal microbial diversity among postmenopausal women with and without hormone replacement therapy. Can J Microbiol 51:777–781.

37. Eschenbach DA, Thwin SS, Patton DL, Hooton TM, Stapleton AE, Agnew K, Winter C, Meier A, Stamm WE. 2000. Influence of the normal menstrual cycle on vaginal tissue, discharge, and microflora. Clin Infect Dis 30:901–907.

38. Farage M, Maibach H. 2006. Lifetime changes in the vulva and vagina. Arch Gynecol Obstet 273:195–202.

39. Gajer P, Brotman RM, Bai G, Sakamoto J, Schutte UME, Zhong X, Koenig SSK, Fu L, Ma Z, Zhou X, Abdo Z, Forney LJ, Ravel J. 2012. Temporal dynamics of the human vaginal microbiota. Sci Transl Med 4:132ra52.

40. Ghartey JP, Smith BC, Chen Z, Buckley N, Lo Y, Ratner AJ, Herold BC, Burk RD. 2014. *Lactobacillus crispatus* dominant vaginal microbiome is associated with inhibitory activity of female genital tract secretions against *Escherichia coli*. PLoS One 9:e96659.

41. Kim J-W, Rajagopal SN. 2001. Antibacterial activities of *Lactobacillus crispatus* ATCC 33820 and *Lactobacillus gasseri* ATCC 33323. J Microbiol 39:146–148.

42. Doerflinger SY, Throop AL, Herbst-Kralovetz MM. 2014. Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human vaginal epithelia in a species-specific manner. J Infect Dis 209:1989–1999.

43. Leizer J, Nasioudis D, Forney LJ, Schneider GM, Gliniewicz K, Boester A, Witkin SS. 2017. Properties of epithelial cells and vaginal secretions in pregnant women when *Lactobacillus crispatus* or *Lactobacillus iners* dominate the vaginal microbiome. Reprod Sci 1–7.

44. Nunn KL, Wang Y-Y, Harit D, Humphrys MS, Ma B, Cone R, Ravel J, Lai SK. 2015. Enhanced trapping of HIV-1 by human cervicovaginal mucus is associated with *Lactobacillus crispatus*-dominant microbiota. MBio 6:e01084-15.

45. Osset J, Bartolomé RM, García E, Andreu A. 2001. Assessment of the capacity of *Lactobacillus* to inhibit the growth of uropathogens and block their adhesion to vaginal epithelial cells. J Infect Dis 183:485–491.

46. Petrova MI, Reid G, Vaneechoutte M, Lebeer S. 2017. *Lactobacillus iners*: Friend or foe? Trends Microbiol 25:182–191.

47. Macklaim JM, Gloor GB, Anukam KC, Cribby S, Reid G. 2011. At the crossroads of vaginal health and disease, the genome sequence of *Lactobacillus iners* AB-1. Proc Natl Acad Sci U S A 108 Suppl 1:4688–4695.

48. France MT, Mendes-Soares H, Forney LJ. 2016. Genomic comparisons of *Lactobacillus crispatus* and *Lactobacillus iners* reveal potential ecological drivers of community composition in the vagina. Appl Environ Microbiol 82:7063–7073.

49. Aroutcheva A, Gariti D, Simon M, Shott S, Faro J, Simoes JA, Gurguis A, Faro S. 2001. Defense factors of vaginal lactobacilli. Am J Obstet Gynecol 185:375–379.

50. Klebanoff SJ, Hillier SL, Eschenbach DA, Waltersdorph AM. 1991. Control of the microbial flora of the vagina by H_2O_2-generating lactobacilli. J Infect Dis 164:94–100.

51. Klebanoff SJ, Coombs RW. 1991. Viricidal effect of *Lactobacillus acidophilus* on human immunodeficiency virus type 1: Possible role in heterosexual transmission. J Exp Med 174:289–292.

52. Ocaña VS, Pesce de Ruiz Holgado AA, Nader-Macías ME. 1999. Selection of vaginal H_2O_2-generating *Lactobacillus* species for probiotic use. Curr Microbiol 38:279–284.

53. O'Hanlon DE, Moench TR, Cone RA. 2011. In vaginal fluid, bacteria associated with bacterial vaginosis can be suppressed with lactic acid but not hydrogen peroxide. BMC Infect Dis 11:200.

54. Tomás MSJ, Bru E, Nader-Macías ME. 2003. Comparison of the growth and hydrogen peroxide production by vaginal probiotic lactobacilli under different culture conditions. Am J Obstet Gynecol 188:35–44.

55. O'Hanlon DE, Lanier BR, Moench TR, Cone RA. 2010. Cervicovaginal fluid and semen block the microbicidal activity of hydrogen peroxide produced by vaginal lactobacilli. BMC Infect Dis 10:120.

56. Boskey ER, Telsch KM, Whaley KJ, Moench TR, Cone RA. 1999. Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. Infect Immun 67:5170–5175.

57. O'Hanlon DE, Moench TR, Cone RA. 2013. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. PLoS One 8:e80074.

58. Amsel R, Totten PA, Spiegel CA, Chen KC, Eschenbach D, Holmes KK. 1983. Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiologic associations. Am J Med 74:14–22.

59. Aldunate M, Tyssen D, Johnson A, Zakir T, Sonza S, Moench T, Cone R, Tachedjian G. 2013. Vaginal concentrations of lactic acid potently inactivate HIV. J Antimicrob Chemother 68:2015–2025.

60. Alakomi H-L, Skyttä E, Saarela M, Mattila-Sandholm T, Latva-Kala K, Helander IM. 2000. Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. Appl Environ Microbiol 66:2001–2005.

61. Mossop H, Linhares IM, Bongiovanni AM, Ledger WJ, Witkin SS. 2011. Influence of lactic acid on endogenous and viral RNA-induced immune mediator production by vaginal epithelial cells. Obstet Gynecol 118:840–846.

62. Hearps AC, Tyssen D, Srbinovski D, Bayigga L, Diaz DJD, Aldunate M, Cone RA, Gugasyan R, Anderson DJ, Tachedjian G. 2017. Vaginal lactic acid elicits an anti-inflammatory response from human cervicovaginal epithelial cells and inhibits production of pro-inflammatory mediators associated with HIV acquisition. Mucosal Immunol 10:1480–1490.

63. Simoes JA, Aroutcheva A, Heimler I, Shott S, Faro S. 2001. Bacteriocin susceptibility of *Gardnerella vaginalis* and its relationship to biotype, genotype, and metronidazole susceptibility. Am J Obstet Gynecol 185:1186–1190.

64. Turovskiy Y, Ludescher RD, Aroutcheva AA, Faro S, Chikindas ML. 2009. Lactocin 160, a bacteriocin produced by vaginal *Lactobacillus rhamnosus*, targets cytoplasmic membranes of the vaginal pathogen, *Gardnerella vaginalis*. Probiotics Antimicrob Proteins 1:67–74.

65. Stoyancheva G, Marzotto M, Dellaglio F, Torriani S. 2014. Bacteriocin production and gene sequencing analysis from vaginal *Lactobacillus* strains. Arch Microbiol 196:645–653.

66. Ojala T, Kankainen M, Castro J, Cerca N, Edelman S, Westerlund-Wikström B, Paulin L, Holm L, Auvinen P. 2014. Comparative genomics of *Lactobacillus crispatus* suggests novel mechanisms for the competitive exclusion of *Gardnerella vaginalis*. BMC Genomics 15:1070.

67. McGroarty JA, Reid G. 1988. Detection of a *Lactobacillus* substance that inhibits *Escherichia coli*. Can J Microbiol 34:974–978.

68. McLean NW, McGroarty JA. 1996. Growth inhibition of metronidazole-susceptible and metronidazole-resistant strains of *Gardnerella vaginalis* by lactobacilli *in vitro*. Appl Environ Microbiol 62:1089–1092.

69. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. 1995. Microbial biofilms. Annu Rev Microbiol 49:711–745.

70. Swidsinski A, Mendling W, Loening-Baucke V, Ladhoff A, Swidsinski S, Hale LP, Lochs H. 2005. Adherent biofilms in bacterial vaginosis. Obstet Gynecol 106:1013–1023.

71. Sadhu K, Domingue PAG, Chow AW, Nelligan J, Bartlett K, Costerton JW. 1989. A morphological study of the *in situ* tissue-associated autochthonous microflora of the human vagina. Microb Ecol Health Dis 2:99–106.

72. Velraeds MM, van der Mei HC, Reid G, Busscher HJ. 1996. Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. Appl Environ Microbiol 62:1958–1963.

73. Heinemann C, van Hylckama Vlieg JE, Janssen DB, Busscher HJ, van der Mei HC, Reid G. 2000. Purification and characterization of a surface-binding protein from *Lactobacillus fermentum* RC-14 that inhibits adhesion of *Enterococcus faecalis* 1131. FEMS Microbiol Lett 190:177–180.

74. Andreu A, Stapleton AE, Fennell CL, Hillier SL, Stamm WE. 1995. Hemagglutination, adherence, and surface properties of vaginal Lactobacillus species. J Infect Dis 171:1237–1243.

75. Boris S, Suárez JE, Vázquez F, Barbés C. 1998. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect Immun 66:1985–1989.

76. Edelman SM, Lehti TA, Kainulainen V, Antikainen J, Kylväjä R, Baumann M, Westerlund-Wikström B, Korhonen TK. 2012. Identification of a high-molecular-mass *Lactobacillus* epithelium adhesin (LEA) of *Lactobacillus crispatus* ST1 that binds to stratified squamous epithelium. Microbiology 158:1713–1722.

77. Reid DG, McGroarty JA, Domingue PAG, Chow AW, Bruce AW, Eisen A, Costerton JW. 1990. Coaggregation of urogenital bacteria *in vitro* and *in vivo*. Curr Microbiol 20:47–52.

78. Reid G. 2001. Probiotic agents to protect the urogenital tract against infection. Am J Clin Nutr 73:437s–443s.

79. Younes JA, van der Mei HC, van den Heuvel E, Busscher HJ, Reid G. 2012. Adhesion forces and coaggregation between vaginal staphylococci and lactobacilli. PLoS One 7:e36917.

80. McMillan A, Dell M, Zellar MP, Cribby S, Martz S, Hong E, Fu J, Abbas A, Dang T, Miller W, Reid G. 2011. Disruption of urogenital biofilms by lactobacilli. Colloids Surf B Biointerfaces 86:58–64.

81. Koumans EH, Sternberg M, Bruce C, McQuillan G, Kendrick J, Sutton M, Markowitz LE. 2007. The prevalence of bacterial vaginosis in the United States, 2001-2004: Associations with symptoms, sexual behaviors, and reproductive health. Sex Transm Dis 34:864–869.

82. Hillier SL, Holmes KK. 1999. Bacterial vaginosis, p. 563–586. *In* Sexually Transmitted Diseases, 3rd ed. McGraw-Hill, New York.

83. Macklaim JM, Fernandes AD, Di Bella JM, Hammond J-A, Reid G, Gloor GB. 2013. Comparative meta-RNA-seq of the vaginal microbiota and differential expression by *Lactobacillus iners* in health and dysbiosis. Microbiome 1:12.

84. Yeoman CJ, Thomas SM, Miller MEB, Ulanov AV, Torralba M, Lucas S, Gillis M, Cregger M, Gomez A, Ho M, Leigh SR, Stumpf R, Creedon DJ, Smith MA, Weisbaum JS, Nelson KE, Wilson BA, White BA. 2013. A multi-omic systems-based approach reveals metabolic markers of bacterial vaginosis and insight into the disease. PLoS One 8:e56111.

85. Ison CA, Easmon CS, Dawson SG, Southerton G, Harris JW. 1983. Non-volatile fatty acids in the diagnosis of non-specific vaginitis. J Clin Pathol 36:1367–1370.

86. van Immerseel F, Ducatelle R, de Vos WM, Boon N, van de Wiele T, Verbeke K, Rutgeerts P, Sas B, Louis P, Flint HJ. 2010. Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. J Med Microbiol 59:141–143.

87. Nugent RP, Krohn MA, Hillier SL. 1991. Reliability of diagnosing bacterial vaginosis is improved by a standardized method of Gram stain interpretation. J Clin Microbiol 29:297–301.

88. Gloor GB, Hummelen R, Macklaim JM, Dickson RJ, Fernandes AD, MacPhee R, Reid G. 2010. Microbiome profiling by Illumina sequencing of combinatorial sequence-tagged PCR products. PLoS One 5:e15406.

89. Dols JAM, Smit PW, Kort R, Reid G, Schuren FHJ, Tempelman H, Bontekoe TR, Korporaal H, Boon ME. 2011. Microarray-based identification of clinically relevant vaginal bacteria in relation to bacterial vaginosis. Am J Obstet Gynecol 204:305.e1-305.e7.

90. Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, Cotch MF, Edelman R, Pastorek JG, Rao AV, McNellis D, Regan JA, Carey JC, Klebanoff MA. 1995. Association between bacterial vaginosis and preterm delivery of a low-birth-weight infant. N Engl J Med 333:1737–1742.

91. McGregor JA, French JI, Jones W, Milligan K, McKinney PJ, Patterson E, Parker R. 1994. Bacterial vaginosis is associated with prematurity and vaginal fluid mucinase and sialidase: results of a controlled trial of topical clindamycin cream. Am J Obstet Gynecol 170:1048–1060.

92. Otsuki K, Tokunaka M, Oba T, Nakamura M, Shirato N, Okai T. 2014. Administration of oral and vaginal prebiotic lactoferrin for a woman with a refractory vaginitis recurring preterm delivery: appearance of *Lactobacillus* in vaginal flora followed by term delivery. J Obstet Gynaecol Res 40:583–585.

93. Hillier SL, Martius J, Krohn M, Kiviat N, Holmes KK, Eschenbach DA. 1988. A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. N Engl J Med 319:972–978.

94. Platz-Christensen JJ, Mattsby-Baltzer I, Thomsen P, Wiqvist N. 1993. Endotoxin and interleukin-1α in the cervical mucus and vaginal fluid of pregnant women with bacterial vaginosis. Am J Obstet Gynecol 169:1161–1166.

95. Mattsby-Baltzer I, Platz-Christensen JJ, Hosseini N, Rosén P. 1998. IL-1β, IL-6, TNFα, fetal fibronectin, and endotoxin in the lower genital tract of pregnant women with bacterial vaginosis. Acta Obstet Gynecol Scand 77:701–706.

96. Martius J, Eschenbach DA. 1990. The role of bacterial vaginosis as a cause of amniotic fluid infection, chorioamnionitis and prematurity—a review. Arch Gynecol Obstet 247:1–13.

97. Sha BE, Zariffard MR, Wang QJ, Chen HY, Bremer J, Cohen MH, Spear GT. 2005. Female genital-tract HIV load correlates inversely with *Lactobacillus* species but positively with bacterial vaginosis and *Mycoplasma hominis*. J Infect Dis 191:25–32.

98. Coleman JS, Hitti J, Bukusi EA, Mwachari C, Muliro A, Nguti R, Gausman R, Jensen S, Patton D, Lockhart D, Coombs R, Cohen CR. 2007. Infectious correlates of HIV-1 shedding in the female upper and lower genital tracts. AIDS 21:755–759.

99. Martin HL, Richardson BA, Nyange PM, Lavreys L, Hillier SL, Chohan B, Mandaliya K, Ndinya-Achola JO, Bwayo J, Kreiss J. 1999. Vaginal lactobacilli, microbial flora, and risk of human immunodeficiency virus type 1 and sexually transmitted disease acquisition. J Infect Dis 180:1863–1868.

100. Cohen CR, Lingappa JR, Baeten JM, Ngayo MO, Spiegel CA, Hong T, Donnell D, Celum C, Kapiga S, Delany S, Bukusi EA. 2012. Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: A prospective cohort analysis among African couples. PLoS Med 9:e1001251.

101. Thurman AR, Kimble T, Herold B, Mesquita PMM, Fichorova RN, Dawood HY, Fashemi T, Chandra N, Rabe L, Cunningham TD, Anderson S, Schwartz J, Doncel

G. 2015. Bacterial vaginosis and subclinical markers of genital tract inflammation and mucosal immunity. AIDS Res Hum Retroviruses 31:1139–1152.

102. Berger EA, Murphy PM, Farber JM. 1999. Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. Annu Rev Microbiol 17:657–700.

103. Osborn L, Kunkel S, Nabel GJ. 1989. Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc Natl Acad Sci U S A 86:2336–2340.

104. Ness RB, Kip KE, Hillier SL, Soper DE, Stamm CA, Sweet RL, Rice P, Richter HE. 2005. A cluster analysis of bacterial vaginosis–associated microflora and pelvic inflammatory disease. Am J Epidemiol 162:585–590.

105. Marrazzo JM, Wiesenfeld HC, Murray PJ, Busse B, Meyn L, Krohn M, Hillier SL. 2006. Risk factors for cervicitis among women with bacterial vaginosis. J Infect Dis 193:617–624.

106. Cherpes TL, Meyn LA, Krohn MA, Lurie JG, Hillier SL. 2003. Association between acquisition of herpes simplex virus type 2 in women and bacterial vaginosis. Clin Infect Dis 37:319–325.

107. Esber A, Miguel RDV, Cherpes TL, Klebanoff MA, Gallo MF, Turner AN. 2015. Risk of bacterial vaginosis among women with herpes simplex virus type 2 infection: A systematic review and meta-analysis. J Infect Dis 212:8–17.

108. Bradshaw CS, Morton AN, Garland SM, Morris MB, Moss LM, Fairley CK. 2005. Higher-risk behavioral practices associated with bacterial vaginosis compared with vaginal candidiasis. Obstet Gynecol 106:105–114.

109. Nilsson U, Hellberg D, Shoubnikova M, Nilsson S, Mårdh PA. 1997. Sexual behavior risk factors associated with bacterial vaginosis and *Chlamydia trachomatis* infection. Sex Transm Dis 24:241–246.

110. Shoubnikova M, Hellberg D, Nilsson S, Mårdh P-A. 1997. Contraceptive use in women with bacterial vaginosis. Contraception 55:355–358.

111. Baeten JM, Nyange PM, Richardson BA, Lavreys L, Chohan B, Martin HLJ, Mandaliya K, Ndinya-Achola JO, Bwayo JJ, Kreiss JK. 2001. Hormonal contraception and risk of sexually transmitted disease acquisition: Results from a prospective study. Am J Obstet Gynecol 185:380–385.

112. Bailey JV, Farquhar C, Owen C. 2004. Bacterial vaginosis in lesbians and bisexual women. Sex Transm Dis 31:691–694.

113. Marrazzo JM, Thomas KK, Agnew K, Ringwood K. 2010. Prevalence and risks for bacterial vaginosis in women who have sex with women. Sex Transm Dis 37:335–339.

114. Gottschick C, Deng Z-L, Vital M, Masur C, Abels C, Pieper DH, Wagner-Döbler I. 2017. The urinary microbiota of men and women and its changes in women during bacterial vaginosis and antibiotic treatment. Microbiome 5:99.

115. Marrazzo JM, Fiedler TL, Srinivasan S, Thomas KK, Liu C, Ko D, Xie H, Saracino M, Fredricks DN. 2012. Extravaginal reservoirs of vaginal bacteria as risk factors for incident bacterial vaginosis. J Infect Dis 205:1580–1588.

116. Liu CM, Hungate BA, Tobian AAR, Ravel J, Prodger JL, Serwadda D, Kigozi G, Galiwango RM, Nalugoda F, Keim P, Wawer MJ, Price LB, Gray RH. 2015. Penile microbiota and female partner bacterial vaginosis in Rakai, Uganda. MBio 6:e00589-15.

117. Joesoef MR, Schmid GP, Hillier SL. 1999. Bacterial vaginosis: Review of treatment options and potential clinical indications for therapy. Clin Infect Dis 28:S57–S65.

118. Greaves WL, Chungafung J, Morris B, Haile A, Townsend JL. 1988. Clindamycin versus metronidazole in the treatment of bacterial vaginosis. Obstet Gynecol 72:799–802.

119. Mayer BT, Srinivasan S, Fiedler TL, Marrazzo JM, Fredricks DN, Schiffer JT. 2015. Rapid and profound shifts in the vaginal microbiota following antibiotic treatment for bacterial vaginosis. J Infect Dis 212:793–802.

120. Koumans EH, Markowitz LE, Hogan V, Group CBW. 2002. Indications for therapy and treatment recommendations for bacterial vaginosis in nonpregnant and pregnant women: A synthesis of data. Clin Infect Dis 35:S152–S172.

121. Bradshaw CS, Morton AN, Hocking J, Garland SM, Morris MB, Moss LM, Horvath LB, Kuzevska I, Fairley CK. 2006. High recurrence rates of bacterial vaginosis over the course of 12 months after oral metronidazole therapy and factors associated with recurrence. J Infect Dis 193:1478–1486.

122. Swidsinski A, Mendling W, Loening-Baucke V, Swidsinski S, Dörffel Y, Scholze J, Lochs H, Verstraelen H. 2008. An adherent *Gardnerella vaginalis* biofilm persists

on the vaginal epithelium after standard therapy with oral metronidazole. Am J Obstet Gynecol 198:97.e1-97.e6.

123. Machado A, Salgueiro D, Harwich M, Jefferson KK, Cerca N. 2013. Quantitative analysis of initial adhesion of bacterial vaginosis-associated anaerobes to ME-180 cells. Anaerobe 23:1–4.

124. Machado A, Cerca N. 2015. Influence of biofilm formation by *Gardnerella vaginalis* and other anaerobes on bacterial vaginosis. J Infect Dis 212:1856–1861.

125. Wang B, Xiao BB, Shang CG, Wang K, Na RS, Nu XX, Liao Q. 2014. Molecular analysis of the relationship between specific vaginal bacteria and bacterial vaginosis metronidazole therapy failure. Clin Microbiol Infect 33:1749–1756.

126. 2001. Joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. FAO/WHO, Cordoba, Argentina.

127. Gardiner GE, Heinemann C, Bruce AW, Beuerman D, Reid G. 2002. Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but not *L. rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. Clin Diagn Lab Immunol 9:92–96.

128. Anukam K, Osazuwa E, Ahonkhai I, Ngwu M, Osemene G, Bruce AW, Reid G. 2006. Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: Randomized, double-blind, placebo controlled trial. Microbes Infect 8:1450–1454.

129. Macklaim JM, Clemente JC, Knight R, Gloor GB, Reid G. 2015. Changes in vaginal microbiota following antimicrobial and probiotic therapy. Microb Ecol Health Dis 26:27799.

130. Reid G, Charbonneau D, Erb J, Kochanowski B, Beuerman D, Poehner R, Bruce AW. 2003. Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: Randomized, placebo-controlled trial in 64 healthy women. FEMS Immunol Med Microbiol 35:131–134.

131. Hummelen R, Changalucha J, Butamanya NL, Cook A, Habbema JDF, Reid G. 2010. *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 to prevent or cure bacterial vaginosis among women with HIV. Int J Gynaecol Obstet 111:245–248.

132. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, Scott K, Stanton C, Swanson KS, Cani PD, Verbeke K, Reid G. 2017. Expert consensus

document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol 14:491–502.

133. Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev 17:259–275.

134. Gibson GR, Roberfroid MB. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 125:1401–1412.

135. Rousseau V, Lepargneur JP, Roques C, Remaud-Simeon M, Paul F. 2005. Prebiotic effects of oligosaccharides on selected vaginal lactobacilli and pathogenic microorganisms. Anaerobe 11:145–153.

136. Coste I, Judlin P, Lepargneur J-P, Bou-Antoun S. 2012. Safety and efficacy of an intravaginal prebiotic gel in the prevention of recurrent bacterial vaginosis: A randomized double-blind study. Obstet Gynecol Int 2012:1–7.

137. Hurley R, de Louvois J. 1979. Candida vaginitis. Postgrad Med J 55:645–647.

138. Peraica M, Radić B, Lucić A, Pavlović M. 1999. Toxic effects of mycotoxins in humans. Bull World Health Organ 77:754–766.

139. Leong Y, Ismail N, Latiff A, Manaf N, Rosma A. 2011. Determination of aflatoxins in commercial nuts and nut products using liquid chromatography tandem mass spectrometry. World Mycotoxin J 4:119–127.

140. Trenk HL, Hartman PA. 1970. Effects of moisture content and temperature on aflatoxin production in corn. Appl Microbiol 19:781–784.

141. Tosun H, Arslan R. 2013. Determination of aflatoxin B1 levels in organic spices and herbs. Sci World J 2013:1–4.

142. Mitchell NJ, Bowers E, Hurburgh C, Wu F. 2016. Potential economic losses to the US corn industry from aflatoxin contamination. Food Addit Contam Part A 33:540–550.

143. IARC. 1993. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. 56. World Health Organization, Lyon.

144. Guengerich FP, Johnson WW, Ueng YF, Yamazaki H, Shimada T. 1996. Involvement of cytochrome P450, glutathione S-transferase, and epoxide hydrolase in the metabolism of aflatoxin B1 and relevance to risk of human liver cancer. Environ Health Perspect 104:557–562.

145. Gong YY, Cardwell K, Hounsa A, Egal S, Turner PC, Hall AJ, Wild CP. 2002. Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: Cross sectional study. BMJ 325:20–21.

146. Jolly PE, Inusah S, Lu B, Ellis WO, Nyarko A, Phillips TD, Williams JH. 2013. Association between high aflatoxin B1 levels and high viral load in HIV-positive people. World Mycotoxin J 6:255–261.

147. Narrod C, Tiongco M, Mahuku G, de Groote H, Hellin J, Bett C, Nzioki H, Collins S, Guantai S, Waliyar F, Ndjeunga J, Bamory D, Ondie K, Lamissa D, Masuoka P, Wu F. 2013. No chance for aflatoxins. Focus 21.

148. Leroy JL, Wang J-S, Jones K. 2015. Serum aflatoxin B_1-lysine adduct level in adult women from Eastern Province in Kenya depends on household socio-economic status: A cross sectional study. Soc Sci Med 1982 146:104–110.

149. Shuaib FMB, Jolly PE, Ehiri JE, Ellis WO, Yatich NJ, Funkhouser E, Person S, Williams JH, Qian G, Wang J-S. 2012. Socio-demographic determinants of aflatoxin B1-lysine adduct levels among pregnant women in Kumasi, Ghana. Ghana Med J 46:179–188.

150. Piekkola S, Turner PC, Abdel-Hamid M, Ezzat S, El-Daly M, El-Kafrawy S, Savchenko E, Poussa T, Woo JCS, Mykkänen H, El-Nezami H. 2012. Characterisation of aflatoxin and deoxynivalenol exposure among pregnant Egyptian women. Food Addit Contam Part A 29:962–971.

151. Smith LE, Mbuya MNN, Prendergast AJ, Turner PC, Ruboko S, Humphrey JH, Nelson RJ, Chigumira A, Kembo G, Stoltzfus RJ. 2017. Determinants of recent aflatoxin exposure among pregnant women in rural Zimbabwe. Mol Nutr Food Res.

152. Polychronaki N, West RM, Turner PC, Amra H, Abdel-Wahhab M, Mykkänen H, El-Nezami H. 2007. A longitudinal assessment of aflatoxin M1 excretion in breast milk of selected Egyptian mothers. Food Chem Toxicol 45:1210–1215.

153. Coulter JB, Lamplugh SM, Suliman GI, Omer MI, Hendrickse RG. 1984. Aflatoxins in human breast milk. Ann Trop Paediatr 4:61–66.

154. Matsiko F, Kanyange C, Ingabire G, Dusingizimana T, Vasanthakaalam H, Kimonyo A. 2017. Detection and quantification of aflatoxin in cassava and maize flour sold in Kigali open markets, Rwanda. Int Food Res J 24:459–464.

155. Nishimwe K, Wanjuki I, Karangwa C, Darnell R, Harvey J. 2017. An initial characterization of aflatoxin B1 contamination of maize sold in the principal retail markets of Kigali, Rwanda. Food Control 73:574–580.

156. Umereweneza D, Kamizikunze T, Muhizi T. 2018. Assessment of mycotoxins types in some foodstuff consumed in Rwanda. Food Control 85:432–436.

157. Sulyok M, Beed F, Boni S, Abass A, Mukunzi A, Krska R. 2015. Quantitation of multiple mycotoxins and cyanogenic glucosides in cassava samples from Tanzania and Rwanda by an LC-MS/MS-based multi-toxin method. Food Addit Contam Part Chem Anal Control Expo Risk Assess 32:488–502.

158. Gelderblom WC, Jaskiewicz K, Marasas WF, Thiel PG, Horak RM, Vleggaar R, Kriek NP. 1988. Fumonisins—novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. Appl Environ Microbiol 54:1806–1811.

159. Medina A, Schmidt-Heydt M, Cárdenas-Chávez DL, Parra R, Geisen R, Magan N. 2013. Integrating toxin gene expression, growth and fumonisin B1 and B2 production by a strain of *Fusarium verticillioides* under different environmental factors. J R Soc Interface 10:20130320.

160. Bhat RV, Shetty PH, Amruth RP, Sudershan RV. 1997. A foodborne disease outbreak due to the consumption of moldy sorghum and maize containing fumonisin mycotoxins. J Toxicol Clin Toxicol 35:249–255.

161. Alizadeh AM, Rohandel G, Roudbarmohammadi S, Roudbary M, Sohanaki H, Ghiasian SA, Taherkhani A, Semnani S, Aghasi M. 2012. Fumonisin B1 contamination of cereals and risk of esophageal cancer in a high risk area in northeastern Iran. Asian Pac J Cancer Prev 13:2625–2628.

162. Gelderblom WC, Abel S, Smuts CM, Marnewick J, Marasas WF, Lemmer ER, Ramljak D. 2001. Fumonisin-induced hepatocarcinogenesis: mechanisms related to cancer initiation and promotion. Environ Health Perspect 109:291–300.

163. Missmer SA, Suarez L, Felkner M, Wang E, Merrill AH, Rothman KJ, Hendricks KA. 2006. Exposure to fumonisins and the occurrence of neural tube defects along the Texas-Mexico border. Environ Health Perspect 114:237–241.

164. Marasas WFO, Riley RT, Hendricks KA, Stevens VL, Sadler TW, Gelineau-van Waes J, Missmer SA, Cabrera J, Torres O, Gelderblom WCA, Allegood J, Martínez C, Maddox J, Miller JD, Starr L, Sullards MC, Roman AV, Voss KA, Wang E, Merrill AH. 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: A potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J Nutr 134:711–716.

165. Abnet CC, Borkowf CB, Qiao Y-L, Albert PS, Wang E, Merrill AH, Mark SD, Dong Z-W, Taylor PR, Dawsey SM. 2001. Sphingolipids as biomarkers of fumonisin exposure and risk of esophageal squamous cell carcinoma in China. Cancer Causes Control 12:821–828.

166. Stockmann-Juvala H, Savolainen K. 2008. A review of the toxic effects and mechanisms of action of fumonisin B1. Hum Exp Toxicol 27:799–809.

167. van der Westhuizen L, Shephard GS, Burger HM, Rheeder JP, Gelderblom WCA, Wild CP, Gong YY. 2011. Fumonisin B1 as a urinary biomarker of exposure in a maize intervention study among South African subsistence farmers. Cancer Epidemiol Biomarkers Prev 20:483–489.

168. Shephard GS, Burger H-M, Gambacorta L, Gong YY, Krska R, Rheeder JP, Solfrizzo M, Srey C, Sulyok M, Visconti A, Warth B, van der Westhuizen L. 2013. Multiple mycotoxin exposure determined by urinary biomarkers in rural subsistence farmers in the former Transkei, South Africa. Food Chem Toxicol 62:217–225.

169. Abia WA, Warth B, Sulyok M, Krska R, Tchana A, Njobeh PB, Turner PC, Kouanfack C, Eyongetah M, Dutton M, Moundipa PF. 2013. Bio-monitoring of mycotoxin exposure in Cameroon using a urinary multi-biomarker approach. Food Chem Toxicol 62:927–934.

170. Ezekiel CN, Warth B, Ogara IM, Abia WA, Ezekiel VC, Atehnkeng J, Sulyok M, Turner PC, Tayo GO, Krska R, Bandyopadhyay R. 2014. Mycotoxin exposure in rural residents in northern Nigeria: A pilot study using multi-urinary biomarkers. Environ Int 66:138–145.

171. Shale K, Mukamugema J, Lues RJ, Venter P. 2012. Toxicity profile of commercially produced indigenous banana beer. Food Addit Contam Part Chem Anal Control Expo Risk Assess 29:1300–1306.

172. Jiménez M, Máñez M, Hernández E. 1996. Influence of water activity and temperature on the production of zearalenone in corn by three *Fusarium* species. Int J Food Microbiol 29:417–421.

173. Frizzell C, Ndossi D, Verhaegen S, Dahl E, Eriksen G, Sørlie M, Ropstad E, Muller M, Elliott CT, Connolly L. 2011. Endocrine disrupting effects of zearalenone, alpha- and beta-zearalenol at the level of nuclear receptor binding and steroidogenesis. Toxicol Lett 206:210–217.

174. Yu Z, Zhang L, Wu D, Liu F. 2005. Anti-apoptotic action of zearalenone in MCF-7 cells. Ecotoxicol Environ Saf 62:441–446.

175. Belhassen H, Jiménez-Díaz I, Arrebola JP, Ghali R, Ghorbel H, Olea N, Hedili A. 2015. Zearalenone and its metabolites in urine and breast cancer risk: A case-control study in Tunisia. Chemosphere 128:1–6.

176. Stack ME, Mislivec PB, Denizel T, Gibson R, Pohland AE. 1983. Ochratoxins A and B, xanthomegnin, viomellein and vioxanthin production by isolates of *Aspergillus ochraceus* from green coffee beans. J Food Prot 46:965–968.

177. Bui-Klimke TR, Wu F. 2015. Ochratoxin A and human health risk: A review of the evidence. Crit Rev Food Sci Nutr 55:1860–1869.

178. Castegnaro M, Canadas D, Vrabcheva T, Petkova-Bocharova T, Chernozemsky IN, Pfohl-Leszkowicz A. 2006. Balkan endemic nephropathy: Role of ochratoxins A through biomarkers. Mol Nutr Food Res 50:519–529.

179. Aldunate M, Srbinovski D, Hearps AC, Latham CF, Ramsland PA, Gugasyan R, Cone RA, Tachedjian G. 2015. Antimicrobial and immune modulatory effects of lactic acid and short chain fatty acids produced by vaginal microbiota associated with eubiosis and bacterial vaginosis. Front Physiol 6:164.

180. Hummelen R, Fernandes AD, Macklaim JM, Dickson RJ, Changalucha J, Gloor GB, Reid G. 2010. Deep sequencing of the vaginal microbiota of women with HIV. PLoS One 5:e12078.

181. Ling Z, Liu X, Chen W, Luo Y, Yuan L, Xia Y, Nelson KE, Huang S, Zhang S, Wang Y, Yuan J, Li L, Xiang C. 2013. The restoration of the vaginal microbiota after treatment for bacterial vaginosis with metronidazole or probiotics. Microb Ecol 65:773–780.

182. Gilbert NM, Lewis WG, Lewis AL. 2013. Clinical features of bacterial vaginosis in a murine model of vaginal infection with *Gardnerella vaginalis*. PLoS One 8:e59539.

183. 1995. A synthesis report of the Africa region - Women, agriculture and rural development. Food and Agriculture Organization of the United Nations, Rome.

184. Gibson GR. 2008. Prebiotics as gut microflora management tools. J Clin Gastroenterol 42:S75–S79.

185. Tester R, Al-Ghazzewi F, Shen N, Chen Z, Chen F, Yang J, Zhang D, Tang M. 2012. The use of konjac glucomannan hydrolysates to recover healthy microbiota in infected vaginas treated with an antifungal agent. Benef Microbes 3:61–66.

186. Nelson TM, Borgogna J-LC, Brotman RM, Ravel J, Walk ST, Yeoman CJ. 2015. Vaginal biogenic amines: Biomarkers of bacterial vaginosis or precursors to vaginal dysbiosis? Front Physiol 6:253.

187. Deepika G, Karunakaran E, Hurley CR, Biggs CA, Charalampopoulos D. 2012. Influence of fermentation conditions on the surface properties and adhesion of *Lactobacillus rhamnosus* GG. Microb Cell Factories 11:116.

188. Mirmonsef P, Hotton AL, Gilbert D, Gioia CJ, Maric D, Hope TJ, Landay AL, Spear GT. 2016. Glycogen levels in undiluted genital fluid and their relationship to vaginal pH, estrogen, and progesterone. PLoS One 11:e0153553.

189. Mirmonsef P, Hotton AL, Gilbert D, Burgad D, Landay A, Weber KM, Cohen M, Ravel J, Spear GT. 2014. Free glycogen in vaginal fluids is associated with *Lactobacillus* colonization and low vaginal pH. PLoS One 9:e102467.

190. Martín R, Soberón N, Vaneechoutte M, Flórez AB, Vázquez F, Suárez JE. 2008. Characterization of indigenous vaginal lactobacilli from healthy women as probiotic candidates. Int Microbiol 11:261–266.

191. Spear GT, French AL, Gilbert D, Zariffard MR, Mirmonsef P, Sullivan TH, Spear WW, Landay A, Micci S, Lee B-H, Hamaker BR. 2014. Human α-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by *Lactobacillus*. J Infect Dis 210:1019–1028.

192. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. 2004. Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr 80:1106–1122.

193. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SSK, McCulle SL, Karlebach S, Gorle R, Russell J, Tacket CO, Brotman RM, Davis CC, Ault K, Peralta L, Forney LJ. 2011. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A 108:4680–4687.

194. Reid G, Younes JA, Van der Mei HC, Gloor GB, Knight R, Busscher HJ. 2011. Microbiota restoration: Natural and supplemented recovery of human microbial communities. Nat Rev Microbiol 9:27–38.

195. Reid G, Brigidi P, Burton JP, Contractor N, Duncan S, Fargier E, Hill C, Lebeer S, Martín R, McBain AJ, Mor G, O'Neill C, Rodríguez JM, Swann J, van Hemert S, Ansell J. 2015. Microbes central to human reproduction. Am J Reprod Immunol 73:1–11.

196. Redondo-Lopez V, Meriwether C, Schmitt C, Opitz M, Cook R, Sobel JD. 1990. Vulvovaginal candidiasis complicating recurrent bacterial vaginosis. Sex Transm Dis 17:51–53.

197. Oleen-Burkey MA, Hillier SL. 1995. Pregnancy complications associated with bacterial vaginosis and their estimated costs. Infect Dis Obstet Gynecol 3:149–157.

198. Srinivasan S, Hoffman NG, Morgan MT, Matsen FA, Fiedler TL, Hall RW, Ross FJ, McCoy CO, Bumgarner R, Marrazzo JM, Fredricks DN. 2012. Bacterial communities in women with bacterial vaginosis: High resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. PLoS One 7:e37818.

199. Blankenstein T, Lytton SD, Leidl B, Atweh E, Friese K, Mylonas I. 2015. Point-of-care (POC) diagnosis of bacterial vaginosis (BV) using VGTest[TM] ion mobility spectrometry (IMS) in a routine ambulatory care gynecology clinic. Arch Gynecol Obstet 292:355–362.

200. Fischbach F, Petersen EE, Weissenbacher ER, Martius J, Hosmann J, Mayer H. 1993. Efficacy of clindamycin vaginal cream versus oral metronidazole in the treatment of bacterial vaginosis. Obstet Gynecol 82:405–410.

201. Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco M-J, Léotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A. 2010. Prebiotic effects: Metabolic and health benefits. Br J Nutr 104:S1–S63.

202. Reid G, Bruce AW, Taylor M. 1995. Instillation of *Lactobacillus* and stimulation of indigenous organisms to prevent recurrence of urinary tract infections. Microecol Ther 23:32–45.

203. Vongsa R, Minerath R, Busch M, Tan J, Koenig D. 2015. *In vitro* evaluation of nutrients that selectively confer a competitive advantage to lactobacilli. Benef Microbes 7:299–304.

204. Sutherland A, Tester R, Al-Ghazzewi F, McCulloch E, Connolly M. 2008. Glucomannan hydrolysate (GMH) inhibition of *Candida albicans* growth in the presence of *Lactobacillus* and *Lactococcus* species. Microb Ecol Health Dis 20:127–134.

205. Foxman B, Wen A, Srinivasan U, Goldberg D, Marrs CF, Owen J, Wing DA, Misra D. 2014. *Mycoplasma*, bacterial vaginosis–associated bacteria BVAB3, race, and risk of preterm birth in a high-risk cohort. Am J Obstet Gynecol 210:e1–e7.

206. Hilbert DW, Smith WL, Chadwick SG, Toner G, Mordechai E, Adelson ME, Sobel JD, Gygax SE. 2016. Development and validation of a highly accurate quantitative real-time PCR assay for the diagnosis of bacterial vaginosis. J Clin Microbiol 54:1017–1024.

207. Machado A, Jefferson KK, Cerca N. 2013. Interactions between *Lactobacillus crispatus* and bacterial vaginosis (BV)-associated bacterial species in initial attachment and biofilm formation. Int J Mol Sci 14:12004–12012.

208. Moynihan PJ, Ferrier S, Blomley S, Wright WG, Russell RR. 1998. Acid production from lactulose by dental plaque bacteria. Lett Appl Microbiol 27:173–177.

209. Sahota SS, Bramley PM, Menzies IS. 1982. The fermentation of lactulose by colonic bacteria. J Gen Microbiol 128:319–325.

210. Cardelle-Cobas A, Corzo N, Olano A, Peláez C, Requena T, Ávila M. 2011. Galactooligosaccharides derived from lactose and lactulose: Influence of structure on *Lactobacillus, Streptococcus* and *Bifidobacterium* growth. Int J Food Microbiol 149:81–87.

211. Hernandez-Hernandez O, Muthaiyan A, Moreno FJ, Montilla A, Sanz ML, Ricke SC. 2012. Effect of prebiotic carbohydrates on the growth and tolerance of *Lactobacillus*. Food Microbiol 30:355–361.

212. Mao B, Li D, Zhao J, Liu X, Gu Z, Chen YQ, Zhang H, Chen W. 2014. *In vitro* fermentation of lactulose by human gut bacteria. J Agric Food Chem 62:10970–10977.

213. Patel CN, Wortham BW, Lines JL, Fetherston JD, Perry RD, Oliveira MA. 2006. Polyamines are essential for the formation of plague biofilm. J Bacteriol 188:2355–2363.

214. Patterson JL, Stull-Lane A, Girerd PH, Jefferson KK. 2010. Analysis of adherence, biofilm formation and cytotoxicity suggests a greater virulence potential of *Gardnerella vaginalis* relative to other bacterial-vaginosis-associated anaerobes. Microbiology 156:392–399.

215. Larsson P-G, Stray-Pedersen B, Ryttig KR, Larsen S. 2008. Human lactobacilli as supplementation of clindamycin to patients with bacterial vaginosis reduce the recurrence rate; a 6-month, double-blind, randomized, placebo-controlled study. BMC Womens Health 8:3.

216. Dirkx M, Boyer MP, Pradhan P, Brittingham A, Wilson WA. 2014. Expression and characterization of a β-fructofuranosidase from the parasitic protist *Trichomonas vaginalis*. BMC Biochem 15:12.

217. Roberfroid MB, Loo JAEV, Gibson GR. 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. J Nutr 128:11–19.

218. Hopkins MJ, Cummings JH, Macfarlane GT. 1998. Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. J Appl Microbiol 85:381–386.

219. Douillard FP, Ribbera A, Kant R, Pietilä TE, Järvinen HM, Messing M, Randazzo CL, Paulin L, Laine P, Ritari J, Caggia C, Lähteinen T, Brouns SJJ, Satokari R, von Ossowski I, Reunanen J, Palva A, de Vos WM. 2013. Comparative genomic and functional analysis of 100 *Lactobacillus rhamnosus* strains and their comparison with strain GG. PLoS Genet 9:e1003683.

220. Kort R. 2014. Personalized therapy with probiotics from the host by TripleA. Trends Biotechnol 32:291–293.

221. Dols JAM, Molenaar D, van der Helm JJ, Caspers MPM, de Kat Angelino-Bart A, Schuren FHJ, Speksnijder AGCL, Westerhoff HV, Richardus JH, Boon ME, Reid G, de Vries HJC, Kort R. 2016. Molecular assessment of bacterial vaginosis by *Lactobacillus* abundance and species diversity. BMC Infect Dis 16:180.

222. Geshnizgani AM, Onderdonk AB. 1992. Defined medium simulating genital tract secretions for growth of vaginal microflora. J Clin Microbiol 30:1323–1326.

223. Aitchison J. 1982. The statistical analysis of compositional data. J R Stat Soc 44:139–177.

224. Lopes dos Santos Santiago G, Tency I, Verstraelen H, Verhelst R, Trog M, Temmerman M, Vancoillie L, Decat E, Cools P, Vaneechoutte M. 2012. Longitudinal qPCR study of the dynamics of *L. crispatus*, *L. iners*, *A. vaginae*, (sialidase positive) *G. vaginalis*, and *P. bivia* in the vagina. PLoS One 7:e45281.

225. De Backer E, Verhelst R, Verstraelen H, Alqumber MA, Burton JP, Tagg JR, Temmerman M, Vaneechoutte M. 2007. Quantitative determination by real-time PCR of four vaginal *Lactobacillus* species, *Gardnerella vaginalis* and *Atopobium vaginae* indicates an inverse relationship between *L. gasseri* and *L. iners*. BMC Microbiol 7:115.

226. Ellison SL, English CA, Burns MJ, Keer JT. 2006. Routes to improving the reliability of low level DNA analysis using real-time PCR. BMC Biotechnol 6:33.

227. Stein SE. 1999. An integrated method for spectrum extraction and compound identification from gas chromatography/mass spectrometry data. J Am Soc Mass Spectrom 10:770–781.

228. Styczynski MP, Moxley JF, Tong L V, Walther JL, Jensen KL, Stephanopoulos GN. 2007. Systematic identification of conserved metabolites in GC/MS data for metabolomics and biomarker discovery. Anal Chem 79:966–73.

229. Kessner D, Chambers M, Burke R, Agus D, Mallick P. 2008. ProteoWizard: open source software for rapid proteomics tools development. Bioinforma Oxf Engl 24:2534–6.

230. Patti GJ, Tautenhahn R, Siuzdak G. 2012. Meta-analysis of untargeted metabolomic data from multiple profiling experiments. Nat Protoc 7:508–16.

231. Anderson DJ, Marathe J, Pudney J. 2014. The structure of the human vaginal stratum corneum and its role in immune defense. Am J Reprod Immunol N Y N 1989 71:618–623.

232. Blaskewicz CD, Pudney J, Anderson DJ. 2011. Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia. Biol Reprod 85:97–104.

233. Witkin SS. 2015. The vaginal microbiome, vaginal anti-microbial defence mechanisms and the clinical challenge of reducing infection-related preterm birth. BJOG 122:213–218.

234. Fields S, Song B, Rasoul B, Fong J, Works MG, Shew K, Yiu Y, Mirsalis J, D'Andrea A. 2014. New candidate biomarkers in the female genital tract to evaluate microbicide toxicity. PLoS One 9:e110980.

235. Barousse MM, Steele C, Dunlap K, Espinosa T, Boikov D, Sobel JD, Fidel PL. 2001. Growth inhibition of *Candida albicans* by human vaginal epithelial cells. J Infect Dis 184:1489–1493.

236. Niu X-X, Li T, Zhang X, Wang S-X, Liu Z-H. 2017. *Lactobacillus crispatus* modulates vaginal epithelial cell innate response to *Candida albicans*. Chin Med J (Engl) 130:273–279.

237. Kucknoor A, Mundodi V, Alderete JF. 2005. *Trichomonas vaginalis* adherence mediates differential gene expression in human vaginal epithelial cells. Cell Microbiol 7:887–897.

238. Sargeant P, Moate R, Harris JE, Morrison GD. 1996. Ultrastructural study of the epithelium of the normal human vulva. J Submicrosc Cytol Pathol 28:161–170.

239. Cotter PD, Ross RP, Hill C. 2012. Bacteriocins — a viable alternative to antibiotics? Nat Rev Microbiol 11:nrmicro2937.

240. Beauregard PB, Chai Y, Vlamakis H, Losick R, Kolter R. 2013. *Bacillus subtilis* biofilm induction by plant polysaccharides. Proc Natl Acad Sci U S A 110:E1621–E1630.

241. Altamimi M, Abdelhay O, Rastall RA. 2016. Effect of oligosaccharides on the adhesion of gut bacteria to human HT-29 cells. Anaerobe 39:136–142.

242. Adebola O, Corcoran O, Morgan WA. 2013. Protective effects of prebiotics inulin and lactulose from cytotoxicity and genotoxicity in human colon adenocarcinoma cells. Food Res Int 52:269–274.

243. Joerger MC, Klaenhammer TR. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J Bacteriol 167:439–446.

244. Bonadè A, Murelli F, Vescovo M, Scolari G. 2001. Partial characterization of a bacteriocin produced by *Lactobacillus helveticus*. Lett Appl Microbiol 33:153–158.

245. Nilsen T, Nes IF, Holo H. 2003. Enterolysin A, a cell wall-degrading bacteriocin from *Enterococcus faecalis* LMG 2333. Appl Environ Microbiol 69:2975–2984.

246. Andreu A, Stapleton AE, Fennell CL, Hillier SL, Stamm WE. 1995. Hemagglutination, adherence, and surface properties of vaginal *Lactobacillus* species. J Infect Dis 171:1237–1243.

247. von Ossowski I, Satokari R, Reunanen J, Lebeer S, de Keersmaecker SCJ, Vanderleyden J, de Vos WM, Palva A. 2011. Functional characterization of a mucus-specific LPXTG surface adhesin from probiotic *Lactobacillus rhamnosus* GG. Appl Environ Microbiol 77:4465–4472.

248. Krausova G, Hyrslova I, Jakubec M, Hynstova I. 2015. *In vitro* evaluation of prebiotics on adherence of lactobacilli. J Microb Biochem Technol 08:450–452.

249. Rajan N, Cao Q, Anderson BE, Pruden DL, Sensibar J, Duncan JL, Schaeffer AJ. 1999. Roles of glycoproteins and oligosaccharides found in human vaginal fluid in bacterial adherence. Infect Immun 67:5027–5032.

250. Rabinovich GA, Cumashi A, Bianco GA, Ciavardelli D, Iurisci I, D'Egidio M, Piccolo E, Tinari N, Nifantiev N, Iacobelli S. 2006. Synthetic lactulose amines: novel class of anticancer agents that induce tumor-cell apoptosis and inhibit galectin-mediated homotypic cell aggregation and endothelial cell morphogenesis. Glycobiology 16:210–220.

251. Fichorova RN, Rheinwald JG, Anderson DJ. 1997. Generation of papillomavirus-immortalized cell lines from normal human ectocervical, endocervical, and vaginal epithelium that maintain expression of tissue-specific differentiation proteins. Biol Reprod 57:847–855.

252. Gelber SE, Aguilar JL, Lewis KLT, Ratner AJ. 2008. Functional and phylogenetic characterization of Vaginolysin, the human-specific cytolysin from *Gardnerella vaginalis*. J Bacteriol 190:3896–3903.

253. Randis TM, Zaklama J, LaRocca TJ, Los FCO, Lewis EL, Desai P, Rampersaud R, Amaral FE, Ratner AJ. 2013. Vaginolysin drives epithelial ultrastructural responses to *Gardnerella vaginalis*. Infect Immun 81:4544–4550.

254. Wilson WA, Roach PJ, Montero M, Baroja-Fernández E, Muñoz FJ, Eydallin G, Viale AM, Pozueta-Romero J. 2010. Regulation of glycogen metabolism in yeast and bacteria. FEMS Microbiol Rev 34:952–985.

255. Alonso-Casajús N, Dauvillée D, Viale AM, Muñoz FJ, Baroja-Fernández E, Morán-Zorzano MT, Eydallin G, Ball S, Pozueta-Romero J. 2006. Glycogen phosphorylase, the product of the glgP gene, catalyzes glycogen breakdown by removing glucose units from the nonreducing ends in *Escherichia coli*. J Bacteriol 188:5266–5272.

256. Jeanningros R, Creuzet-Sigal N, Frixon C, Cattaneo J. 1976. Purification and properties of a debranching enzyme from *Escherichia coli*. Biochim Biophys Acta 438:186–199.

257. Rogers D, Yu SH. 1962. Substrate specificity of a glucose permease of *Escherichia coli*. J Bacteriol 84:877–881.

258. Szmelcman S, Schwartz M, Silhavy TJ, Boos W. 1976. Maltose transport in *Escherichia coli* K12. Eur J Biochem 65:13–19.

259. Wylie JG, Henderson A. 1969. Identity and glycogen-fermenting ability of lactobacilli isolated from the vagina of pregnant women. J Med Microbiol 2:363–366.

260. Spiegel CA, Amsel R, Eschenbach D, Schoenknecht F, Holmes KK. 1980. Anaerobic bacteria in nonspecific vaginitis. N Engl J Med 303:601–607.

261. Yeoman CJ, Yildirim S, Thomas SM, Durkin AS, Torralba M, Sutton G, Buhay CJ, Ding Y, Dugan-Rocha SP, Muzny DM, Qin X, Gibbs RA, Leigh SR, Stumpf R, White BA, Highlander SK, Nelson KE, Wilson BA. 2010. Comparative genomics of *Gardnerella vaginalis* strains reveals substantial differences in metabolic and virulence potential. PLoS One 5.

262. Safwat MS, Mahmoud SA, Attia RM, Abd-El-Nasser M, Ali FS. 1983. Bacterial alpha-amylase inhibitors. Zentralbl Mikrobiol 138:145–150.

263. Balkus JE, Mitchell C, Agnew K, Liu C, Fiedler T, Cohn SE, Luque A, Coombs R, Fredricks DN, Hitti J. 2012. Detection of hydrogen peroxide-producing *Lactobacillus* species in the vagina: a comparison of culture and quantitative PCR among HIV-1 seropositive women. BMC Infect Dis 12:188.

264. Wilks M, Wiggins R, Whiley A, Hennessy E, Warwick S, Porter H, Corfield A, Millar M. 2004. Identification and H2O2 production of vaginal lactobacilli from pregnant women at high risk of preterm birth and relation with outcome. J Clin Microbiol 42:713–717.

265. Rampersaud R, Planet PJ, Randis TM, Kulkarni R, Aguilar JL, Lehrer RI, Ratner AJ. 2011. Inerolysin, a cholesterol-dependent cytolysin produced by *Lactobacillus iners*. J Bacteriol 193:1034–1041.

266. Brotman RM, Ravel J, Cone RA, Zenilman JM. 2010. Rapid fluctuation of the vaginal microbiota measured by Gram stain analysis. Sex Transm Infect 86:297–302.

267. Thoma ME, Gray RH, Kiwanuka N, Aluma S, Wang M-C, Sewankambo N, Wawer MJ. 2011. The short-term variability of bacterial vaginosis diagnosed by Nugent Gram stain criteria among sexually active women in Rakai, Uganda. Sex Transm Dis 38:111–116.

268. Witkin SS, Mendes-Soares H, Linhares IM, Jayaram A, Ledger WJ, Forney LJ. 2013. Influence of vaginal bacteria and D- and L-lactic acid isomers on vaginal extracellular matrix metalloproteinase inducer: implications for protection against upper genital tract infections. MBio 4.

269. Debruères J, Sedallian A. 1985. [Isolation and identification of *Gardnerella vaginalis*]. Pathol Biol (Paris) 33:687–692.

270. Ali FS, Abdel-Moneim AA. 1989. Effect of chemicals on fungal alpha-amylase activity. Zentralbl Mikrobiol 144:623–628.

271. Huffman RD, Nawrocki LD, Wilson WA, Brittingham A. 2015. Digestion of glycogen by a glucosidase released by *Trichomonas vaginalis*. Exp Parasitol 159:151–159.

272. Marín S, Albareda X, Ramos AJ, Sanchis V. 2001. Impact of environment and interactions of *Fusarium verticillioides* and *Fusarium proliferatum* with *Aspergillus parasiticus* on fumonisin B1 and aflatoxins on maize grain. J Sci Food Agric 81:1060–1068.

273. Thompson C, Henke SE. 2000. Effect of climate and type of storage container on aflatoxin production in corn and its associated risks to wildlife species. J Wildl Dis 36:172–179.

274. Wagacha JM, Muthomi JW. 2008. Mycotoxin problem in Africa: Current status, implications to food safety and health and possible management strategies.pdf. Int J Food Microbiol 124:1–12.

275. Shephard GS. 2008. Impact of mycotoxins on human health in developing countries. Food Addit Contam 25:146–151.

276. Wilson DM, King JK. 1995. Production of aflatoxins B1, B2, G1, and G2 in pure and mixed cultures of *Aspergillus parasiticus* and *Aspergillus flavus*. Food Addit Contam 12:521–525.

277. Goto T, Wicklow DT, Ito Y. 1996. Aflatoxin and cyclopiazonic acid production by a sclerotium-producing *Aspergillus tamarii* strain. Appl Environ Microbiol 62:4036–4038.

278. Ross RK, Yu MC, Henderson BE, Yuan J-M, Qian G-S, Tu J-T, Gao Y-T, Wogan GN, Groopman JD. 1992. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. The Lancet 339:943–946.

279. Johnson WW, Guengerich FP. 1997. Reaction of aflatoxin B1 exo-8,9-epoxide with DNA: Kinetic analysis of covalent binding and DNA-induced hydrolysis. Proc Natl Acad Sci U S A 94:6121–6125.

280. Ramsdell HS, Parkinson A, Eddy AC, Eaton DL. 1991. Bioactivation of aflatoxin B1 by human liver microsomes: role of cytochrome P450 IIIA enzymes. Toxicol Appl Pharmacol 108:436–447.

281. Sitas F, Parkin M, Chirenje Z, Stein L, Mqoqi N, Wabinga H. 2006. Cancers, p. . *In* Jamison, DT, Feachem, RG, Makgoba, MW, Bos, ER, Baingana, FK, Hofman, KJ, Rogo, KO (eds.), Disease and Mortality in Sub-Saharan Africa, 2nd ed. World Bank, Washington (DC).

282. Newton R, Ngilimana PJ, Grulich A, Beral V, Sindikubwabo B, Nganyira A, Parkin DM. 1996. Cancer in Rwanda. Int J Cancer 66:75–81.

283. Krishnamachari KA, Bhat RV, Nagarajan V, Tilak TB. 1975. Hepatitis due to aflatoxicosis. An outbreak in Western India. Lancet Lond Engl 1:1061–1063.

284. Azziz-Baumgartner E, Lindblade K, Gieseker K, Rogers HS, Kieszak S, Njapau H, Schleicher R, McCoy LF, Misore A, DeCock K, Rubin C, Slutsker L. 2005. Case–control study of an acute aflatoxicosis outbreak, Kenya, 2004. Environ Health Perspect 113:1779–1783.

285. Bankole S, Schollenberger M, Drochner W. 2006. Mycotoxins in food systems in Sub Saharan Africa: A review. Mycotoxin Res 22:163–169.

286. Hueza IM, Raspantini PCF, Raspantini LER, Latorre AO, Górniak SL. 2014. Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound. Toxins 6:1080–1095.

287. Zinedine A, Soriano JM, Moltó JC, Mañes J. 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. Food Chem Toxicol 45:1–18.

288. Kristensen P, Andersen A, Irgens LM. 2000. Hormone-dependent cancer and adverse reproductive outcomes in farmers' families — effects of climatic conditions favoring fungal growth in grain. Scand J Work Environ Health 26:331–337.

289. Warth B, Braun D, Ezekiel CN, Turner PC, Degen GH, Marko D. 2016. Biomonitoring of mycotoxins in human breast milk: Current state and future perspectives. Chem Res Toxicol 29:1087–1097.

290. Chen G, Gong YY, Kimanya ME, Shirima CP, Routledge MN. 2017. Comparison of urinary aflatoxin M1 and aflatoxin albumin adducts as biomarkers for assessing aflatoxin exposure in Tanzanian children. Biomarkers 0:1–6.

291. Routledge MN, Kimanya ME, Shirima CP, Wild CP, Gong YY. 2014. Quantitative correlation of aflatoxin biomarker with dietary intake of aflatoxin in Tanzanian children. Biomark Biochem Indic Expo Response Susceptibility Chem 19:430–435.

292. Oda Y, Huang K, Cross FR, Cowburn D, Chait BT. 1999. Accurate quantitation of protein expression and site-specific phosphorylation. Proc Natl Acad Sci U S A 96:6591–6596.

293. Taylor PJ. 2005. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography–electrospray–tandem mass spectrometry. Clin Biochem 38:328–334.

294. Schuhmacher J, Zimmer D, Tesche F, Pickard V. 2003. Matrix effects during analysis of plasma samples by electrospray and atmospheric pressure chemical ionization mass spectrometry: practical approaches to their elimination. Rapid Commun Mass Spectrom RCM 17:1950–1957.

295. Schlittenbauer L, Seiwert B, Reemtsma T. 2015. Matrix effects in human urine analysis using multi-targeted liquid chromatography-tandem mass spectrometry. J Chromatogr A 1415:91–99.

296. Waikar SS, Sabbisetti VS, Bonventre JV. 2010. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int 78:486–494.

297. Mitchell NJ, Kumi J, Johnson NM, Dotse E, Marroquin-Cardona A, Wang J-S, Jolly PE, Ankrah N-A, Phillips TD. 2013. Reduction in the urinary aflatoxin M1

biomarker as an early indicator of the efficacy of dietary interventions to reduce exposure to aflatoxins. Biomarkers 18:391–398.

298. Obuseh FA, Jolly PE, Jiang Y, Shuaib FMB, Waterbor J, Ellis WO, Piyathilake CJ, Desmond RA, Afriyie-Gyawu E, Phillips TD. 2010. Aflatoxin B1 albumin adducts in plasma and aflatoxin M1 in urine are associated with plasma concentrations of vitamins A and E. Int J Vitam Nutr Res Int Z Vitam- Ernahrungsforschung J Int Vitaminol Nutr 80:355.

299. Awuor AO, Yard E, Daniel JH, Martin C, Bii C, Romoser A, Oyugi E, Elmore S, Amwayi S, Vulule J, Zitomer NC, Rybak ME, Phillips TD, Montgomery JM, Lewis LS. 2017. Evaluation of the efficacy, acceptability and palatability of calcium montmorillonite clay used to reduce aflatoxin B1 dietary exposure in a crossover study in Kenya. Food Addit Contam Part Chem Anal Control Expo Risk Assess 34:93–102.

300. Gerding J, Cramer B, Humpf H-U. 2014. Determination of mycotoxin exposure in Germany using an LC-MS/MS multibiomarker approach. Mol Nutr Food Res 58:2358–2368.

301. Solfrizzo M, Gambacorta L, Visconti A. 2014. Assessment of multi-mycotoxin exposure in southern Italy by urinary multi-biomarker determination. Toxins 6:523–538.

302. Food and Agriculture Organization of the United Nations Statistics Database. 2014. Crop Production. FAOSTAT.

303. Butte NF, Wong WW, Treuth MS, Ellis KJ, Smith EO. 2004. Energy requirements during pregnancy based on total energy expenditure and energy deposition. Am J Clin Nutr 79:1078–1087.

304. Piers LS, Diggavi SN, Thangam S, van Raaij JM, Shetty PS, Hautvast JG. 1995. Changes in energy expenditure, anthropometry, and energy intake during the course of pregnancy and lactation in well-nourished Indian women. Am J Clin Nutr 61:501–513.

305. Voss KA, Plattner RD, Riley RT, Meredith FI, Norred WP. 1998. In vivo effects of fumonisin B1-producing and fumonisin B1-nonproducing Fusarium moniliforme isolates are similar: fumonisins B2 and B3 cause hepato- and nephrotoxicity in rats. Mycopathologia 141:45–58.

306. Shier WT, Abbas HK, Mirocha CJ. 1981. Toxicity of the mycotoxins fumonisins B1 and B2 and *Alternaria alternata* f. sp. *lycopersici* toxin (AAL) in cultured mammalian cells. Mycopathologia 116:97–104.

307. Mislivec PB, Bruce VR, Gibson R. 1983. Incidence of toxigenic and other molds in green coffee beans. J Food Prot 46:969–973.

308. Stanifer JW, Jing B, Tolan S, Helmke N, Mukerjee R, Naicker S, Patel U. 2014. The epidemiology of chronic kidney disease in sub-Saharan Africa: a systematic review and meta-analysis. Lancet Glob Health 2:e174–e181.

309. Yang W-C, Hsu T-C, Cheng K-C, Liu J-R. 2017. Expression of the *Clonostachys rosea* lactonohydrolase gene by *Lactobacillus reuteri* to increase its zearalenone-removing ability. Microb Cell Factories 16:69.

310. Lee A, Cheng K-C, Liu J-R. 2017. Isolation and characterization of a *Bacillus amyloliquefaciens* strain with zearalenone removal ability and its probiotic potential. PLoS One 12:e0182220.

311. Northolt MD, Bullerman LB. 1982. Prevention of mold growth and toxin production through control of environmental conditions. J Food Prot 45:519–526.

312. McMillan A, Renaud JB, Burgess KMN, Orimadegun AE, Akinyinka OO, Allen SJ, Miller JD, Reid G, Sumarah MW. Aflatoxin exposure in Nigerian children with severe acute malnutrition. Food Chem Toxicol Ahead of print.

313. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, Brown M, Knowles JD, Halsall A, Haselden JN, Nicholls AW, Wilson ID, Kell DB, Goodacre R, Human Serum Metabolome (HUSERMET) Consortium. 2011. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. Nat Protoc 6:1060–1083.

314. McCoy LF, Scholl PF, Schleicher RL, Groopman JD, Powers CD, Pfeiffer CM. 2005. Analysis of aflatoxin B_1-lysine adduct in serum using isotope-dilution liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 19:2203–2210.

315. Hill PG, Wells TN. 1983. Bromocresol purple and the measurement of albumin. Falsely high plasma albumin concentrations eliminated by increased reagent ionic strength. Ann Clin Biochem 20 (Pt 5):264–270.

316. Rolker HB, Maldonado A, Soto-Méndez M-J, Melse-Boonstra AB, Solomons NW. 2017. Comparative evaluation of the performance of urinary biomarkers of hydration status among lactating women in the Western Highlands of Guatemala. FASEB J 31:650.2-650.2.

317. Armstrong LE, Maresh CM, Castellani JW, Bergeron MF, Kenefick RW, LaGasse KE, Riebe D. 1994. Urinary indices of hydration status. Int J Sport Nutr 4:265–279.

318. DiGiulio DB, Callahan BJ, McMurdie PJ, Costello EK, Lyell DJ, Robaczewska A, Sun CL, Goltsman DSA, Wong RJ, Shaw G, Stevenson DK, Holmes SP, Relman DA. 2015. Temporal and spatial variation of the human microbiota during pregnancy. Proc Natl Acad Sci U S A 112:11060–11065.

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

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Amendment Approval Notice

Principal Investigator: Dr. Gregor Reid
Department & Institution: Schulich School of Medicine and Dentistry\Microbiology & Immunology, Western University

Review Type: Expedited
HSREB File Number: 106089
Study Title: Acquisition and analysis of vaginal swabs from healthy woman and those with bacterial vaginosis
Sponsor: Kimberly Clark Corp

HSREB Amendment Approval Date: June 15, 2016
HSREB Expiry Date: January 13, 2017

Documents Approved and/or Received for Information:

Document Name	Comments	Version Date
Revised Letter of Information & Consent	Revised ICF clean	2016/05/19
Revised Western University Protocol		2016/06/14

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 Guideline 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 IRB 00000940.

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer: Erika Basile __ Katelyn Harris __ Nicola Kaniki __ Grace Kelly __ Vikki Tran __ Karen Gopaul __

Western University, Research, Support Services Bldg., Rm. 5150
London, ON, Canada N6G 1G9 t. 519.661.3036 f. 519.850.2466 www.uwo.ca/research/ethics

Appendix A.2: University of Western Ontario HSREB approval for Rwanda study

Western

Research Ethics

Use of Human Participants - Ethics Approval Notice

Principal Investigator:Dr. Gregor Reid
File Number:102886
Review Level:Full Board
Approved Local Adult Participants:0
Approved Local Minor Participants:0
Protocol Title:Pilot study investigating bacteria (microbiota), their by-products (metabolome) 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

Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Western University Protocol		
Letter of Information & Consent	Sample Collection Group Only	
Letter of Information & Consent	Probiotic Trial Group	

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/ICH 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 REB's as defined in Division 5 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.

Member 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 Serbices under the IRB registration number IRB 00000940.

Signature

Ethics Officer to Contact for Further Information

Janice Sutherland	Grace Kelly	Shentel Walcott
		

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

Western University, Support Services Bldg Rm. 5150 London, ON, Canada N6A 3K7
t. 519.661.3036 f. 519.850.2466 www.uwo.ca/research/ethics

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

CENTRE HOSPITALIER UNIVERSTAIRE

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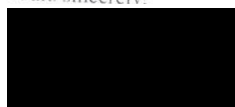
Dr George NTAKIYIRUTA.
The Vice President, Ethics Committee,
Kigali University Teaching Hospital

<<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 Tél. Fax : 00 (250) 576638E-mail : chuk.hospital@chukigali.org

Curriculum Vitae

STEPHANIE LYN 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

1. Collins SL and Reid G. 2016. Distant site effects of ingested prebiotics. Nutrients 8:e523.

2. Collins SL, McMillan A, Seney S, van der Veer C, Kort R, Sumarah MW, and Reid G. 2018. Promising prebiotic candidate established by evaluation of lactitol, lactulose, raffinose, and oligofructose for maintenance of a *Lactobacillus*-dominated vaginal microbiota. Appl Environ Microbiol 84.

3. Daisley B, Trinder M, McDowell T, Collins SL, Sumarah M, and Reid G. Microbiota-mediated modulation of organophosphate insecticide toxicity by species-dependent lactobacilli interactions in a *Drosophila melanogaster* insect model. *Submitted to Appl Environ Microbiol.*

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

1. <u>Collins SL</u>. *Oral presentation.* Metabolomics for pro- and pre-biotics research. ISAPP SFA meeting, Chicago, USA. June 2017.

2. <u>Collins SL</u>, McMillan A. Seney S, Sumarah M, and Reid G. *Poster presentation.* Lactulose stimulates commensal vaginal lactobacilli and an acidic pH to improve microbial homeostasis. ISAPP SFA meeting, Chicago, USA. June 2017.

3. <u>Collins SL</u>. *Oral presentation.* A student's perspective on the future. Lawson Executive Retreat, London, CA. May 2017.

4. <u>Collins SL</u>, McMillan A, and Reid G. *Oral presentation.* Multi-mycotoxin biomarker detection in a cohort of reproductive-age Rwandan women: an aflatoxin perspective. Winter Blues Workshop, London, CA. March 2017.

5. <u>Collins SL</u>, McMillan A, Seney S, Sumarah M, and Reid G. *Oral presentation.* Lactulose is fermented by commensal microbes to maintain vaginal health. Infection and Immunity Research Forum, London, CA. September 2016.

6. <u>Collins SL</u>, McMillan A, Seney S, Sumarah M, and Reid G. *Poster presentation.* Lactulose, a gut prebiotic, can maintain vaginal symbiosis. ISAPP SFA meeting, Turku, FI. June 2016.

7. <u>Collins SL</u>, Sumarah M, and Reid G. *Poster presentation*. Evaluating the efficacy of prebiotics to maintain homeostasis in the vaginal microbiome. Infection and Immunity Research Forum, London, CA. November 2015.

8. <u>Collins SL</u>, Seney S, and Reid G. *Poster presentation.* The effect of prebiotics on vaginal microbes. Biochemistry Research Showcase, London, CA. January 2015.

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