Developing and Applying Microdroplet Co-Cultivation Technology for Elucidating Bacterial Interspecies Interactions in the Human Vaginal Microbiome

by

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Contents

Acknowledgments **ii**

List of Figures **xiv**

List of Tables **xvii**

Abstract **xviii**

Chapter

1 Background and Motivation **1**

1.1 The human vaginal microbiome composition 1

1.2 Polymicrobial disorders related to the human vaginal microbiome . . 6

1.3 Microbial competition in the HVM 10

1.4 Microbe-host interactions in the human vaginal microbiome 13

1.5 Microdroplets: advantages and biological applications 15

1.6 Dissertation Overview: Developing and applying a microdroplet platform to elucidate the HVM 20

2 Extending a Technology Pipeline for Co-cultivating Bacteria and Characterizing Vaginal Bacterial Interactions in Microdroplets **25**

2.1 Summary 25

2.2 Introduction 26

2.2.1 Human health and disease 26
2.2.2 Employing microfluidics for biological applications 28
2.2.3 Overview 29
2.3 Results and discussion 31
2.3.1 Overall approach 31
2.3.2 Flask co-culture of *L. jensenii* and *G. vaginalis* 31
2.3.3 Encapsulation and co-cultivation of *L. jensenii* and *G. vaginalis* 33
2.3.4 qPCR on pooled microdroplets of *L. jensenii* and *G. vaginalis* 35
2.3.5 *G. vaginalis* and *L. jensenii* in isolated microdroplets 38
2.3.6 Comparison of assays 41
2.3.7 *E. faecalis* and *L. jensenii* in co-culture 42
2.4 Conclusion 44
2.5 Methods 45
2.5.1 Bacteria strains and cultivation protocols 45
2.5.2 Methods for flask culture experiments 45
2.5.3 Methods for fabrication and preparation of microfluidic devices 46
2.5.4 Methods for microdroplet generation, incubation, and isolation 47
2.5.5 Methods for droplet imaging 48
2.5.6 Methods for qPCR on pooled microdroplets 49

3 Investigating Interspecies Interactions Between *Lactobacillus* Species in Synthetic Laboratory Media 54
3.1 Summary 54
3.2 Introduction 56
3.2.1 Transitions in the HVM 56
3.3 Results and discussion 62
3.3.1 Overview 62

3.3.2 Effects of 2,2'-bipyridyl and Fe(II)SO_4 63
3.3.3 Competition experiments between *L. crispatus* and *L. iners* . 68
3.3.4 Spent-medium experiments between *L. crispatus* and *L. iners* 71
3.3.5 Competition experiments between *L. crispatus* and *L. gasseri* 76
3.3.6 Spent-medium experiments between *L. crispatus* and *L. gasseri* 79
3.4 Conclusion 82
3.5 Material and methods 83
3.5.1 Bacterial strains and cultivation protocols 83
3.5.2 Methods for determining the effects of 2,2'-bipyridyl and Fe(II)SO_4 at 0 and 24 hours 84
3.5.3 Methods for examining effects of 2,2'-bipyridyl and Fe(II)SO_4 on *L. iners* 85
3.5.4 Methods for culturing *L. iners* in *L. crispatus*-spent MNC broth 85
3.5.5 Methods for co-culturing *L. crispatus* and *L. iners* 86
3.5.6 Methods spent-medium experiments between *L. crispatus* and *L. iners* 87
3.5.7 Methods for co-culturing *L. crispatus* and *L. gasseri* 87
3.5.8 Methods for spent-medium experiments between *L. crispatus* and *L. gasseri* 88

4 Technology development for cultivating vaginal bacteria in micro-droplets using human cervicovaginal fluid 89

4.1 Summary 89
4.2 Introduction 90
4.2.1 Culture-media simulating cervicovaginal fluid 90
4.2.2 Vaginal metabolome and microbial composition 92

4.2.3 Human cervicovaginal fluid to cultivate lactobacilli in microdroplets . . . 94
4.3 Results and discussion . . . 96
4.3.1 Overview . . . 96
4.3.2 Collecting human vaginal bacteria and CVF . . . 97
4.3.3 Analyzing vaginal samples . . . 98
4.3.4 Creating pooled CVF . . . 99
4.3.5 Culturing in pooled CVF . . . 101
4.4 Conclusion . . . 105
4.5 Material and methods . . . 106
4.5.1 Bacterial strains and cultivation protocols . . . 106
4.5.2 Methods for recruiting donors . . . 106
4.5.3 Criteria for donors . . . 106
4.5.4 Methods for collecting vaginal samples . . . 107
4.5.5 Methods for processing Softdiscs™ . . . 108
4.5.6 Methods for separating CVF and CVM . . . 109
4.5.7 Methods for processing and submitting vaginal swabs for 16S rRNA gene sequencing . . . 109
4.5.8 Methods for analyzing 16S rRNA gene sequencing data . . . 110
4.5.9 Methods for pooling and characterizing pooled CVF . . . 114
4.5.10 Method for generating CVF-microdroplets with pure cultures 114
4.5.11 Methods for observing fluorescence of *L. crispatus* ATCC 33820 pT1-aFP . . . 114
4.5.12 Method for enumerating pure cultures from CVF-microdroplets 115

5 Concluding Remarks and Future Directions **117**

5.1 Concluding remarks . . . 117

5.2 Future directions 121
5.2.1 Iron requirements for *Lactobacillus iners* 121
5.2.2 Competition experiments in synthetic media 122
5.2.3 Optimizing chemically defined medium for vaginal bacteria . . 122
5.2.4 Cervicovaginal fluid microdroplets with vaginal epithelial cells 123
5.2.5 Comparing metabolomic profiles of *Lactobacillus crispatus* and *L. iners*-dominated communities from the human vagina . . . 124

References **125**

List of Figures

1.1 Bacterial populations of 394 non-pregnant women of reproductive age clustered by community state types (CSTs) I thru V 3

1.2 A schematic of the vaginal environment during alternative states of eubiosis. 12

1.3 Schematic of diagrams of planar microfluidic droplet generator 17

1.4 Schematic for soft lithography 19

2.1 Schematic of the overall approach used to study interspecies interactions of human vaginal bacteria in microdroplets 30

2.2 Fold change of *G. vaginalis* (left) and *L. jensenii* (right) in monoculture and co-culture after 17 hours of anaerobic cultivation in flasks . . 32

2.3 Anaerobic co-cultivation of *L. jensenii* and *G. vaginalis* in microdroplets. 34

2.4 The average number of cells per microdroplet observed, compared to that predicted from the inoculum density and Poisson distribution assumption 35

2.5 Standard curves of *L. jensenii* and *G. vaginalis* from approximately 1,000 pooled microdroplets after PCR 36

2.6 Estimated cell numbers of *G. vaginalis* (left) or *L. jensenii* (right) in monoculture vs. co-culture microdroplets after 12 hours of anaerobic incubation 37

2.7 Number of droplets isolated into an individual microplate well 38

2.8 Representative isolated microdroplets with *G. vaginalis, L. jensenii*, and both species in co-culture after 8 hours of anaerobic incubation . 40
2.9 CFUs of *G. vaginalis* (left) and *L. jensenii* (right) in individual microdroplets that were isolated after 8 hours of anaerobic cultivation . 40
2.10 Study of a second model system consisting of *E. faecalis* and *L. jensenii* 51
2.11 Representative isolated microdroplets with *E. faecalis, L. jensenii*, and both species in co-culture after 12 hours of anaerobic incubation . . . 52
2.12 *Enterococcus faecalis* and *Lactobacillus jensenii* plated onto an MRS agar plate. 53
3.1 Net change in OD after 24 hours of (a) *L. iners* and (b) *L. crispatus* in MNC 66
3.2 Effects of Fe(II)SO_4 and 2,2'-bipyridyl on *L. iners* 67
3.3 Serial dilution co-cultures and relative abundances of *L. crispatus* ATCC 33820 pT1-aFP and *L. iners*ATCC 55195 at different initial seed densities 72
3.4 Spent-medium experiments for *L. iners* in *L. iners* (LI)-spent MNC media, *L. iners* in *L. crispatus* (LC)-spent MNC media, *L. crispatus* in LI-spent MNC media, and *L. crispatus* in LC-spent MNC media . 74
3.5 Serial dilution co-cultures and relative abundances of *L. crispatus* ATCC 33820 pT1-aFP and *L. gasseri* JV-V03 at different initial seed densities 77
3.6 Spent-medium experiment for *L. gasseri* in *L. gasseri* (LG)-spent MRS media, *L. gasseri* in *L. crispatus* (LC)-spent MRS media, *L. crispatus* in LG-spent MRS media, and *L. crispatus* in LC-spent MNC media 81
4.1 Antimicrobial activity of vaginal fluid. 95

4.2 Relative abundances of different taxa in the vaginal microbiome of four donors 99
4.3 Absolute and relative abundances of reads from vaginal samples of donors. 100
4.4 *Lactobacillus crispatus* ATCC 33820/pT1-aFP in pooled *L. crispatus*-dominated CVS 102
4.5 Pure cultures of *Lactobacillus* species in pooled LC-CVS at 0 hours and 12 hours. 103
4.6 Colony forming units (CFUs) of *Lactobacillus* species enumerated on agar after being cultured in LC-CVS in microdroplets. 116
5.1 *L. jensenii* JV-V16, *G. vaginalis* ATCC 49145, and *L. iners* ATCC 55195 in medium simulating vaginal fluid (MSVF) with twenty amino acids 123

List of Tables

3.1 List of 15 strains of *L. crispatus* and 15 of *L. iners* 59

4.1 Comparison of the chemical compositions of vaginal fluid vs. recipes of chemically defined medium (CDM), vaginal fluid simulant (VFS), and medium-simulating vaginal fluid (MSVF) 91

Abstract

The role of the human vaginal microbiome (HVM) has gained increased recognition due to recent technological advancements that helped link community composition and women's health risks. However, the ecological roles of members of the HVM and microbe-microbe-host interactions remain unclear. Current approaches for investigating these mechanisms have been low-throughput, require large cultivation volumes and utilize chemically indistinct media diverging from the *in vivo* condition. Microdroplet-based co-cultivation is a new technology for overcoming these challenges by confining and performing sensitive assays at the nano-liter scale. This dissertation aims to: (i) develop new methods for elucidating interactions in the HVM through co-cultivation in microdroplets; (ii) test hypotheses that reduced iron limits the growth of *L. iners* and study interactions between lactobacilli in co-culture in laboratory media; and (iii) further extend the microdroplet technology for culturing vaginal bacteria in pooled cervicovaginal fluid (CVF).

First, we adapted and extended a microdroplet co-cultivation technology pipeline to investigate the HVM and tested it using two pairwise model systems. In one case, *Lactobacillus jensenii* JV-V16, a lactic-acid bacterium, and *Gardnerella vaginalis* ATCC 49145, a putative pathogen, were cultured in microdroplets as pure cultures and co-cultures. Then, qPCR was used to quantify the bacteria in pooled

microdroplets, and individual microdroplets were isolated and cells within each were plated on agar media. We demonstrated that *L. jensenii* inhibits *G. vaginalis* in microdroplets, which concurs with flask cultivation studies. We further demonstrated a second model system consisting of *L. jensenii* and another potential pathogen, *Enterococcus faecalis*. Our findings suggest that microdroplets can detect microbial interactions.

Second, we determined the effects of iron on the growth of the most common lactobacilli, *L. iners* and *L. crispatus*, and investigated pairwise interactions between lactobacilli using laboratory media. We measured the growth of *L. iners* and *L. crispatus* in spent-media supplemented with Fe(II)SO_4 or 2,2'-dipyridyl. Results show that higher concentrations of 2,2'-dipyridyl reduced the growth of *L. iners*, but not that of *L. crispatus*. We conducted serial dilutions on co-cultures of *L. crispatus* and *L. iners*, and *L. crispatus* and *L. gasseri*. As observed, one species became the most dominant in each co-culture. Spent-medium experiments indicated that no interference competition existed between these lactobacilli. Future investigation is needed to identify mechanisms for resource competition.

Third, we extended our technology to cultivate vaginal bacteria in microdroplets using CVF and investigated whether *L. crispatus, L. gasseri*, or *L. iners* could grow in pooled CVF. We analyzed 16S rRNA genes of 49 vaginal samples collected from healthy reproductive-age women. Of them, 16 were selectively pooled to create *L. crispatus* (LC)-dominated CVF. Using microdroplets, we subsequently confined and axenically cultured *L. crispatus*, *L. iners*, and *L. gasseri* in LC-CVF. We observed that *L. iners* grew in LC-CVF at pH 7 but was killed at pH 4. Our results indicate how vaginal pH may influence *L. iners* growth. *L. crispatus* survived and *L. gasseri*

decreased in viability in LC-CVF at pH 4.

In conclusion, this dissertation demonstrates methods for investigating interactions in the HVM through co-cultivation in microdroplets in a high-throughput manner. We have also shown the utilization of small volumes of human samples in cultivating vaginal bacteria while simulating the natural condition of the vagina. Further extension of this approach and its future applications hold tremendous potential for elucidating microbial interactions and how they impact human health.

Chapter 1

Background and Motivation

1.1 The human vaginal microbiome composition

The role of the human vaginal microbiome (HVM) in women's health has gained increased recognition due to the ability to characterize community composition and relate it to health risks. [1] For instance, bacterial vaginosis (BV), the most common polymicrobial disorder in reproductive-age women, is characterized by a depletion of lactobacilli and an overgrowth of strict anaerobes. [2–14] Consequently, women with BV may have an increased risk of acquiring sexually transmitted diseases and adverse pregnancy outcomes. [2, 5, 7, 9–11, 13, 14] An increased understanding has been attributed to recent technological advancements in culture-independent DNA sequencing. [15, 16] With the help of sequencing, four vaginal *Lactobacillus* species have been identified as common inhabitants of the vagina, three of which are associated with health. [17–20]

A high prevalence of vaginal *Lactobacillus* species have been associated with promoting health in the HVM in various ways. [17, 19, 21, 22] For instance, vaginal *Lactobacillus* species are known to decrease the vaginal pH to 4 by producing lactic acid, and this acidic pH is thought to preclude pathogenic microbes from colonizing

the vagina. [1,23] Most *Lactobacillus* species are also known to produce both D and L-lactic acid. [24] Other putative mechanisms of exclusion that *Lactobacillus* species may use against pathogenic microbes are the production of bacteriocins, adherence to vaginal epithelial cells (VECs) and others. [20,25] Bacteriocins are proteinaceous antimicrobial molecules that are produced by lactic acid bacteria and are known to have a killing effect against species with a similar ecological niche. [25] Some bacteriocin gene clusters have been putatively reported, but much information about their mechanisms remain unknown. [26,27] In the case of adherence, *L. crispatus* is speculated to adhere to VECs using S-layer proteins that form a paracrystaline structure on cell surfaces. [27] *Lactobacillus* species also promote health by slowing the transmission of HIV; Cervicovaginal mucus (CVM) that was predominated by *L. crispatus* has been reported to have an enhanced ability to trap the HIV-1 virions compared to CVM from women who were dominated by *L. iners*. [28]

The high phylogenetic resolution of culture-independent sequencing has aided in advancing our knowledge of the vaginal microbial composition worldwide. [1,29–32] Microbial composition has also been shown to differ among women. [1] In 2011, Ravel *et al.* reported a cross-sectional study comparing the microbial compositions of 394 reproductive-age women in the United States to their community state types (CSTs) as shown in Figure 1.1. [1] Overall, five CSTs were reported, namely, *Lactobacillus crispatus* (CST-I), *L. gasseri* (CST-II), *L. iners* (CST-III), *L. jensenii* (CST-V), and a group of facultative and strict anaerobes collectively known as the diversity group (CST-IV), also known as bacterial vaginosis-associated bacteria bacterial vaginosis-associated bacteria are composed of various facultative and strictly anaerobic bacteria. [1] Species in CST-IV include *Gardnerella, Mobiluncus, Atopobium, Prevotella, Finegoldia, Sneathia, Peptoniphilus, Megasphaera, Eggerthella, Corynebac-*

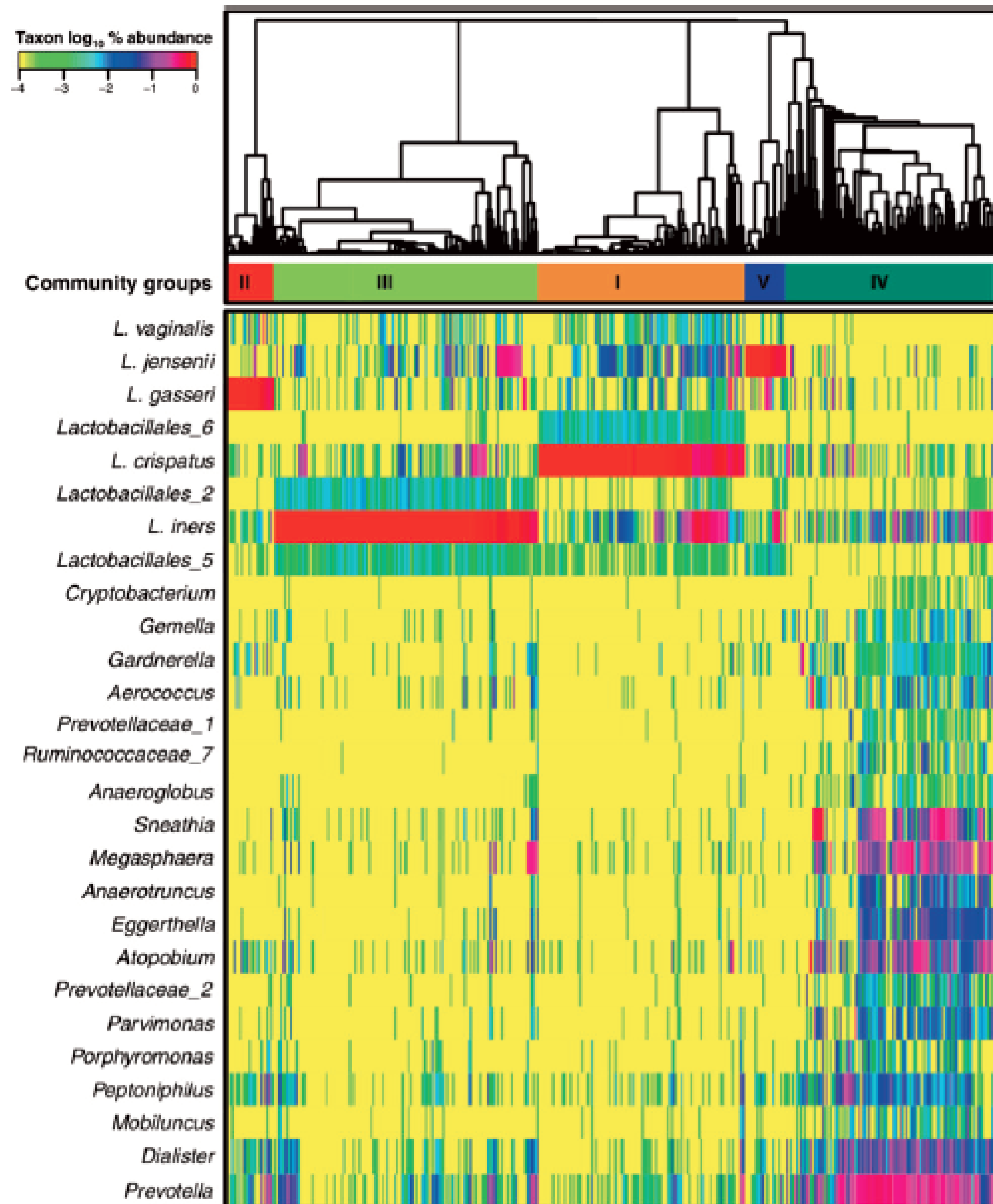


Figure 1.1: Bacterial populations of 394 non-pregnant women of reproductive age clustered by community state types (CSTs) I thru V. Bacterial populations were classified based on the V1-V2 hypervariable regions of the 16S rRNA gene. Heatmap shows relative abundance (see colored key) of bacterial taxa. Reproduced from [1].

terium, Aerococcus, and others. [1, 4, 33] Ravel *et al.* also detected commonalities between microbial composition and ethnicity, reporting a higher microbial diversity and vaginal pH in Hispanic and Black women compared with that of White and Asian women. [1] The implications of Ravel's study suggest that differences within and between women of different ethnicities call for a more refined way to categorize the kinds of microbial communities that are normally found in healthy women. [1] Ravel's study also acknowledges the need to appreciate differences between the microbial structures of individuals so that they can be considered during risk assessments and diagnostics.

Additional cross-sectional studies have assessed species composition of non-pregnant and presumably healthy women of reproductive age in Sweden, Nigeria, China, Japan, and the United States. [1, 29–32] In 2002, Vasquez *et al.* conducted a study on a cohort of 23 Swedish women and reported that subjects had a high prevalence of *L. crispatus, L. jensenii, L. gasseri*, and for the first time reported, *L. iners*. [29] In 2006, Anukam *et al.* reported that 64% of 241 vaginal swabs collected from Nigerian women in Benin City, were colonized mostly by *L. iners*. [30] In 2009, Shi *et al.* recruited five healthy Chinese women and stated they were mostly dominated by *L. crispatus* and *L. iners*. [31] In 2010, Zhou *et al.* recruited 73 Japanese women and similar to Vasquez *et al.*, also reported that their cohort was mostly colonized by *L. crispatus, L. jensenii, L. gasseri, and L. iners*. [29, 32] Thus, the unanimous detection of four *Lactobacillus* species in the HVM suggests that certain factors from the host may facilitate in the selection of specific *Lactobacillus* species that can adapt to the dynamic vaginal environment. [33, 34]

Longitudinal studies have shown that the vagina is a dynamic environment that

may facilitate changes in both community structure and composition. [34] In 2012, Gajer *et al.* conducted a study on a cohort of 32 women of reproductive-age that assessed the microbial composition of vaginal swabs that were collected biweekly for 16 weeks. As vaginal swabs were collected, subjects were tasked with reporting their days of menstruation and behaviors that included but were not limited to douching, oral and vaginal coitus. [34] Gajer *et al.* reported that some microbial communities appeared more stable than others and could change based on time in the menstrual cycle, community composition, and sometimes sexual activity. In 2012, Gajer *et al.* also observed two types of diversity groups; CST-IV-A and CST-IV-B. [34] Modest proportions of *Lactobacillus* species and lower proportions of anaerobic bacteria were characteristic of CST-IV-A. In contrast, higher amounts of strict and fastidious anaerobes were characteristic of CST-IV-B. CST-IV has been associated with BV due to its concomitance with BV and its ability to maintain a neutral vaginal pH, albeit, CST-IV has been detected in many healthy women. [34] In another study that included a cohort of 25 women with symptomatic BV (SBV), asymptomatic BV (ASB), and no BV, Ravel *et al.* reported highly personalized community dynamics in women with and without SBV and ABV. [33] Ravel *et al.* also observed some levels of consistency in the communities of women without BV, but they were not always stable and would fluctuate between *Lactobacillus* species and *Bifidobacterium*. [35,36] Sensibly, the role of bacterial vaginosis-associated bacteria surrounds a myriad of unanswered questions as to how these species maintain health and lead to diseases. [13, 37]

1.2 Polymicrobial disorders related to the human vaginal microbiome

Contrary to the vaginal microbiome of non-human primates that have low levels of lactobacilli and to the diverse microbiome of the human gut, microbial diversity in the HVM has been associated with vaginal dysbiosis. [12, 13, 35, 36, 38, 39] Vaginal dysbiosis is characterized by fewer *Lactobacillus* species and higher abundances of diverse bacteria. [40] Communities that are dominated by diverse bacteria or bacterial vaginosis-associated bacteria and are less resilient may encounter disturbances that can eventually lead to dysbiosis. [41] When dysbiosis occurs, the host is more subject to polymicrobial infections. [41] Polymicrobial infections are not caused by a single pathogen as explicated in the framework of Koch's postulates nor are they defined by the classical case of an exogenous microorganism that invades a community. Rather, the etiology of polymicrobial infections is concomitant with an increase in the abundance of a mixture of indigenous microorganisms that are typically rare. Such changes in the population can be due to modulations from the host such as a change in nutrients, or disturbances that change competitive dynamics. [40] Examples of polymicrobial infections in the HVM are bacterial vaginosis (BV) and aerobic vaginosis (AV). [9, 42]

BV is the most common disorder in women of reproductive age and is characterized by increased abundances of bacterial vaginosis-associated bacteria. [13, 40] Increased amounts of bacterial vaginosis-associated bacteria contribute to a neutral pH in the vagina. [40, 43] Other studies have drawn connections between bacterial vaginosis-associated bacteria and higher susceptibility for adverse pregnancy outcomes such as spontaneous abortion, preterm birth, as well as acquiring sexually transmitted

infections (STIs). [9,43] Goldenberg *et al.* reported more preterm births and spontaneous abortions occurred with increased microbial diversity in the vagina. [44] Callahan *et al.* reported an increased risk of preterm birth with more diverse bacteria concerning Caucasian and African-American women; however, weaknesses in their study included an inability to distinguish between progesterone treatment and prior history of preterm birth, and geographical socioeconomics were different for both cohort populations. [45] Nunn *et al.* also suggested that bacterial vaginosis-associated bacteria that dominate CVM might increase the risks of acquiring other STIs. [28] Consequently, not only do bacterial vaginosis-associated bacteria increase the risk of STIs, but if STIs are left untreated, they can progress to pelvic inflammatory disease (PID), which can result in infertility. [2]

Gardnerella vaginalis has been associated with BV since its discovery in 1955 due to its high prevalence in women with BV. [46] Gardner and Duke, who discovered *G. vaginalis*, first associated *Gardnerella vaginalis* as a causative agent of BV since its discovery, but *Gardnerella* and other bacterial vaginosis-associated bacteria have not been shown to cause BV. [46] Schwebke *et al.* developed a model that postulated *Gardnerella* was solely sexually transmitted between human beings. [47] Nonetheless, this claim was disputed by Hickey *et al.* who detected significant proportions of *Gardnerella* in adolescent females who were pre-menarche, and self-reported that they had not encountered coitus. [48] Moreover, *Gardnerella* has frequently been detected in adolescent premenarcheal girls, BV-negative women of reproductive age, and postmenopausal women. [1,49] *G. vaginalis* is also the only species that is classified under *Gardnerella* and other researchers have shown there to be variation between strains. [49–51] Hickey *et al.* clustered 35 strains of *Gardnerella vaginalis* into four different clades by using 16S rRNA sequences, with pairwise similarity rang-

ing from 98.7 to 100%. [49] Some strains of *Gardnerella* are more pathogenic than others due to their virulence factors. [14, 50, 52, 53] For instance, *Gardnerella* strains are known to produce biofilms, sialidase, and vaginolysin. [54] More work is needed to elucidate the role and function of bacteria and their communication with the host to understand the mechanisms of BV.

Another polymicrobial infection that is less common is aerobic vaginitis (AV), which affects the urogenital tract. [42] AV is diagnosed in patients when vaginal smears are deficient in lactobacilli, positive for cocci or coarse bacilli, positive for parabasal epithelial cells, and positive for vaginal leukocytes. [42] Symptoms of AV include red inflammation, yellow discharge, vaginal dysparaeunia, of which the latter is pain during sexual intercourse. [42] AV results in burning, itching, and irritation of the vulva area. AV is also associated with increased amounts of Escherichia coli, group B *Streptococci, Staphylococcus aureus, Enterococcus faecalis*, and *Trichomonas vaginalis*. [42]

Similar to BV, patients with AV have lactate concentrations that are substantially reduced. AV also initiates a higher immune response than BV, which results in the production of immune cells such as interleukin (IL)-6, IL-1β, and leukemia inhibitory factors in cervicovaginal fluid. [42, 53, 55] AV is associated with adverse pregnancy outcomes such as a ruptured membrane, preterm delivery, and ascending chorioamnionitis. AV is understudied and more research needs to be done to understand how species interact with each other and with the host.

Lactobacillus iners, one of the four vaginal *Lactobacillus* species, has many unique characteristics, which aid in more speculation about its role in the HVM. [53, 55]

"Iners", which translates to inert or lazy, has the smallest genome, ranging from 1.24 - 1.37 Mbp, which is within the range of symbionts and parasites. [56] Although it has the smallest genome, it is the most widely detected species in the HVM. [22,57] Unlike the three other *Lactobacillus* species, *Lactobacillus iners* only produces L-lactic acid as opposed to D-lactic acid. [24] A high prevalence of L. iners has been detected when the vaginal pH has increased, during periods of BV, menses, and during metrodonizale treatment, of which the latter is an antibiotic used to treat BV. [5,55,58,59] It is speculated that *L. iners* could be a transitional species for the HVM. [5,38,55,57]

Conversely, *L. iners* has characteristics that are similar to pathogens. *L. iners* produces inerolysin, a pore-forming cholesterol-dependent cytolysin (CDC) that has 68% gene similarity to vaginolysin, a CDC that is produced by *G. vaginalis.* [55,60] Inerolysin was likely acquired by horizontal gene transfer and is most active at 4.5 pH although it is active across a pH range of 4.5 to 6.0. [55] Inerolysin was thought by Macklaim *et al.* to have been produced by all strains of *L. iners*, but a recent study showed inerolysin production might be strain-dependent. [61] In addition, iron is an essential nutrient for bacterial pathogens that binds to hemoglobin, the latter of which are in red blood cells. [62,63] It is understood that iron is used for many biochemical processes including DNA replication, energy generation, oxygen transport, and protection against oxidative stress, [62,63] but most lactobacilli have been reported to use other metals such as cobalt and manganese instead. [64] While most lactobacilli grow on MRS agar, which contains manganese, *L. iners* only grew on MRS agar after the addition of 1-5% sheep and human blood. [55,56] *L. iners* was also reported to show alpha hemolysis on tryptic soy agar that was supplemented with 5% sheep blood but did not show alpha hemolysis with 5% or 10% human

blood. [55] The compound(s) in sheep and horse blood that have stimulated the growth of *L. iners* remain(s) unknown. However, we speculate that iron may be a contributing factor to the growth of *L. iners* because it is present in blood and serum, and is an essential nutrient for many microorganisms.

The unique characteristics of *L. iners* present some uncertainty as to its role and function and the way it interactions in the HVM. Research has been conducted to characterize microbial interactions, but further investigation is needed to further elucidate the underlying mechanisms of these interactions between members of the HVM community.

1.3 Microbial competition in the HVM

Interspecies interactions can be ecologically complex and categorized in several ways. [65] For instance, competition is a type of interaction that is generally categorized by biologists into two groups: resource competition and interference competition. Resource competition is when two or more species indirectly compete for one or more resources. [65] Interference competition is when one or both microbial populations release chemicals that have a toxic effect on another population. [65] Interference competition can also be divided into two groups: antagonism is when both species excrete substances that intoxicate each other; amensalism is when only one species excretes substances that intoxicate a population. [18, 23, 24, 66, 67] Examples of each kind of competition in relation to the HVM are described below.

Temporal fluctuations in the abundances and types of vaginal microbial community types are indicative of niche overlap and potentially resource competition. [68] Temporal studies have shown frequent transitions between *L. iners* and *L. crispa-*

tus-dominated communities, the two most dominant species in the HVM. [33, 34] Frequent transitions between both *L. crispatus* and *L. iners*-dominated may also suggest niche partitioning. [69] One potential mechanism for niche partitioning is conditional differentiation, when the growth of a population is influenced by the availability of abiotic or biotic factors. [68] Such factors may originate from the host, bacteria, or the environment. In 2016, France *et al.* compared the core and accessory genes of several strains of *L. iners* and *L. crispatus* and postulated that differences in their genomes could potentially facilitate partitioning of their shared niche space; [68] Partitioning of niche space has been reported to occur when microorganisms compete for resources. [69]

Lactobacilli may partake in resource competition when metabolizing sugars that have been derived from glycogen-degraded products. In the vagina, glycogen is stored in vaginal epithelial cells (VECs) until it is released, and is eventually degraded by alpha-amylase and metabolized by lactobacilli as shown in Figure 1.2. [67] Alpha-amylase has been reported to degrade glycogen into sugars like maltose, maltotriose, maltopentaose and alpha-limited dextrins maltodextrins. [70]

France *et al.* predicted that both *L. iners* and *L. crispatus* could metabolize glucose and maltose, giving evidence that some resources are shared between them. [68] Although both species may metabolize the same sugars, they likely metabolize them at different rates, which also influences competitive ability. [67, 70–73]

Conversely, different studies have demonstrated interference competition by measuring the killing effect of vaginal lactobacilli on bacterial vaginosis-associated bacteria through the production of lactic acid. [18, 23, 74] In 2011, O'Hanlon *et al.* demon-

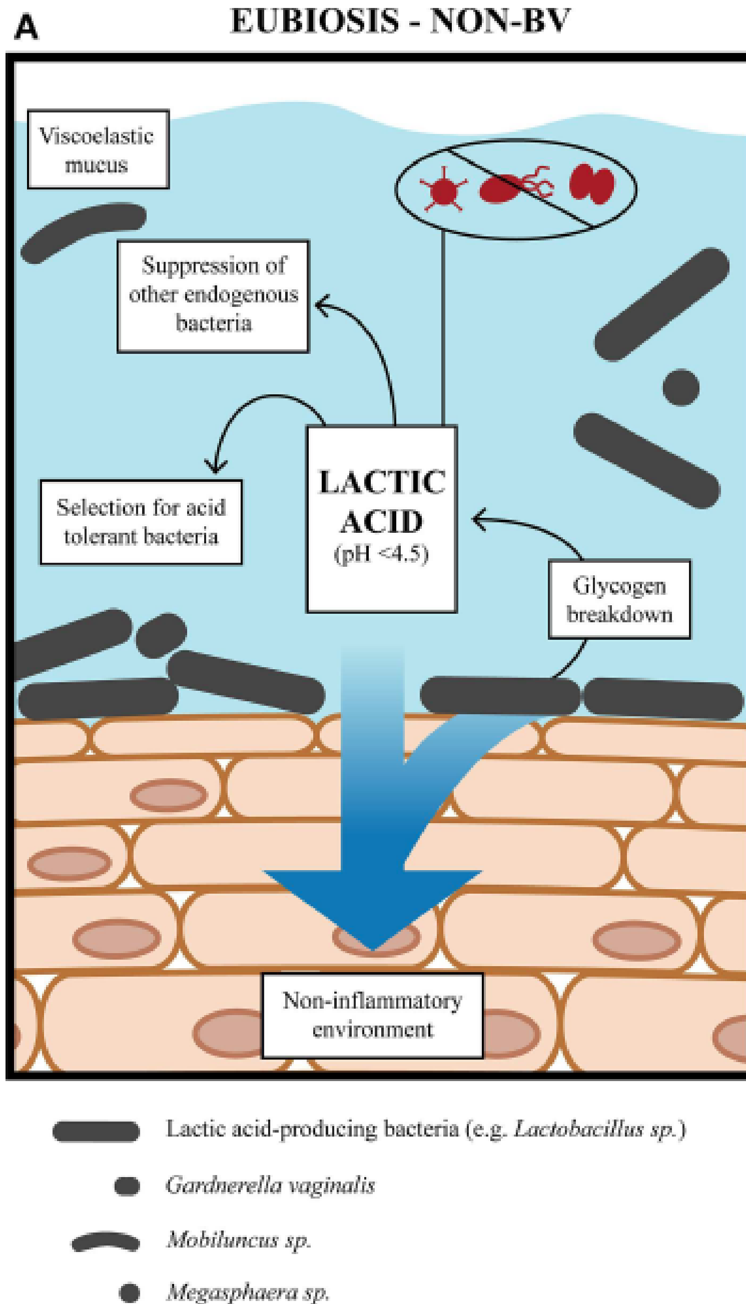


Figure 1.2: A schematic of the vaginal environment during alternative states of eubiosis. (a) During eubiosis, *Lactobacillus* species are producing lactic acid, metabolizing degraded products of glycogen, and endogenous bacteria have been suppressed. (b) During BV, short chain fatty acids (SCFA) are produced which are pro-inflammatory to vaginal epithelial cells (VECs), degraded products of glycogen are metabolized by endogenous bacteria into SCFA, amines produce a malodor, and the number of anaerobic bacteria is increased, mucin breaks down to form thin homogenous discharge. Reproduced from [67].

strated how lactic acid can kill 17 bacterial vaginosis-associated bacteria. [18, 74] In 2006, Atassi *et al.* showed how spent media from *Lactobacillus gasseri* KS 120.1 had a severe killing effect on *G. vaginalis* within one hour. [18] In 2015, Breshear *et al.* showed that *L. crispatus* inhibited *G. vaginalis* and Neisseria gonorrhea by using a porcine mucosa. [23] *Lactobacillus* species are also known to produce biosurfactants that can inhibit pathogens. [68,75–77] *L. iners* has not been shown to severely inhibit BV-associated bacteria. [58] A paper showed its inability to preclude colonization of *G. vaginalis* compared to *Lactobacillus reuteri* RC-14, a potential probiotic. [34, 58] It seems to be a commensal with such species as it is often found with other species from CST-IV-B. [34]

As we continue to invest interactions between vaginal bacteria, it is becoming increasingly evident that factors from the host are essential for truly elucidating microbial interactions in the HVM. Microbes depend on the host for nutrients, and a place to adhere. Such important considerations are essential for investigating interactions in the future.

1.4 Microbe-host interactions in the human vaginal microbiome

Influences from the host have major effects on microbial fitness, function, and their ability to interact with each other and with the host. [78, 79] For instance, by closely regulating and limiting the availability of free iron, the human body can reduce the chances of infection from pathogens, which require iron to replicate and maintain virulence. [63] Iron is an essential nutrient to many microorganisms, but not for all species of lactobacilli. [64] A study by France reported the presence of ABC

transporter genes in the core genome of *L. crispatus* that were unique to *L. iners* and may give *L. crispatus* a competitive advantage when sequestering iron. [19,55,60] As previously mentioned, *L. iners* has been shown to grow on MRS agar only after the addition of 1-5% sheep and human blood and in MRS broth that contains serum. [56, 80] Increased concentrations of iron during menses may select for a particular species. [55]

Vaginal samples from different hosts have been reported to vary largely due to differences in the immune system, microbial composition, and other factors from the individual hosts. [78] In 2002, Valore *et al.* demonstrated the wide range of antimicrobial activity of five donors against bacteria, most of which were considered exogenous to the HVM. [78] If the variation between host samples is not controlled, samples that were collected from different hosts can introduce biases and hinder the reproducibility of data.

Few studies have utilized host vaginal fluid or vaginal mucosa when investigating interactions between bacteria in the HVM. Of these studies, Breshears *et al.* used explants of a porcine vaginal mucosa to evaluate the inhibiting effects of *L. crispatus* on *G. vaginalis* and *Neisseria gonorrhoeae*. [23] O'Hanlon *et al.* incubated 17 bacterial vaginosis-associated bacteria in vaginal fluid with H_2O_2 and lactic acid. [74] O'Hanlon *et al.* found that just 1% vaginal fluid blocked the microbicidal activity of 1M H_2O_2 against bacterial vaginosis-associated bacteria, but were killed in 111 mM lactic acid with 10% lactic acid. [74]

Although studies have used porcine mucosa and vaginal fluid to emulate a vaginal environment, conventional cultivation techniques have severe limitations. Experi-

ments that use porcine mucosa have a limited number of replicates and vary based on the animals that were used to collect explants. [23, 78] When using vaginal fluid, each sample was diluted after it was collected, then lyophilized to return the volume and concentration to its approximate initial volume and concentration. After, each replicate contained a small volume of only 30 μL and may be difficult to reproduce as the results included five donors.

Conventional cultivation technique can reduce the reproducibility and throughput of experiments. Many questions remain regarding the influence of the host and its effect on the role, function, and interactions of ecological members of the HVM community. By conducting experiments that include factors from the host while employing technology to manipulate small volumes of fluid, we can greatly enhance our knowledge of how vaginal microbes interact in a natural setting.

1.5 Microdroplets: advantages and biological applications

As opposed to microwells, which can compartmentalize small volumes of fluid, microdroplets provide a distinct capability for stochastic confinement, screening, sorting, splitting, mixing, and reagent injection. [81] Exploring the advantageous capabilities of microdroplets can add additional advantages including co-cultivation. High-throughput microdroplet co-cultivation offers a promising new approach for detecting microbial interactions that occur in natural microbial communities such as the HVM.

Compared to conventional cultivation techniques and microarrays, microdroplet generation is cost-effective, time-efficient, and can generate approximately 1 - 1,000 monodisperse microdroplets per second. [82] Each microdroplet is a compartmentalized mini bioreactor that is suspended in a water-in-oil emulsion. Rectangular

microchannels in the device, as well as confinement, also modulate droplet breakup in a microfluidic device. [82, 83]

Microdroplets are formed by tangential stresses that are applied to the fluid where the distribution of normal forces maintains the spherical shape of the droplet. The formation of droplets takes place in rectangular microchannels that are driven by shear forces and surface tension, which can be scaled by the Capillary number (Ca) as shown in equation 1.1:

$$Ca = \frac{\eta U_0}{\gamma} \tag{1.1}$$

In this equation, η is the dynamic viscosity of the aqueous phase, U_o is the velocity of the aqueous phase, and γ is the interfacial tension. [82, 83] Low Ca numbers (Ca < 0.1) correspond to squeezing regimes; fluid interfaces become narrow, pinch off and the interfacial force dominates the viscous force. High Ca numbers (Ca > 0.1) correspond to the dripping regimes; Inner fluid is dispersed into small droplets, and the viscous force dominates the interfacial force. [82, 84] Each of the different droplet breakup regimes; "squeezing", "thread formation", "dripping", and "jetting", are shown in Figure 1.3. [82, 84]

Microdroplets can be formed in different planar dimensions such as coflow, cross-flow, and flow-focusing geometries. [85] Flow-focusing geometry is when a geometric element, such as an orifice, causes the streams to accelerate, thus narrowing the inner fluid thread. [86] Microdroplets are generated using laminar flow, which is characteristic of smooth fluid particles flowing in smooth paths in layers with little to no mixing and a low Reynolds number. Low-Reynolds number, low-capillary

number flows, and continuous phase flow rate influence breakup regime and droplet size.

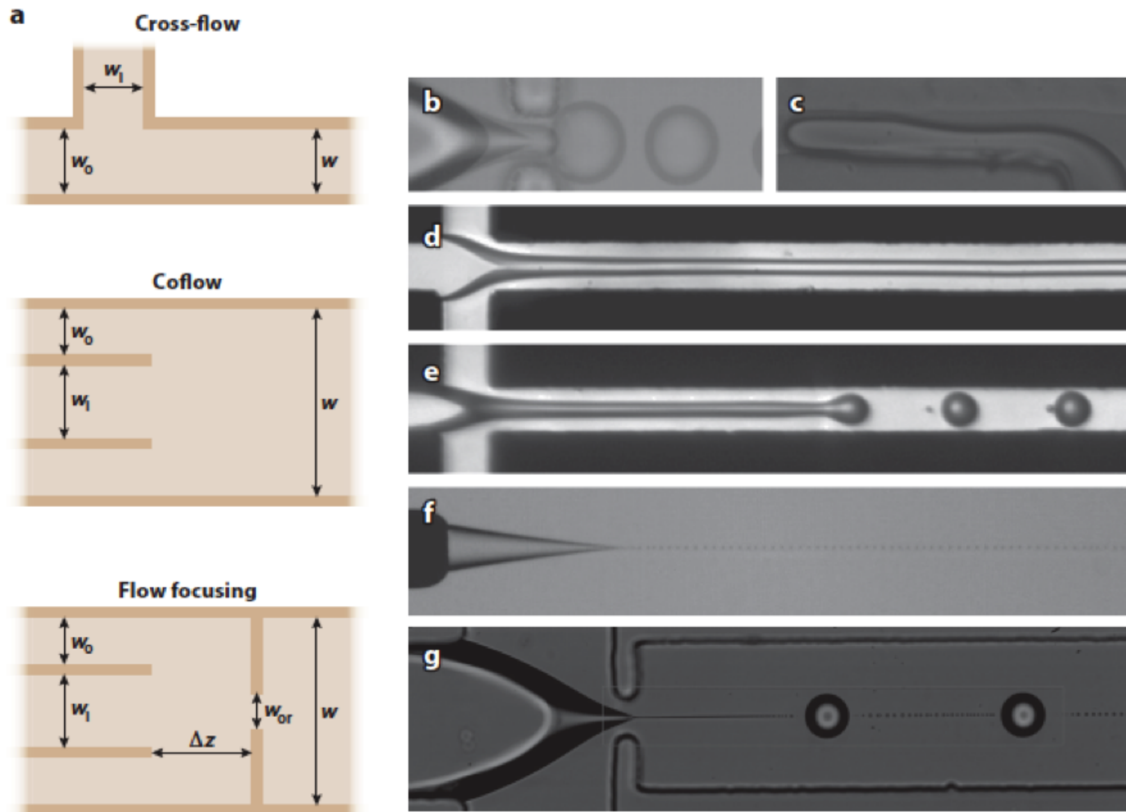


Figure 1.3: Schematic of diagrams of planar microfluidic droplet generator: (a) cross-flow, coflow, and flow focusing geometries.21 (b-g) show images of droplet-generation modes. (b) squeezing mode of droplet breakup in flow focusing geometry, (c) squeezing mode of droplet breakup in a cross-flow geometry, (d) jet formation in a confined flow-focusing geometry, (e) dripping mode of jet breakup in a flow-focusing geometry, (f) fine thread formation in coflow geometry, and (g) tip streaming in a flow-focusing geometry. Reproduced from [74, 87].

Microchannel geometries in generation devices are designed using the process of soft lithography. [87–89] Droplet generation devices can be fabricated from glass or poly(dimethylsiloxane) (PDMS). Albeit, glass devices have certain advantageous capabilities, but they are not elastomeric and not as readily reproducible as PDMS. Any particle of debris located between a layer of glass can easily prevent proper

bonding between the layers. PDMS has many advantageous properties such as being optically clear, chemically inert, non-toxic, elastomeric, and readily reproducible material, which would enable easy bonding between layers. [87–89] Steps for fabricating a droplet generation device are described in Figure 1.4. [87] High-throughput microdroplet co-cultivation offers a promising new approach for detecting microbial interactions that occur in natural microbial communities such as the HVM.

Microdroplets can be used for stochastic confinement of particles and cells. The Poisson distribution controls for the number of particles and cells in a droplet. [81, 87, 90] In addition, there are several advantages of microdroplets; they are high-throughput, high-parallel, handle small amounts of fluid, reduce required space and personnel, and reduce time and cost. [91]

Over the years, several studies have employed microdroplets to conduct high-throughput and highly-sensitive biological assays at a single-cell level. [91–95] In 2011, Park *et al.*, a former member of the Lin lab, developed and applied a microfluidic device to observe the effects of cross-feeding between two *E. coli* auxotrophs in microdroplets. [91] Park *et al.* encapsulated two *E. coli* auxotrophs in singleton microdroplets that did not grow after 18 hours, but in doubleton droplets, both auxotrophs grew after 18 hours of cultivation. This experiment demonstrated mutualism between the two *E. coli* auxotrophs. Another study by Liu *et al.* used microdroplets to identify rare, slow-growing microorganisms from environmental samples. [92] Guo *et al.* used droplets to build a library and screen small compounds to determine whether droplets were infected with virions, and conducted a droplet-based antibody screen. [93] Lam *et al.* demonstrated bacterial transformation between two strains of *Streptococcus pneumoniae* in femtoliter microdroplets. [95]

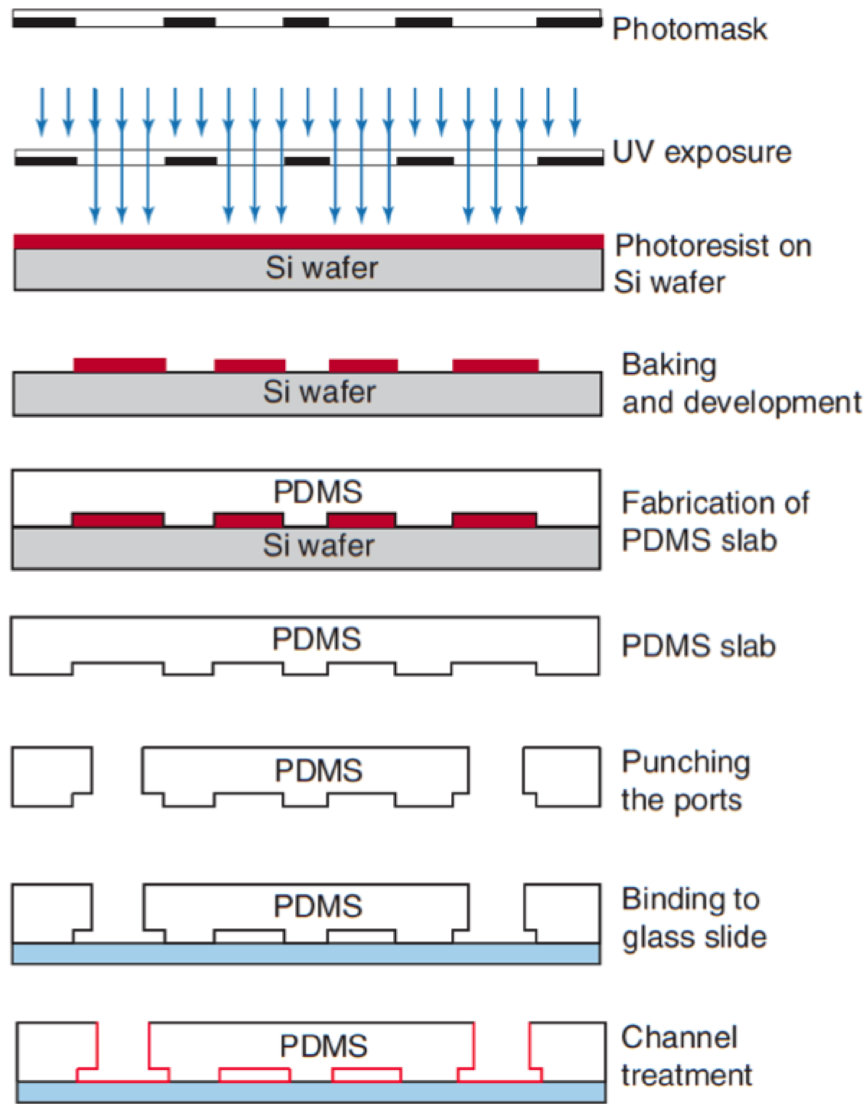


Figure 1.4: Schematic for soft lithography. First, a photomask undergoes UV exposure. Then, photoresist is added to Silicon (Si) wafer. The photoresist is developed onto the Si wafer by baking. Then, a PDMS slab is fabricated by poured PDMS onto the mold on the Si wafer. The PDMS slab is removed. Then holes can be punched into PDMS ports. PDMS can be bound to a glass or PDMS slide. The microchannels can be chemically treated to increase hydrophobicity in the channels. Reproduced from [68].

Additional members of the Lin lab have also employed microdroplets as a tool to increase throughput and assay sensitivity. Saleski *et al.* used fluorescent automated cell sorting (FACS) to screen microdroplets to demonstrate a Syntrophic Co-culture Amplification of Production phenotype (SnoCAP) screening framework between two co-cultured auxotrophic strains of E. coli to produce two target molecules; 2-ketoisovalerate and L-tryptophan. [96] Sida *et al.* used microdroplets to amplify gDNA from *E. coli* and *Pseudomonas putida* in microdroplets. [97] Carruthers et. al. showed positively interacting algal bi-culture that produced increased biomass and negatively interacting co-culture that produced less biomass. [98] In this dissertation, we aim to employ microdroplets as a new technique to elucidate microbial interactions in the HVM. [99] In doing so, we can increase throughput, reduce time and cost, increase the sensitivity of our assays, and manipulate small amounts of vaginal fluid that can be used to culture vaginal bacteria and implement a more natural environment.

1.6 Dissertation Overview: Developing and applying a microdroplet platform to elucidate the HVM

Scientific investigation of the HVM has become increasingly important due to recent advances in culture-independent sequencing, which have established a connection between community composition and health risks including the acquisition of BV or acquiring sexually transmitted infections like HIV. However, the roles and functions of different species and their influence on interactions mostly remain unclear. Current approaches for elucidating them have been low-throughput, require large culture volumes and utilize standardized and chemically indistinct media that is divergent from the *in vivo* condition. Microdroplet-based co-cultivation has emerged as a new tool that can stochastically confine cells and perform sensitive assays at the

nano-liter scale. Collectively, this dissertation aims to: (i) develop a new method to recapitulate interactions in microdroplets that have been previously reported; (ii) test the hypothesis that reduced iron can limit the growth of *L. iners* ATCC 55195, (iii) investigate whether interference and resource competition can be detected between *Lactobacillus* species in co-culture in standardized media; (iv) and implement a new cultivation media to culture three *Lactobacillus* species and determine which species can grow in pooled cervicovaginal fluid (CVF).

The objective of Chapter 2 is to show that microdroplets can be used to co-culture and characterize pairwise interactions between species from the HVM. In this model, we chose *L. jensenii* JV-V16 because it represents one of four *Lactobacillus* species, it is associated with health, it produces D-lactic acid, and previous reports have shown that several strains of *L. jensenii* have an inhibiting effect on *G. vaginalis.* [18] *G. vaginalis* ATCC 49145 was selected because it is associated with bacterial vaginosis. [52, 54] Based on previous studies that were conducted at the bench-scale, [18] we anticipate that *L. jensenii* would inhibit *G. vaginalis* in microdroplets. We have tested this hypothesis by comparing the growth in axenic cultures and co-cultures by enumerating colonies that grew from cultures in flasks and also from cultures in individual microdroplets. We analyzed pooled droplets using qPCR to quantify the estimated number of cells based on DNA content. We have demonstrated that *L. jensenii* inhibits *G. vaginalis* in pooled microdroplets and cultures grown in flasks. We have also found that *G. vaginalis* inhibits *L. jensenii* in pooled microdroplets, but we acknowledge the high level of sensitivity in qPCR and the overall differences between this method and enumerating colonies on agar medium. To further demonstrate the general applicability of our microdroplet co-cultivation platform, we co-cultured *L. jensenii* and *Enterococcus faecalis* ATCC 19433 in microdroplets

and characterized their interactions in individual microdroplets. Amensalism was detected between these two species as *L. jensenii* inhibited *E. faecalis* in individual microdroplets and cultures grown in flasks.

After demonstrating how microdroplets could be applied to help elucidate microbial interactions in the HVM, we wanted to investigate the effects of host cervicovaginal fluid (CVF) on microbial growth with further implications for conducting competition experiments in CVF. Although the literature reports the inhibiting effects of vaginal lactobacilli on bacterial vaginosis-associated bacteria, [18, 23] there exists little to no empirical data on the competitive interactions between lactobacilli. Competitive interactions between lactobacilli were of particular interest because recent work by Ravel *et al.* [1,33], and Gajer *et al.* [34] reported that *L. iners* and *L. crispatus* were the most widely detected species in the HVM. Their work also showed that species often transition from one community state to another, which gives reason to speculate that competition may be occurring. France *et al.* compared the genomes of several strains of *L. iners* and *L. crispatus* and hypothesized that differences in their genomes may facilitate niche partitioning between these species. [68] Another interesting observation from France *et al.* was the presence of ABC transporters in the core genome of *L. crispatus*, which may provide *L. crispatus* with a competitive ability for acquiring iron. [68] However, these ABC transporters were absent from the core genome of *L. iners*. [68] *L. iners* has been reported to only grow on MRS agar after the addition of 1-5% sheep or horse blood, which contains iron and other compounds. [55] The specific compounds in the sheep or horse blood that stimulated the growth of *L. iners* are unknown. However, we hypothesized that the limited availability of iron would reduce the growth of *L. iners*.

In Chapter 3, we evaluate the effect of the availability of iron on the growth of *L. iners* and *L. crispatus* and determine whether interference or resource competition can be detected between pairwise co-cultures of vaginal *Lactobacillus* species. We also measured the growth of *L. iners* and *L. crispatus* in fresh and spent media that was supplemented with Fe(II)SO_4 and 2,2'-dipyridyl, an iron chelator, and measured with a microplate photometer. We have found that increasing concentrations of 2,2'-bipyridyl can reduce the growth of *L. iners*, which concurs with our hypothesis that reduced availability of iron can hamper the growth of *L. iners*. Two pairwise competition experiments were conducted between *L. iners* ATCC 55195 and *L. crispatus* ATCC 33820 pT1-aFP using MNC broth and also between *L. crispatus* and *L. gasseri* JV-V03 using MRS broth. We conducted serial-dilution co-culture experiments and spent-medium experiments for both pairwise model systems. After the serial-dilution co-culture experiment between *L. crispatus* and *L. iners*, we report that *L crispatus* consistently dominated the co-culture, which we speculate is due to the faster growth rate of *L. crispatus* compared to that of *L. iners*. The differences in the microbial composition between *L. crispatus* and *L. gasseri* in co-culture were less distinct, but both populations showed uninhibited growth. From our experiments, we have no evidence for interference competition between either model systems. The associated mechanisms for resource competition were undetected for both pairs and further investigation is required to understand their interactions.

To further employ microdroplets to confine vaginal bacteria into small volumes of CVF, we collected 49 vaginal samples from healthy women of reproductive age. These samples included a Softdisc™ for the collection of CVF, and included vaginal swabs used to collect bacteria that were submitted for 16S rRNA gene sequencing. After analyzing 49 vaginal samples, the CVF samples were sterilized with a 0.22 μ filter.

Sixteen samples were predominated by *L. crispatus* and were selected to become part of the pooled *L. crispatus* (LC)-dominated CVF. By employing microdroplets, we subsequently confined and axenically cultured *L. crispatus*, *L. iners*, and *L. gasseri* in this pooled CVF. One of our hypotheses from Chapter 4 was that *L. crispatus* would grow in LC-dominated CVF. We report that *L. iners* grew in LC-CVF at pH 7 but was killed at pH 4. *L. crispatus* survived and the viability of *L. gasseri* colony-forming units decreased by approximately one log in LC-CVF at pH four. Our results indicate that vaginal pH influences the growth and fitness of *L. iners*. We hypothesize that *L. crispatus*' lack of growth may be due to a depletion of essential nutrients and/or the absence of constitutively expressed signaling molecules from the host.

In conclusion, this dissertation demonstrated several techniques for investigating microbial interactions in the HVM by co-cultivating species in microdroplets in a high-throughput manner. We also demonstrated the utilization of small volumes of human samples while simulating the *in vivo* environment. More information about our conclusions are described in Chapter 5. Further extension of our approach and its potential applications can hold tremendous potential for investigating how microbial interactions help define the ecology of the HVM and impact the host's health.

Chapter 2

Extending a Technology Pipeline for Co-cultivating Bacteria and Characterizing Vaginal Bacterial Interactions in Microdroplets

2.1 Summary

The human vaginal microbiome (HVM) plays a fundamental role in women's reproductive health. [1,13,43] For instance, bacterial vaginosis (BV) is the leading cause of healthcare visits for women of reproductive age and is characterized by a depletion of *Lactobacillus* species and an increase of strict and facultative anaerobes. Vaginal lactobacilli are associated with promoting health, and strict anaerobes such as *Gardnerella vaginalis*, are not. The mechanism(s) underlying BV remain unclear and current approaches for investigating them have severe limitations. [11,13,67] We previously developed a high-throughput co-cultivation technology platform based on the co-cultivation of bacteria in microdroplets to dissect interspecies interactions in microbial communities. [91] Here, we adapt and extend this technology to recapitulate interspecies interactions associated with polymicrobial infections such as BV. [99] In one case *L. jensenii* JV-V16 and *G. vaginalis* ATCC 49145 were cultured in microdroplets as axenic cultures and co-cultures. We then used two assays to analyze their growth in microdroplets: First, qPCR was used to quantify bacteria in pooled

microdroplets; Second, cells in individual microdroplets were plated and enumerated on agar media. Using these methods, we have demonstrated the inhibiting effect of *L. jensenii* on *G. vaginalis*, which is in concordance with previous literature. We validated the general applicability of our technology platform with a second co-culture model system that demonstrates amensalism between *L. jensenii*, which was used in the previous model system, and *Enterococcus faecalis* 19433. [99]

In this work, we have demonstrated effective ways to investigate interactions between vaginal bacteria based on co-cultivation in microdroplets using two model systems each consisting of a *Lactobacillus* species and a potential pathogen. This approach can be applied and extended in future studies for elucidating microbe-microbe-host interactions of complex microbial ecosystems.

All of the work presented in this chapter has been published in the following article: Corine M Jackman, Kyle W Deans, Larry J Forney, Xiaoxia Nina Lin, Microdroplet co-cultivation and interaction characterization of human vaginal bacteria, Integrative Biology, Volume 11, Issue 3, March 2019, Pages 69-78. The caption for each figure. describes whether each figure was published in the main article or supplementary information.

2.2 Introduction

2.2.1 Human health and disease

The human vaginal microbiome (HVM) has profound effects on women's reproductive health. Culture-independent studies have shown that either one of four *Lactobacillus* species or a diverse group of strictly anaerobic bacteria typically dominate the

HVM of most reproductive age women. [1, 34, 43, 76] These *Lactobacillus* species, namely *L. crispatus, L. gasseri, L. iners*, and *L. jensenii* are thought to preclude the colonization of urogenital pathogens by various means that include acidification of the environment by producing lactic acid, by secreting bacteriocins, and other means. [1, 74, 76, 100]

In contrast, a significant proportion of women harbor another type of microbial community that has far fewer lactic acid-producing bacteria and much greater proportions of strict anaerobes. [1] In some instances, communities of this type are accompanied by symptoms of bacterial vaginosis (BV), which according to Amsel's clinical criteria, include an elevated pH, an amine odor, vaginal discharge, and the presence of epithelial cells with high numbers of adherent bacteria. [101] There is also an alternative method for diagnosing BV called the Nugent criteria, which uses a scoring system to quantify the presence of *Lactobacillus* morphotypes, *Gardnerella* and *Bacteroides* species morphotypes, and curved gram-variable rods. [102] Compared to women without BV, women with BV are likely at a higher risk of acquiring human immunodeficiency virus (HIV-1). [28] However, women with high prevalences of *L. crispatus* and other lactobacilli were reported to have enhanced trapping and immobilization of the HIV-1 virus. [28, 103]

Another common polymicrobial syndrome in the vagina is aerobic vaginitis (AV), which is characterized by fewer *Lactobacillus* species and the proliferation of facultative anaerobes such as *Enterococcus faecalis*, a urogenital pathogen that is known to cause urinary tract infections. [42] Other species that are commonly associated with AV are Group B *Streptococci, Escherichia coli, Staphylococcus aureus*, and *Trichomonas vaginalis.* [42] Women with AV complain of inflammation, yellow dis-

charge, and vaginal dyspareunia. [42]

Several studies demonstrate the ability of *Lactobacillus* species to inhibit putative urogenital pathogens. [18,23,104] For example, O'Hanlon *et al.* reported a severe reduction of *G. vaginalis* and other bacterial vaginosis-associated species after just two hours of exposure to lactic acid. [74] Atassi *et al.* also observed decreased viability of *G. vaginalis* after four hours of co-culture with 10 of 13 vaginal *Lactobacillus* isolates. [18] Similarly, Breshears *et al.* found that spent media containing metabolites from *L. crispatus* inhibited the growth of *G. vaginalis* on explants of normal porcine vaginal mucosa. [23] Another study reported a decreased ability of *E. faecalis* to adhere to glass in the presence of biosurfactants produced by *Lactobacillus acidophilus* RC14. [104]

2.2.2 Employing microfluidics for biological applications

Traditionally, microbes are cultivated the laboratory using flasks or tubes with liquid media. [105] These conventional cultivation methods have uncovered significant interactions between microbial species and strains, yet they have severe limitations when they are used to study complex microbial communities. [37] These limitations include their low throughput, the requirement of relatively large culture volumes, and low assay sensitivities. A promising approach for overcoming these limitations is to use nanoliter-scale microdroplets for co-cultivation of bacteria. [96,98,99,106] Microdroplet encapsulation enables monodisperse compartmentalization of cells in an ultra-high-throughput manner, decreases the amount of time needed to reach a detection threshold, reduces requirements on cost, space, and volume of chemical reagents, and enables cultivation and analysis of single cells and mixtures of cells. [93,107–109]

Microfluidic devices for biomedical applications are commonly made of polydimethylsiloxane (PDMS), which is easy to work with during fabrication and is air-permeable, nontoxic, and optically clear. [88, 110] Cultivation in microdroplets has been used in the development of new biological assays including screening for biomass production, gene-targeted cultivation, and more specifically, single-cell confinement. [92, 93, 98, 107, 111] Most relevant to this work, Park *et al.* previously demonstrated mutualism between two cross-feeding amino acid auxotrophs of *Escherichia coli* in a synthetic model system of symbiosis. [91]

2.2.3 Overview

In this work, we adapt and extend a microfluidic co-cultivation technology platform to co-cultivate vaginal bacteria in microdroplets and examine interspecies interactions. The species selected were *Lactobacillus jensenii*, a lactic acid-producing vaginal bacterium, and *Gardnerella vaginalis*, a bacterium whose virulence is ecotype-dependent; the latter has a high prevalence among women with BV. [3, 49] The interaction between these two species was examined using conventional cultivation methods and their co-culture in anaerobic microdroplets. Subsequently, two assays were used to characterize growth in microdroplets. In one assay, the abundance of bacteria in pooled microdroplets was determined using qPCR. In the second, individual microdroplets were isolated and plated on an agar medium to estimate the number of viable cells in individual microdroplets. Our results showed that *L. jensenii* JV-V16 severely inhibited the growth of *G. vaginalis* ATCC 49145, which was in agreement with the findings of a previous experiment that was conducted at the bench-scale. [18] To demonstrate its general applicability, we further validated the technology with a second co-culture system that consisted of *L. jensenii* JV-V16 and *Enterococcus faecalis* ATCC 19433. These results show that microdroplet

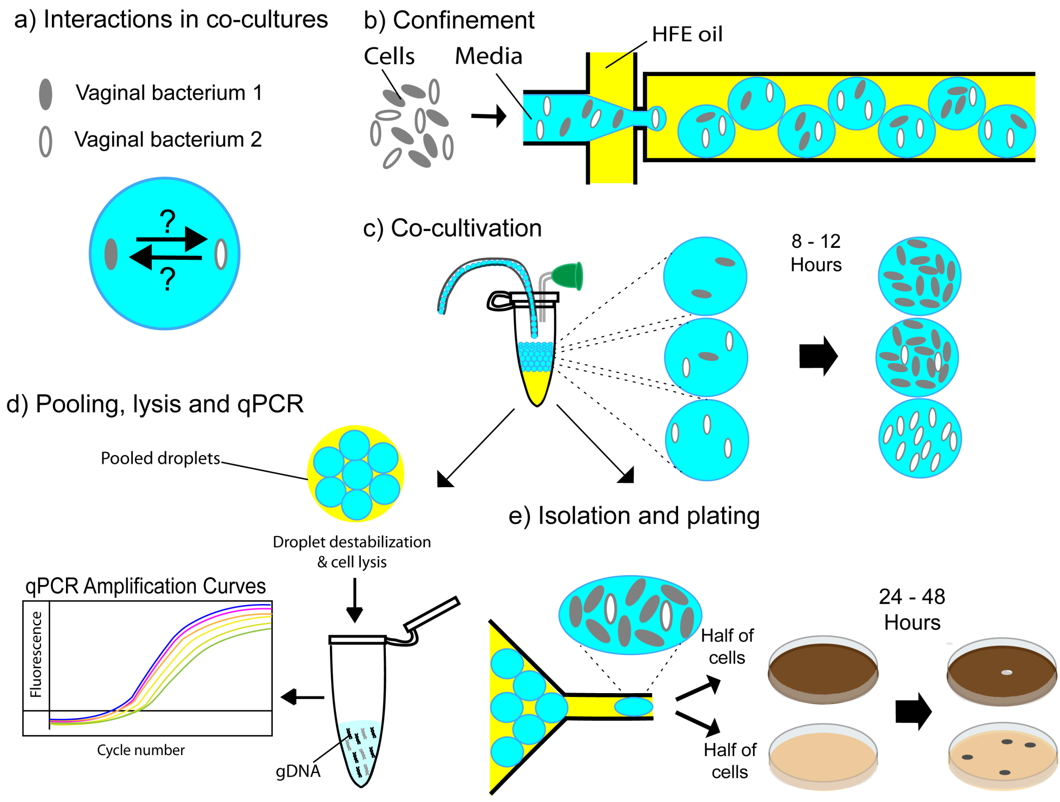


Figure 2.1: Schematic of the overall approach used to study interspecies interactions of human vaginal bacteria in microdroplets. (a) Unknown interspecies interactions between two different bacterial species co-cultured in a microdroplet. Pairwise interactions can be inferred based on the differential growth of each species in monocultures and co-cultures. (b) Microdroplet generation resulting in randomized confinement of cells in a flow-focusing microfluidic device. (c) Microdroplets incubated in a microcentrifuge tube and possible outcomes in three different types of droplets containing each monoculture and the co-culture, respectively. Gray ovals represent *Lactobacillus* cells and white ovals are non-*Lactobacillus* cells. (d) A pool of microdroplets analyzed by qPCR after microdroplets were destabilized and cells were lysed. (e) Individual microdroplets were isolated, destabilized, and plated onto agar medium and incubated.

co-cultivation systems can be characterized using both cultivation and molecular methods. This technology platform provides an effective method for discovering and studying interspecies interactions in microbial communities such as those found in the HVM.

2.3 Results and discussion

2.3.1 Overall approach

Building on our previous work that used microdroplets to co-cultivate *E. coli* auxotrophs, in this work, we created a technology pipeline for co-cultivating vaginal bacteria in microdroplets and characterizing interspecies interactions (Figure 2.1(a)). [91] To test and validate the technological pipeline described above, we applied it to a model system consisting of two vaginal bacteria, *Lactobacillus jensenii* and *Gardnerella vaginalis*. This model system was selected because both are representative species in the HVM, can be cultivated in the laboratory, and existing literature suggests that *L. jensenii* is capable of inhibiting *G. vaginalis*. [18, 23, 74]

2.3.2 Flask co-culture of *L. jensenii* and *G. vaginalis*

To establish the pair of *L. jensenii* and *G. vaginalis* as a valid model system for our study, we first conducted cultivation experiments with the monocultures and co-culture, using flasks. We inoculated monocultures of *G. vaginalis* and *L. jensenii* at seed densities of $(6.2 \pm 1.1)\times10^5$ CFU/ml and $(5.7 \pm 1.4) \times 10^4$ CFU/ml, respectively. For *G. vaginalis*, its density increased to $(1.8 \pm 0.07) \times 10^8$ CFU/ml in the monoculture, which was over 200-fold higher than the initial seed density (Figure 2.2). Whereas, when co-cultured with *L. jensenii*, the density of *G. vaginalis* decreased to $(3.5 \pm 3) \times 10^3$ CFU/ml, which was over 100-fold lower than the initial (Figure 2.2).

The unpaired t-test found a significant difference between the change of *G. vaginalis* density in the monoculture and that in the co-culture ($P < 0.0001$). This observation was consistent with previous work showing inhibition of *G. vaginalis* by several different vaginal *Lactobacillus* species. [18] For *L. jensenii*, after 17 hours of incubation, its density increased to $(8.6 \pm 0.8) \times 10^5$ CFU/ml in the monoculture and to $(9.1 \pm 2) \times 10^5$ CFU/ml in the co-culture with *G. vaginalis*, both of which were about 10-fold higher than the initial (Figure 2.2). Based on these results, we concluded that the growth of *L. jensenii* was not significantly affected by *G. vaginalis*.

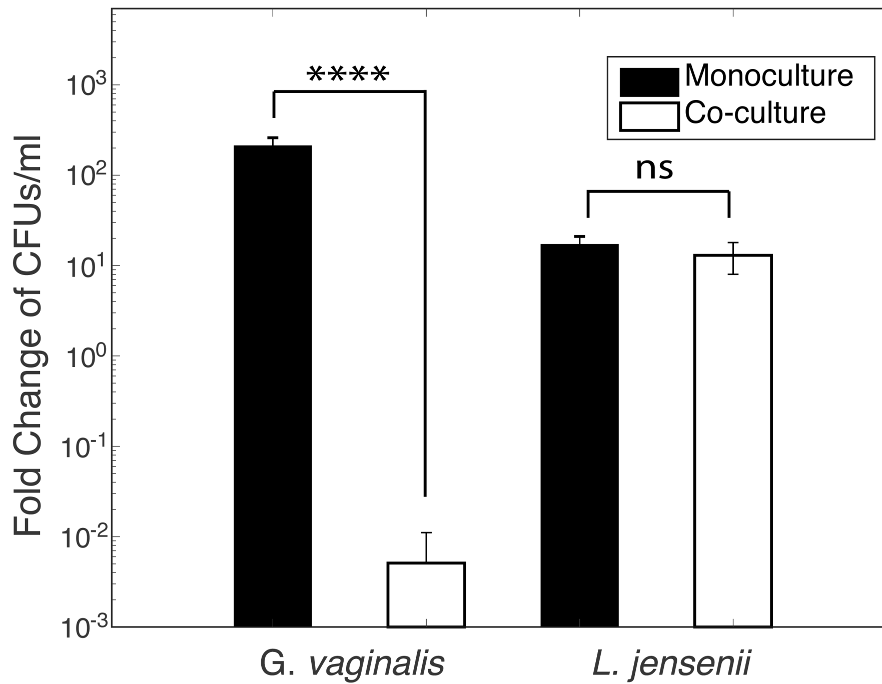


Figure 2.2: Fold change of *G. vaginalis* (left) and *L. jensenii* (right) in monoculture and co-culture after 17 hours of anaerobic cultivation in flasks. The monoculture is represented by the black bar and the co-culture is shown by the white bar. Error bars are standard deviations from four biological replicates. The unpaired t-test shows $P < 0.0001$ for *G. vaginalis*. This figure was published in the main article.

2.3.3 Encapsulation and co-cultivation of *L. jensenii* and *G. vaginalis*

We then applied the microdroplet co-cultivation technology to this pairwise model system of *L. jensenii* and *G. vaginalis.* To generate microdroplets for encapsulation of cells, we injected an oil phase and an aqueous phase containing a bacterial cell suspension into the inlets of a PDMS microfluidic device (Figure 2.3(a)). Shear forces at the orifice caused the formation of monodispersed cell-containing microdroplets in a continuous oil phase (Figure 2.3(b)). Using devices with microchannels of 50 μm in height, we generated microdroplets that shaped roughly as cylinders with a height of approximately 50 μm and a diameter of approximately 130 μm. The generation rate was approximately 100 microdroplets sec-1. We prepared the inoculum cultures so that the average number of cells per microdroplet would be 10 for each monoculture and 20 for the co-culture assuming a Poisson distribution from random encapsulation. Close examination of microdroplet images from time zero before incubation confirmed that this assumption was reasonable. Across 10 microdroplets, we averaged 8 $\pm$ 2.0, and 10 $\pm$ 1.9 cells for *G. vaginalis* and *L. jensenii* respectively, and 21 $\pm$ 2.6 cells for the co-culture (Figure 2.4). The microdroplets were then incubated anaerobically at 37°C for 12 hours. Figure 2.3(c-h) illustrates the monoculture and co-culture microdroplets at the initial and final time points. We observed the distinctive cell morphology of *L. jensenii*, which were aggregated rod-shaped cells (Figure 2.3(c,d)), and *G. vaginalis*, which appeared as coccus-shaped cells (Figure 2.3(g,h)). In the co-cultures, the cell morphologies observed at the final time point (Figure 2.3(f)) resembled those of *L. jensenii*, suggesting the inhibition of *G. vaginalis* by *L. jensenii.* It should be noted that we observed relatively large variation in the outcomes across microdroplets, which are likely due to the non-uniform distribution of cells across microdroplets (i.e. the Poisson distribution) and inherent cell-to-cell variation. [112]

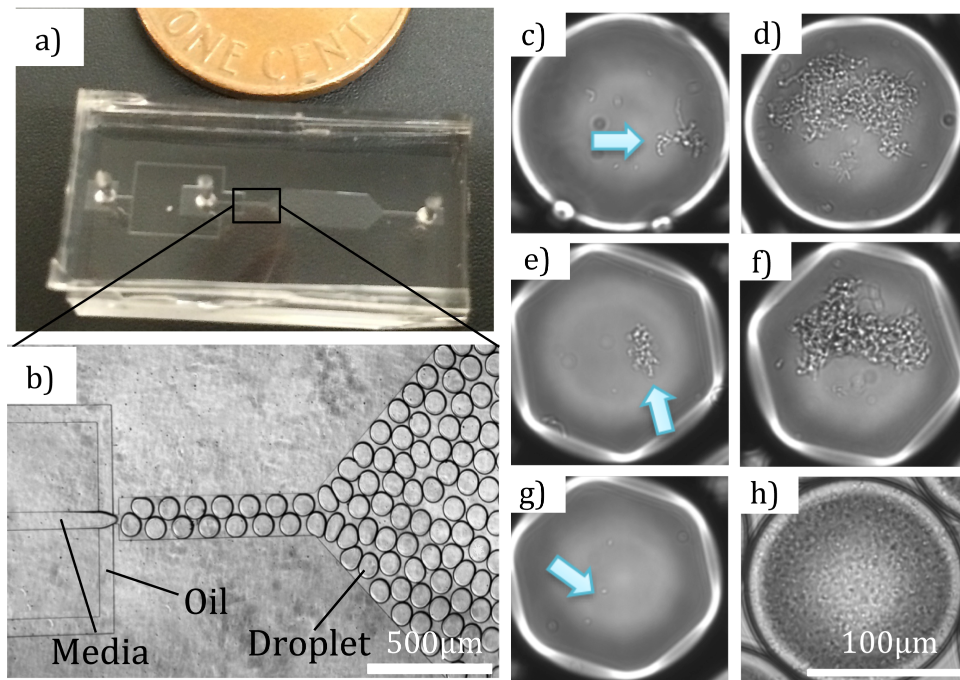


Figure 2.3: Anaerobic co-cultivation of *L. jensenii* and *G. vaginalis* in microdroplets. (a) Photo of a PDMS microfluidic flow-focusing device for droplet generation. (b) Generation of monodisperse water-in-oil microdroplets as the media (i.e. aqueous phase) and oil flow through a narrow orifice on the microfluidic device. (c-f) Representative images of cells in microdroplets: *L. jensenii* monoculture at 0 hours (c) and 12 hours (d); *L. jensenii* and *G. vaginalis* bi-culture at 0 hours (e) and 12 hours (f); *G. vaginalis* monoculture at 0 hours (g) and 12 hours (h). Blue arrows indicate some of the cells at 0 hours. This figure was published in the main article.

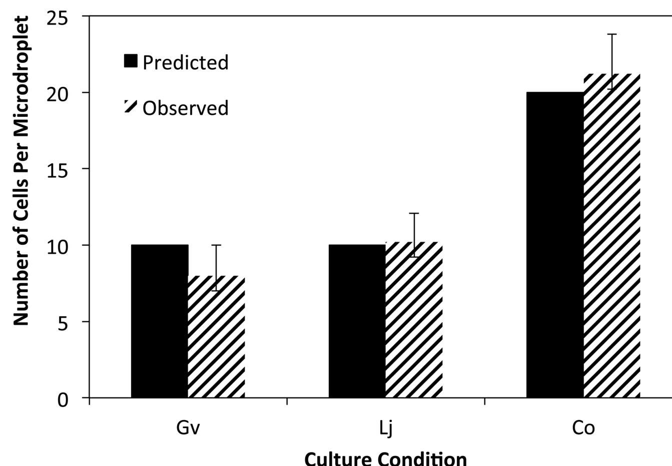


Figure 2.4: The average number of cells per microdroplet observed, compared to that predicted from the inoculum density and Poisson distribution assumption. Microdroplets containing *Gardnerella vaginalis* (Gv) monoculture, *Lactobacillus jensenii* (Lj) monoculture, and their co-culture (Co) had approximately 10, 10, and 20 cells per microdroplet, respectively, at the beginning of cultivation. This figure was published as supplementary information.

2.3.4 qPCR on pooled microdroplets of *L. jensenii* and *G. vaginalis*

As the first method for analyzing microdroplets downstream of co-cultivation, we developed a qPCR-based protocol for estimating the cell number of each bacterium in pooled microdroplets. The PCR primers used were specific for the 16S rRNA genes of *L. jensenii* and *G. vaginalis.* Due to the limited sensitivity of qPCR, we pooled microdroplets for the analysis and used 1 μL of microdroplets (i.e. approximately 1,000 microdroplets) for each assay. After pooling, the microdroplets were destabilized and gDNA was extracted to provide templates for qPCR. A standard curve was developed for each bacterium that correlates the number of cells used to generate DNA templates and the threshold cycle number (Ct), taking into account the procedure of microdroplet destabilization, cell lysis, and gDNA extraction (see details in Methods). The standard curves covered the range of 2.1×10^4 to 1.3×10^7 cells for *G. vaginalis* (Figure 2.5(a)) and 4.1×10^3 to 1.3×10^7 cells for *L. jensenii*

(Figure 2.5(b)). The gene copy number was accounted for in each standard curve.

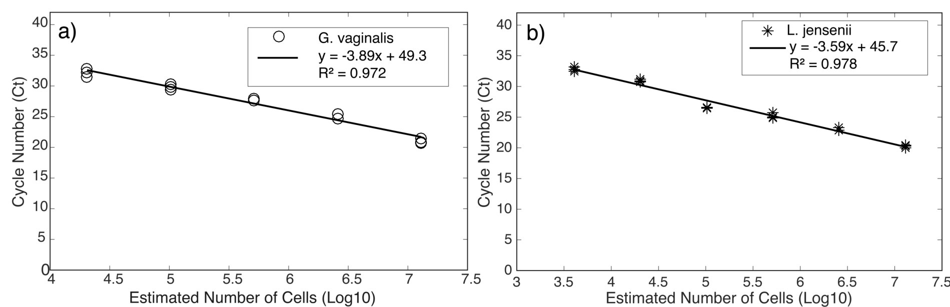


Figure 2.5: Standard curves of *L. jensenii* and *G. vaginalis* from approximately 1,000 pooled microdroplets after PCR. (a) The standard curve correlates the estimated number of cells (log10) to the cycle threshold number for (a) *G. vaginalis* shown in circles and (b) *L. jensenii* shown in stars. This figure was published as supplementary information.

qPCR analysis of the microdroplets with monocultures and the co-culture confirmed the inhibition of *G. vaginalis* by *L. jensenii*. The microdroplet pool with *G. vaginalis* in monoculture had 6.07 $\pm$ 0.03 log10 cells, compared to 4.67 $\pm$ 0.02 log10 cells when co-cultured with *L. jensenii* (Figure 2.6). The unpaired t-test revealed a significant difference between these two conditions ($P < 0.0001$). This result agreed with our previous observation of inhibition of *G. vaginalis* by *L. jensenii* in flask experiments. On the other hand, qPCR analysis of *L. jensenii* cells in microdroplets of monoculture and co-culture showed that the growth of *L. jensenii* was also inhibited in the co-culture (Figure 2.6). A decrease in *L. jensenii* in co-culture is likely detected due to the high sensitivity of our qPCR assay, and because we have increased the signal-to-noise ratio of the pooled microdroplets by combining them instead of evaluating the inherent variation for each microdroplet. Variability from each mi-

crodroplet stems from stochastic cell confinement and phenotypic differences within each cell. [87] The pool of microdroplets with *L. jensenii* in monoculture contained 5.44 ± 0.07 log10 cells, compared to 5.13 ± 0.05 log10 cells when co-cultured with *G. vaginalis*. The small standard deviations resulted in a significant difference between the monoculture and co-culture of *L. jensenii* (P = 0.003) according to the unpaired t-test. It should be noted that the variation described was observed in technical replicates of qPCR conducted on microdroplets from the same microcentrifuge tube we used for storage and incubation of microdroplets. This result appears different from our observation in experiments conducted using flasks and further discussion will be provided in a later section.

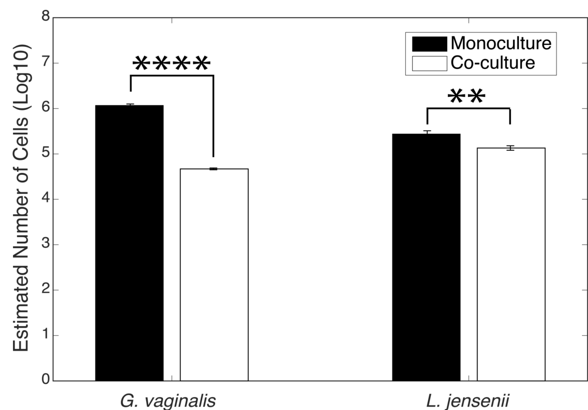


Figure 2.6: Estimated cell numbers of *G. vaginalis* (left) or *L. jensenii* (right) in monoculture vs. co-culture microdroplets after 12 hours of anaerobic incubation, based on qPCR of approximately 1000 pooled microdroplets. Monoculture conditions are represented by black bars and the co-culture by white bars. The error bar is the standard deviation of three pools of approximately 1,000 microdroplets, which are treated as three technical replicates for qPCR. The unpaired t-test for *G. vaginalis* showed a statistically significant difference ($P < 0.0001$) as did the unpaired t-test for *L. jensenii* (P = 0.003). This figure was published in the main article..

2.3.5 *G. vaginalis* and *L. jensenii* in isolated microdroplets

As a second method for analyzing the bacteria cultured in microdroplets, we used a previously developed microfluidic device to isolate individual microdroplets [97] and then plated cells from each microdroplet on Petri dishes to determine the number of viable cells for each species. To optimize and verify our technique for microdroplet isolation, we dispensed individual fluorescein-labeled microdroplets into 20 separate wells in a black glass-bottom 96-well microplate. Under optimal conditions, 75% of the wells contained single microdroplets, 15% contained none, and 10% of the wells contained more than one microdroplet (Figure 2.7).

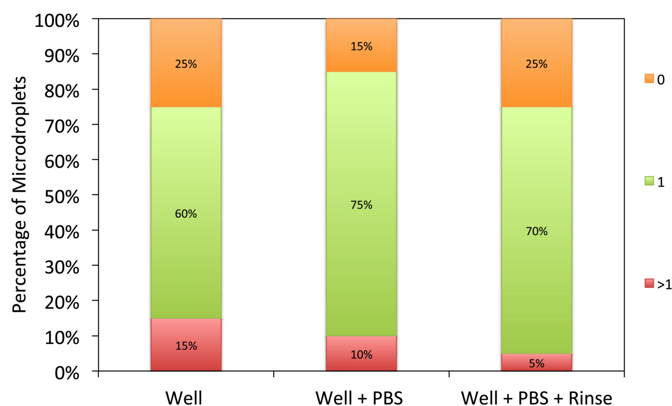


Figure 2.7: Number of droplets isolated into an individual microplate well, with three variants of a deposition protocol. 0, 1 and >1 in the legend represent the number of microdroplets in a single well that was observed via microscopy. Well: microdroplet deposited into a dry well; Well + PBS: microdroplet deposited into a well containing 200 μL PBS; Well + PBS + Rinse: microdroplet deposited into a well containing 200 μL PBS plus rinsing of the tip of the dispensing tube with additional PBS. Data obtained by isolation and deposition of fluorescein-labeled microdroplets into 20 wells in a 96-well glass-bottom plate. Well+PBS was found to be the optimal protocol and used in subsequent experiments. This figure was published as supplementary information.

For each monoculture and co-culture, approximately 10 microdroplets were analyzed.

Each microdroplet was isolated, destabilized, and plated onto two different types of agar media, which were then incubated for 8 hours. Images of the microdroplets that were isolated and plated can be found in Figure 2.8. Based on the plate count data, we again concluded that *G. vaginalis* was severely inhibited when co-cultured with *L. jensenii*. We can also observe the aggregation of *Lactobacillus* colony-forming units in Figure 2.8. To account for aggregation, the cell content was suspended in liquid, vortexed, and the cell content was referred to as colony-forming units as opposed to individual cells to compensate for the effects of residual aggregation.

G. vaginalis averaged 218 CFUs per microdroplet in monoculture and 0 in co-culture microdroplets (Figure 2.9). This was consistent with previous results from experiments in flasks, and analysis of pooled microdroplets using qPCR. *L. jensenii*, on the other hand, averaged 273 CFUs per microdroplet in monoculture and 608 CFUs per microdroplet when co-cultured with *G. vaginalis* (Figure 2.9). One microdroplet of *G. vaginalis* in monoculture was estimated to contain 2241 CFUs, was deemed to be an outlier and hence was not included in the t-test.

Yet, the variations across microdroplets under each condition remain large and consequently, the difference between monoculture and co-culture was not found to be significant for either bacterium. Variations across microdroplets are larger than those associated with conventional flask experiments due to stochastic effects that arose from the small numbers of cells in individual microdroplets. Two levels of unpredictability may be considered. First, the distribution of cells per microdroplet followed a Poisson distribution and there was substantial variation in the cell number from one microdroplet to another. Second, individual cells have inherent differences and the growth phenotype can vary significantly from one cell to another. [112]

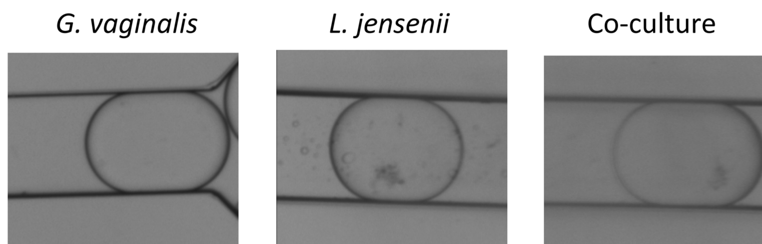


Figure 2.8: Representative isolated microdroplets with *G. vaginalis, L. jensenii*, and both species in co-culture after 8 hours of anaerobic incubation. This figure was published as supplementary information.

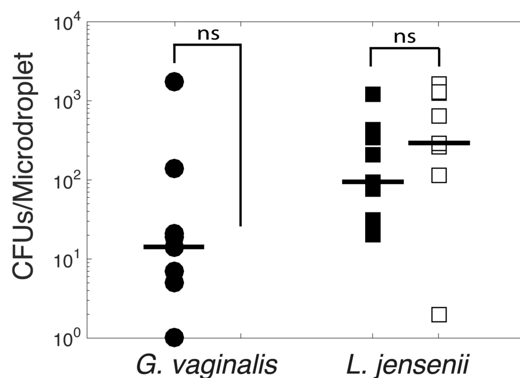


Figure 2.9: CFUs of *G. vaginalis* (left) and *L. jensenii* (right) in individual microdroplets that were isolated after 8 hours of anaerobic cultivation. Black circles: *G. vaginalis* in monoculture; white circles: *G. vaginalis* in co-culture, all at zero; black squares: *L. jensenii* in monoculture; white squares: *L. jensenii* in co-culture. The number of microdroplets: *G. vaginalis* in monoculture, 9; *L. jensenii* in monoculture, 10; *G. vaginalis* in co-culture and *L. jensenii* in co-culture, 9. Each horizontal line represents the median of the corresponding microdroplets. This Figure was published in the main article.

2.3.6 Comparison of assays

There were similarities and differences across the results we obtained from conventional flask cultivation and microdroplet experiments that are described above. Most importantly, the growth of *G. vaginalis* was inhibited by *L. jensenii* in both flask cultures and microdroplets (Figure 2.2, Figure 2.6, Figure 2.9). In contrast, the effect of *G. vaginalis* on *L. jensenii* was less conclusive. In conventional flask experiments, there was no significant difference in the growth of *L. jensenii* in monoculture versus when it was co-cultured with *G. vaginalis.* In microdroplet experiments, *L. jensenii* appeared to have a reduced cell number when co-cultured with *G. vaginalis*, compared to that when grown in monoculture, when we analyzed pools of approximately 1,000 microdroplets by qPCR. Yet, no difference was observed when approximately 10 individual microdroplets were plated to determine the number of viable cells. Multiple factors could have accounted for this difference. First, qPCR measured the total 16S rRNA gene content and had the limitation of not distinguishing between viable and non-viable cells. Thus the different results could be caused by the disparity between the total number of cells and the number of viable cells. Second, the qPCR assay we employed was conducted on a pool of 1000 microdroplets, each of which could be considered as a biological replicate of micro-bioreactor cultivation. Thus the qPCR analysis essentially provided an estimate of the average behavior of a very large number of biological replicates, canceling out variations across the microdroplets.

Variations in the pooled microdroplet analysis reported above reflected the technical replicates of qPCR conducted on the same population of microdroplets held in an incubation tube. These variations were therefore of different natures, compared to

those of biological replicates in flask cultivation and individual microdroplet analysis. The qPCR assay may have been more sensitive because each assay included a much larger number of biological replicates than those in the flask cultivation (i.e. 3-4) and individual microdroplet analysis (i.e. approximately 10), whereby the signal to noise ratio of the resulting data was increased considerably. When interspecies interactions were strong, as was the case of *L. jensenii* inhibiting *G. vaginalis*, we expected that all three means of cultivation and analysis would be able to detect the relationship when a sufficiently large number of microdroplets (i.e. biological replicates) were examined.

In this study, *L. jensenii* JV-V16 and *G. vaginalis* ATCC 49145 were selected for our first model system. However, the intensity of the killing effects were dependent upon the strains of *L. jensenii* and *G. vaginalis*. In 2006, Atassi *et al.* selected there strains of *L. jensenii* in addition to other species. However, strains of *L. jensenii* expressed different killing effects for *G. vaginalis* and ranged between 5.30 ± 0.30 to 6.82 ± 0.78 log CFU/ml. As for *G. vaginalis*, there are four different clades of *G. vaginalis* and some of them have different levels of association with BV. *G. vaginalis* ATCC 49145 is in clade 1 which positively associates with BV. [113] Including *G. vaginalis* ATCC 49145 was very relevant because it demonstrates the effects of a lactic acid-producing bacterium on a putative pathogen in the HVM.

2.3.7 *E. faecalis* and *L. jensenii* in co-culture

To verify the general applicability of the approach described above, we examined the interaction of *L. jensenii* and *Enterococcus faecalis*, the latter is a bacterium associated with the urogenital tract. We started with flask cultivation experiments and observed that the growth of *E. faecalis* was inhibited when it was co-cultured

with *L. jensenii*. We inoculated monocultures of *E. faecalis* and *L. jensenii* at seed densities of $(1.0 \pm 0.3) \times 10^4$ CFU/ml and $(1.2 \pm 0.4) \times 10^5$ CFU/ml, respectively. The co-culture was inoculated at similar seed densities of the two bacteria. All the cultures were incubated for 12 hours. *E. faecalis* increased to $(1.1 \pm 0.3) \times 10^9$ CFU/ml in the monoculture, which was over 10^5-fold higher than the initial (Figure 2.10(a)). Whereas, in a co-culture with *L. jensenii*, the density of *E. faecalis* was increased to $(3.8 \pm 0.9) \times 10^7$ CFU/ml, which was about 4×10^3-fold higher than the initial (Figure 2.10(a)). The difference between the change of *E. faecalis* in monoculture and that in the co-culture with *L. jensenii* was found to be statistically significant ($P < 0.0001$), suggesting that the growth of *E. faecalis* was inhibited by *L. jensenii*. For *L. jensenii*, after 12 hours of incubation, its density increased to $(3.2 \pm 0.5) \times 10^8$ CFU/ml in the monoculture, about 2.5×10^3-fold higher than the initial, and to $(2.5 \pm 0.7) \times 10^8$ CFU/ml in the co-culture with *E. faecalis*, about 1.8 $\times$ 10^3-fold higher than the initial (Figure 2.10(a)). The growth of *L. jensenii* was not significantly affected by *E. faecalis*.

Next, we encapsulated monocultures of *L. jensenii, E. faecalis*, and their co-culture in microdroplets as shown in Figure 2.10(b,d,f), and grew them for 12 hours as shown in Figure 2.10(c,e,g). For quantitative analysis, we isolated several microdroplets for each condition (see images in Figure 2.11) and plated the cells onto MRS agar plates (Figure 2.12). As shown in Figure 2.10(h), we determined that the number of *E. faecalis* CFUs in an individual microdroplet was $(6.6 \pm 5.0) \times 10^2$ in monoculture and $(2.8 \pm 2.0) \times 10^2$ in co-culture. The unpaired t-test found this difference to be statistically significant ($P = 0.039$). Nonetheless, the inhibiting effect of *L. jensenii* was more strongly detected against *G. vaginalis* than *E. faecalis*, thereby demonstrating the high sensitivity of our technology platform.

2.4 Conclusion

In this work, by adapting a previously developed method for co-cultivating bacteria in microdroplets and extending with a generally applicable analysis module, we created a technology pipeline for the co-cultivation and characterization of cells in microdroplets to study interspecies interactions in the vaginal microbiome. We validated the technology by testing it using two model systems: the first included *L. jensenii* and *G. vaginalis*; while the second included *L. jensenii* and *E. faecalis. L. jensenii* was found to inhibit the growth of *G. vaginalis* in co-cultures, in flasks and microdroplets when analyzed through pooling followed by qPCR. Additionally, *L. jensenii* was found to inhibit *E. faecalis* in flasks and microdroplets when analyzed through isolation of individual droplets followed by agar plating. We conclude that co-cultivation in microdroplets and characterization of cells in them can effectively recapitulate interspecies interactions observed in flask cultures as shown in Figure 2.10(a).

Further development and application of this technology presents an opportunity to elucidate diverse microbial ecosystems. For instance, the nanoliter microdroplet based technologies will render the use of host body fluids with or without supplementation to be used as culture media, enabling *ex vivo* growth experiments that more closely mimic the *in vivo* environment. Extending the general approach also illustrated in this work with multiplex droplet sequencing [89, 114] or fluorescence-activated droplet sorting [115, 116], one will be able to study millions of droplets simultaneously to identify interspecies interactions between different members of complex microbial communities.

2.5 Methods

2.5.1 Bacteria strains and cultivation protocols

Lactobacillus jensenii JV-V16, a lactic acid bacterium that produces D-lactic acid and *Enterococcus faecalis* ATCC 19433, a putative pathogen associated with UTIs, were streaked onto ATCC medium 416 (*Lactobacilli* MRS agar) plates and incubated overnight. *Gardnerella vaginalis* ATCC 49145 was streaked onto ATCC medium 2119 (chocolate agar) plates without IsoVitalex enrichment and incubated for 2 to 3 days. Single colonies from these plates were used as inocula for liquid cultures. The co-culture of *G. vaginalis* and *L. jensenii* was grown in ATCC medium 1685 (NYC III medium) while the co-culture of *E. faecalis* and *L. jensenii* was grown in ATCC medium 416. All cultures were grown anaerobically without agitation at 37° C.

2.5.2 Methods for flask culture experiments

Liquid seed cultures of *L. jensenii* and *G. vaginalis* were harvested during the exponential phase and four biological replicates were prepared for each of the monocultures and co-cultures. For each monoculture replicate, 9.9 mL of fresh medium was dispensed into a sterile glass flask and was inoculated with 0.1 mL of the seed culture. Each co-culture replicate contained 9.8 mL fresh medium and was inoculated with 0.1 mL of each bacterium. Cultures were incubated anaerobically using GasPaks from Becton Dickinson (Franklin Lakes, NJ, USA). At the initial and final time points, single-plate serial dilution spotting was used to determine the abundance of bacteria. [117] Colonies from different species were able to be distinguished on agar media. Colonies of *L. jensenii* were counted on MRS agar medium as *G. vaginalis* does not grow on this medium. Colonies of *G. vaginalis* were counted on chocolate agar medium, which allows both bacteria to grow but gives rise to different mor-

phologies between *G. vaginalis* and *L. jensenii*. The number of colony-forming units (CFUs) for *G. vaginalis* and *L. jensenii* were determined in triplicate. The detection limit for *G. vaginalis* was 1.3x10^2 CFU/ml. Assays without detectable bacteria were reported as ND (not detectable). The standard deviation was calculated using data from all four biological replicates.

Co-culture experiments of *E. faecalis* and *L. jensenii* were carried out in ways similar to those of *G. vaginalis* and *L. jensenii* as described above, except ATCC medium 416 was used instead of ATCC media 1685 and 2119. The CFUs of *E. faecalis* and *L. jensenii* were determined with four replicates.

2.5.3 Methods for fabrication and preparation of microfluidic devices

Flow-focusing devices were fabricated for microdroplet generation as described previously by Carruthers *et al.* [98] However, the polydimethylsiloxane (PDMS) mixture was cured at 80° C for 1h and treated with oxygen plasma for six seconds. The devices were also treated with trichloro(1,1,2,2-perfluorocytl) silane to increase hydrophobicity and were not used until at least two days after fabrication.

Spacing devices were fabricated using PDMS, as first developed by Wang *et al.*. [97] Specifically, the valve (top) and channel (bottom) layers used a mixture of 10:1 monomer to curing agent. The PDMS mixture was poured onto the mold, degassed under a vacuum, and solidified by heating to 80°C for 1 hour. The devices were cut out and a hole was punched into the valve on the top layer so that air can flow into the valve. A membrane between the valve and the channel layers was made from PDMS with a ratio of 15:1 monomer to curing agent. The PDMS mixture was degassed, poured onto a glass wafer, spun at 1,000 RPM until the thickness of the

membrane was 50 μ m, and then it was heated to 80° C for 15 minutes. The top layer was bonded to the membrane using oxygen plasma. The remaining outlets and inlets for the channels were punched through both the top layer and the bonded membrane. The top layer and membrane were aligned with the bottom layer. The bottom layer was bonded to the other side of the membrane using oxygen plasma.

2.5.4 Methods for microdroplet generation, incubation, and isolation

Aqueous microdroplets dispersed in a continuous oil phase were formed in a microfluidic device with a flow-focusing geometry. The oil phase was 2% 008-FluoroSurfactant from RAN Biotechnologies (Beverly, MA, USA) in Novec^{TM} HFE 7500 Engineered Fluid from 3M (Maplewood, MN, USA). The aqueous phase was NYC III or MRS media containing bacteria. The distribution of bacterial cells across microdroplets follows the Poisson distribution, of which the λ parameter corresponds to the average number of cells per microdroplet. [87] λ values were selected to be 10 and 20 for monocultures and co-cultures, respectively. MASTECH DC Power Supply HY5003 provided voltage that was controlled by LabVIEW^{TM} software to adjust the flow rates of each phase. Typical flow rates of the oil and aqueous phases were 3.5 μL min^{-1} and 4.2 μL min^{-1}, respectively. Each pneumatic pump was connected to a 2.5 mL syringe barrel from Nordson Engineering Fluid Dispensing (East Providence, RI, USA) that was assembled to other connectors in the following order: a female Luer from Cole-Parmer (Vernon Hills, IL, USA), polytetrafluoroethylene (PTFE) E-3603 flexible tubing from Fisher Scientific (Hampton, NH, USA), a male Luer (Cole-Parmer), and a fifteen gauge precision tip (Nordson EFD), which was inserted in the inlets of the microfluidic device. All tubing and connectors were sterilized with ethanol and by exposure to UV light.

Microdroplets were collected into 1.5 mL microcentrifuge tubes. For preparation of the collection tube, two small holes were drilled into the lids of 1.5 mL microcentrifuge tubes. Then, 0.022" inside diameter (ID) PTFE tubing (Cole-Parmer) and eighteen gauge 90°C bend precision tips (Nordson EFD) were inserted into the lids of tubes and then sealed with an epoxy glue. After microdroplets were transferred into a collection tube, they were incubated anaerobically using GasPaks from Becton Dickinson (Franklin Lakes, NJ, USA) at 37°C for 8 hours (*G. vaginalis and L. jensenii* co-culture) or 12 hours (*E. faecalis* and *L. jensenii* co-culture).

For isolation of individual microdroplets, we used a layered spacing device. It had two inlets, a side port for the air valve and one outlet. HFE oil with 0.5% fluorosurfactant was injected into the outer most inlet for microdroplet spacing. PTFE tubing of 0.022" ID was used to connect the microcentrifuge tube containing microdroplets to the second inlet of the microfluidic device. An air-dispensing connector was added to the air valve to manually control the flow of microdroplets. More PTFE tubing (0.012"ID, Cole-Parmer) was used to dispense microdroplets from the spacing device. The tubing was treated with Rain-X by flushing the tubing with Rain-X solution followed by HFE-7500 (3M) oil. Microdroplets were retrieved at the rate of one microdroplet every two minutes. Each retrieved microdroplet was dispensed into a separate 1.5 mL microcentrifuge tube with 100 μL 1x PBS, which was chosen because of its isotonic and hydrophilic properties.

2.5.5 Methods for droplet imaging

To image microdroplets, 10 μL of microdroplet suspension was transferred to a disposable hemocytometer (Fisher Scientific) and was sealed with an epoxy glue, and imaged using Fly Capture 2 Camera Selection 2.10.3.169 software at 200x magnifi-

cation using a Nikon Eclipse Ti microscope.

2.5.6 Methods for qPCR on pooled microdroplets

We used qPCR to determine the number of *L. jensenii* and *G. vaginalis* cells based on the amount of specific 16S rRNA gene fragments in the genomic DNA (gDNA) extracted from the corresponding cells. We designed primers for *L. jensenii* and *G. vaginalis* targeting the V5-V6 and V1 regions of the 16S rRNA gene, respectively, using Primer3 software with appropriate specifications. [118, 119] The primers were as follows:

L. jensenii

5'-AGCTGTCGCTAAAGGATGGA-3' (94F-Ljen)

5'-GGCCGATCAGTCTCTCAACT-3' (193R-Ljen)

G. vaginalis

5'-CTCTTGGAAACGGGTGGTAA-3' (26F-Gvag49145)

5'-AAGACATGCGTCAAGTTGGA-3' (79R-Gvag49145).

Both PCR reactions use the following set-up: stage 1, 95°C (10 min); stage 2, 60 cycles of 95°C (15 s), 55°C (5 s), and 68°C (10 s); and stage 3, 72°C (20 s). SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA, USA) was used.

To quantify the number of bacterial cells in monocultures and co-cultures based on qPCR of the 16S rRNA gene, we started by transferring 1 μL aliquots of pooled microdroplets to separate microcentrifuge tubes. Then, these microdroplets were destabilized with 1-3 μL of 1H, 1H, 2H, 2H-perfluoro-1-octanol from Sigma-Aldrich, (St. Louis, MO, USA). Afterward, 250 μL of a sterile mixture of 10 mM Tris-HCl: 50 mM EDTA (pH 8) was added and the aqueous phase containing cell suspension was transferred to a new oil-free 1.5 mL microcentrifuge tube. DNA extraction was carried out as described in a previous study (method 2) by Yuan *et al.* [120] The number of bacterial cells present in the sample was determined by qPCR using 1 μL of DNA template in a 20 μL reaction mixture. The standard curve correlating cell numbers and threshold cycle numbers (Ct) was created using the same protocol, except that 1 μL of empty microdroplets and a known number of cells were mixed before the mixture of 10 mM Tris-HCl: 50 mM EDTA (pH 8) was added. Five and six serial dilutions of known cell numbers were used to create the standard curves for *G. vaginalis* and *L. jensenii*, respectively.

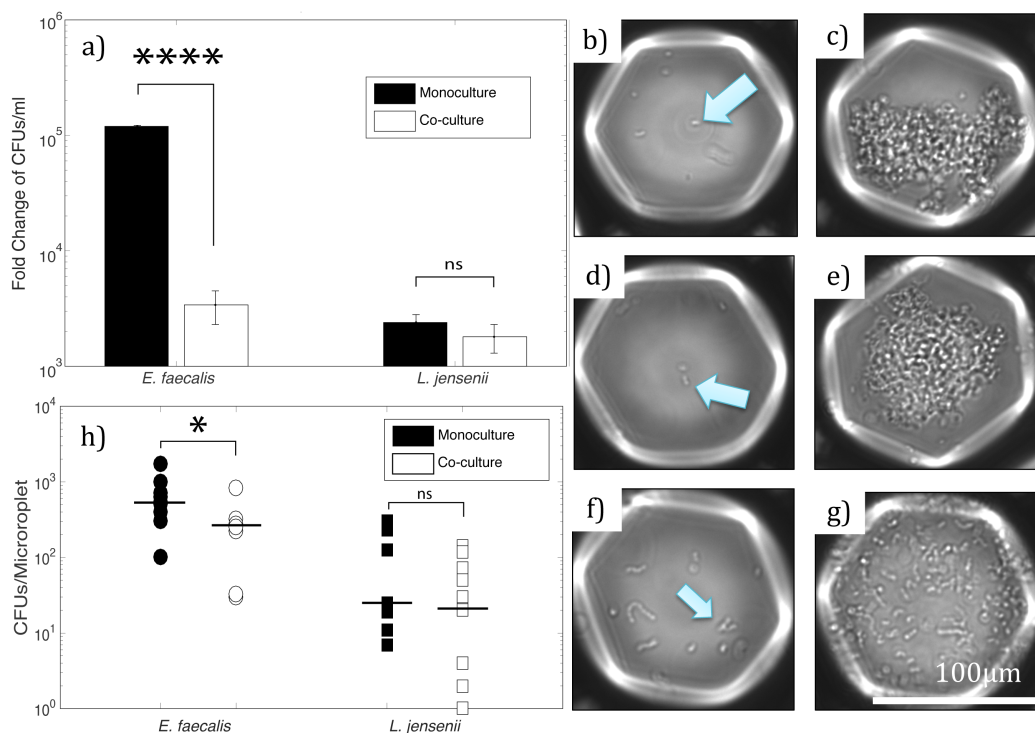


Figure 2.10: Study of a second model system consisting of *E. faecalis* and *L. jensenii*. (a) Fold increase of CFUs for *E. faecalis* and *L. jensenii* after cultivation in flasks. Monocultures are represented by black bars and co-culture by white bars. The difference between the monoculture and co-culture for *E. faecalis* is statistically significant ($P < 0.0001$). (b-g) Representative images of microdroplets containing *E. faecalis* and *L. jensenii* at 0 and 12 h: (b) *L. jensenii* in monoculture at 0 h, $\lambda = 10$; (c) *L. jensenii* in monoculture at 12 h; (d) *L. jensenii* and *E. faecalis* in co-culture at 0 h, $\lambda = 20$; (e) *L. jensenii* and *E. faecalis* in co-culture at 12 h; (f) *E. faecalis* in monoculture at 0 h, $\lambda = 10$; and (g) *E. faecalis* in monoculture at 12 h. Blue arrows illustrate cells in microdroplets at 0 h. (h) CFUs of individual microdroplets after 12 h of cultivation, as determined by plating on agar medium. Black circles: *E. faecalis* in monoculture; white circles: *E. faecalis* in co-culture; black squares: *L. jensenii* in monoculture; white squares: *L. jensenii* in co-culture. Number of analyzed microdroplets: *L. jensenii* in monoculture, 7; *E. faecalis* in monoculture, 9; and co-culture, 10. Each horizontal line represents the median under each condition. The difference between monoculture and co-culture microdroplets for *E. faecalis* was found to be statistically significant ($P = 0.0359$). This figure. was published in the main article.

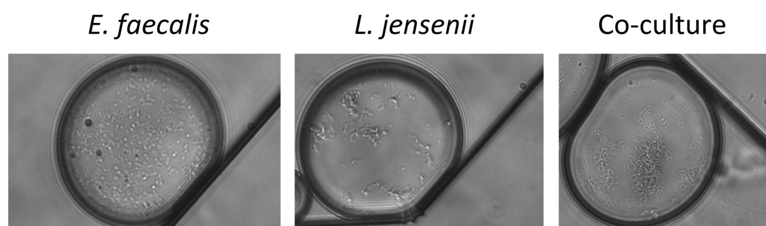


Figure 2.11: Representative isolated microdroplets with *E. faecalis, L. jensenii*, and both species in co-culture after 12 hours of anaerobic incubation. This Figure was published as supplementary information.

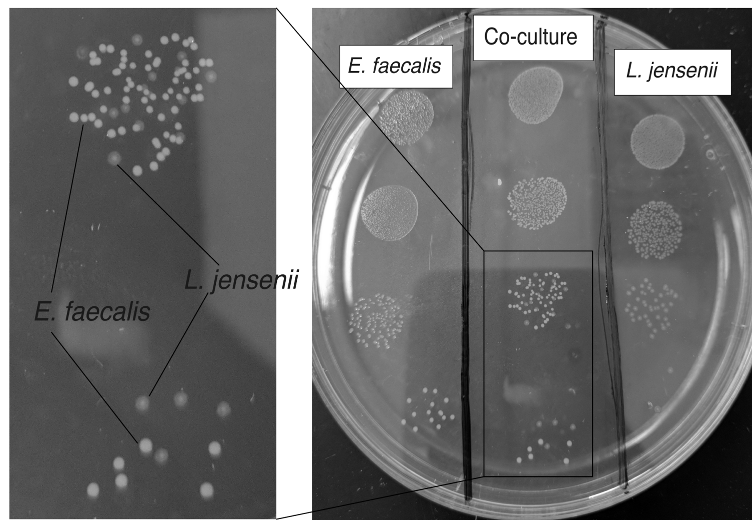


Figure 2.12: *Enterococcus faecalis* and *Lactobacillus jensenii* plated onto an MRS agar plate. *E. faecalis* colonies are opaque with a yellow tint. *L. jensenii* is translucent with a small opaque circle at the center of the colony. This figure was published as supplementary information.

Chapter 3

Investigating Interspecies Interactions Between *Lactobacillus* Species in Synthetic Laboratory Media

3.1 Summary

Longitudinal studies of the vaginal microbiome in individual hosts have shown temporal fluctuations between the most widely detected vaginal *Lactobacillus* species. [33,34] Causes for these fluctuations remain elusive. One related hypothesis is conditional differentiation, a phenomenon that can occur when species competitive ability is influenced by the availability of environmental or biotic resources. [68] However, there exists very little published data from empirical studies of mechanisms for microbial competition between *Lactobacillus* species.

Iron is an essential nutrient and is required by most microorganisms. [63] However, most lactobacilli uptake manganese or cobalt instead. [62–64,121] A study that compared the core and accessory genes of the two most common bacteria in the HVM, *L. iners* and *L. crispatus*, identified ABC transporter genes in the core genome of *L. crispatus*, which suggests that *L. crispatus* may have a competitive advantage for acquiring iron compared to *L. iners*. [68] However, *L. iners* has been reported to

grow on MRS agar after the addition of sheep or horse blood, which both contain iron. [55] Nonetheless, the number and types of compounds in the blood that helped stimulate the growth of *L. iners* remain unknown. The objective of the work in this chapter is to determine how limited concentrations of iron affects the growth of *L. iners* ATCC 55195 and *L. crispatus* ATCC 33820 pT1-aFP. We also aim to characterize pairwise interactions between vaginal lactobacilli in synthetic laboratory media by conducting co-cultivation experiments and spent-medium experiments.

In this work, we have detected reduced growth of *L. iners* ATCC 55195 at high concentrations of 2,2'-dipyridyl, an Fe^{2+} chelator that forms the complex, $[Fe(bipy)_3]^{2+}$. However, 2,2'-bipyridyl had no apparent effect on the growth of *L. crispatus* ATCC 33820 pT1-aFP. Based on our results, we speculate that the limited availability of free iron reduces the growth of *L. iners*, but *L. crispatus* is unaffected; possible explanations for the latter are that *L. crispatus* does not require iron and/or that it can sequester iron from its environment. *L. iners* also did not grow in *L. crispatus*-spent media that was supplemented with iron and glucose, which indicates iron and glucose may not have been the only limiting resources for *L. iners* in the spent media from *L. crispatus*.

In the second part of this work, our objective is to investigate whether resource competition or interference competition exists between pairs of lactobacilli using serial-dilution co-culture experiments and spent-medium experiments. Competition experiments were conducted between *L. crispatus* and *L. iners* in MNC broth, and *L. crispatus* and *L. gasseri* JV-V03 in MRS broth. *L. gasseri* has been selected because it is one of the four most dominant vaginal lactobacilli. In the serial-dilution co-culture experiment between *L. crispatus* and *L. iners*, we observed that *L crispatus*

dominated the co-culture. The ability of *L. crispatus* to dominate in co-culture may be due to its fast growth rate compared to that of *L. iners*. In the second model system, *L. crispatus* and *L. gasseri* both grew in co-culture. Further investigation is required to identify the limiting nutrients for resource competition for each model system. Interference competition was not observed in either model system based on results from the spent medium experiments.

All of the work presented in this chapter was previously unpublished.

3.2 Introduction

3.2.1 Transitions in the HVM

Cross-sectional studies have identified *L. crispatus* and *L. iners* as the most widely detected species in the HVM. [1] In 2011, Ravel *et al.* identified *Lactobacillus crispatus* (CST-I), *L. gasseri* (CST-II), *L. iners* (CST-III), *L. jensenii* (CST-V), and the diversity group (CST-IV) as five CSTs in a cohort of 394 women using 16S rRNA sequencing. [1] However, 73% of the cohort was dominated by one or more lactobacilli with at least 50% of all sequences obtained. [1] *L. iners, L. crispatus, L. gasseri*, and *L. jensenii* dominated 108 (34.1%), 104 (26.2%), 25 (6.3%), and 21 (5.3%) of 394 vaginal communities, respectively.

Gajer *et al.*'s longitudinal study in 2012 assessed the temporal dynamics of vaginal microbial communities in a cohort of 32 women and observed different frequencies in the number of transitions between certain CSTs. [34] In this study, 936 vaginal samples were collected and analyzed via 16S rRNA gene sequencing; samples were collected from each woman biweekly for 16 consecutive weeks. Afterward, a total

of 905 CST transitions were recorded from every two consecutive time points. The highest frequencies for *Lactobacillus* CSTs were those that maintained resilience. For instance, CST II remained CST II for 50 transitions (89.3% of CST II transitions); CST III remained CST III for 314 transitions (85.8%), and; CST I remained CST I for 133 transitions (84.2%). Interestingly, CST II had zero transitions to and from CST I and only two transitions to and from CST III, 3.6% and 0.5%, respectively. However, the most frequent transitions between alternate *Lactobacillus* CSTs occurred when CST I (*L. crispatus*) transitioned 12 times (7.6%) to CST III (*L. iners*) and CST III transitioned 10 times (2.7%) to CST I, deeming the most transitions between alternate CSTs to be between *L. iners* and *L. crispatus*. In addition to discovering patterns between transitions, Gajer *et al.*'s study revealed that although *Lactobacillus* CSTs may co-occur, they can also transition from one CST to another. Transitions between community states may be indicative of fundamental niche overlap. [1, 34] Currently, no published studies have empirically demonstrated microbial competition between *Lactobacillus* species.

Several different potential resources vary in the dynamic environment of the HVM that could potentially be a limiting resource for lactobacilli. Glycogen is present in vaginal epithelial cells, and when it is released, it is degraded by α-amylase, which breaks down the glycogen into smaller subunits that can be metabolized by lactobacilli. [70, 71, 122] These compounds include maltose, maltotriose, other sugars and alpha-limited dextrins. [72] Although multiple vaginal lactobacilli can metabolize a sugar such as maltose, [68] they also metabolize nutrients at different rates. Iron is another potential nutrient that becomes available during menses. [55] Many lactobacilli have been shown to uptake cobalt and manganese as an alternative to iron, but recent data revealed that the growth of *L. iners* was stimulated after 5%

horse or sheep blood was added, which contains iron among other compounds. [55] *L. crispatus* was also predicted to have ABC transporter genes, which may increase its competitive ability for sequestering iron. [68] However, the exact compounds that stimulated the growth of *L. iners* are unknown. More research and empirical data may help to gain a sense of how vaginal lactobacilli interact with each other.

In 2016, France *et al.*'s study combined ecological theory with comparative genomics to predict potential factors that could facilitate temporal fluctuations in *L. crispatus* and *L. iners*. [68] In this study, Frances characterized and compared 15 strains of *L. iners* and 15 strains of *L. crispatus*. They reported differences in genome size and functional makeup. Strains of *L. iners* appeared to have a much smaller average genome size that is within the range of parasites and symbionts, 1.28 Mbp. It is speculated that environmental factors aid in their survival of *L. iners*. [19, 22] *L. crispatus*, on the other, hand has a larger average genome size, 2.25 Mbp. According to the literature, *L. iners* has been shown to grow during an increased pH, coitus, and menses, whereas populations of *L. crispatus* under those conditions are typically not as prevalent. [17, 19, 57, 60, 123] Variation in competitive ability on the strain and species level may reflect the expression of both core and accessory genes. The genome sizes of each strain that was included in France's study can be seen in Table 3.1.

France *et al.* conducted a study that compared the core and accessory genomes of *L. iners* and *L. crispatus*. [68] These two species were likely selected because they are the most widely detected species in the HVM and because transitions occur often between these CSTs. [34] To conduct this study, several strains were selected for each species. During the analysis, France found that the core genome of *L. crispatus*

was larger with 1,442 genes compared to 993 core genes for *L. iners*. *L. crispatus* also contained 2,884 accessory genes of which 45% of the genes were present in one strain while the accessory gene of *L. iners* only contained 1,233 genes, which was 56% of the genes. Differences in the number of accessory and core genes reflect *L. crispatus*'capability to have greater metabolic ability in the HVM.

TABLE 1 Bacterial genome data

Strain	Source	Genome size (Mbp)	No. of contigs	No. of ORFs
L. crispatus				
JV-V01	Human vagina	2.22	86	2,151
MV-1A-US	Human vagina	2.25	7	2,383
125-2-CHN	Human vagina	2.30	30	2,196
MV-3A-US	Human vagina	2.44	76	2,458
CTV-05	Human vagina	2.36	25	2,425
FB049-03	Human vagina	2.46	5	2,474
FB077-07	Human vagina	2.70	10	2,688
SJ-3C-US	Human vagina	2.09	201	2,199
214-1	Human vagina	2.07	187	2,100
2029	Human vagina	2.19	295	2,545
EM-LC1	Human vagina	1.83	63	1,839
ST1	Chicken crop	2.04	1	2,060
VCM6	Human vagina	2.34	253	2,397
VCM7	Human vagina	2.10	247	2,108
VCM8	Human vagina	2.33	255	2,427
L. iners				
UPII 60-B	Human vagina	1.32	31	1,288
UPII 143-D	Human vagina	1.26	21	1,213
LactinV 01V1-a	Human vagina	1.29	92	1,506
LactinV 03V1-b	Human vagina	1.30	67	1,454
LactinV 09V1-c	Human vagina	1.31	35	1,384
LactinV 11V1-d	Human vagina	1.31	27	1,362
LEAF 2052A-d	Human vagina	1.32	28	1,268
LEAF 2053A-b	Human vagina	1.37	37	1,288
LEAF 2062A-h1	Human vagina	1.30	24	1,278
LEAF 3008A-a	Human vagina	1.27	25	1,216
SPIN 1401G	Human vagina	1.28	52	1,224
SPIN 2503 V10-D	Human vagina	1.28	31	1,293
ATCC 55195	Human vagina	1.24	7	1,152
AB-1	Human vagina	1.29	7	1,230
DSM 13335	Human urine	1.28	12	1,212

Table 3.1: List of 15 strains of *L. crispatus* and 15 of *L. iners* their source of origin, genome size, number of contigs, and number of open reading frames. Reproduced from [68].

The availability of carbon sources such as glucose and maltose in the HVM is pos-

tulated to be byproducts of degraded glycogen. Glycogen can be found inside of vaginal epithelial cells (VECs). When VECs are sloughed it has been hypothesized that they release glycogen. No reports have shown that *Lactobacillus* species can degrade glycogen. Reports have shown that α-amylase can degrade glycogen into maltodextrins and simple sugars that can be metabolized by lactobacilli. [71, 72] No published studies have reported the origin of α-amylase, but it is speculated to have derived from VECs. The amount of VECs and thus potential glycogen availability are positively proportional to estradiol levels, which may affect the competitive ability of *L. iners*.

Iron is an essential nutrient that is used by most microorganisms except for several lactobacilli [63] and is used by pathogens to replicate DNA and maintain virulence. [19, 55] To decrease the chances of infection, the human host keeps iron tightly regulated and bound to limit its availability, but iron becomes available in the vagina during menses. [124] Menses, which are mostly blood and other matter that has been discharged from the uterus, contain hemoglobin, which contains iron. On the other hand, *L. crispatus* has unique ABC transporter genes, which can acquire iron by using siderophores, but whether *L. crispatus* requires iron is still debatable as *L. crispatus* has not been included in studies that examined *Lactobacillus* species' requirement for iron. [68, 121, 125] Thus, genes have been identified, but their roles have only been postulated.

Studies suggest differences between the ecological roles of *L. crispatus* and *L. iners*. Reasons for speculation are partially due to adverse conditions that enable the persistence of either *L. iners* or *L. crispatus* during eubiosis, a stable state, and only *L. iners* and other bacterial vaginosis-associated bacteria during dysbiosis, an unstable

state. [19, 61, 67, 123] Not only is *L. iners* able to persist during outbreaks of bacterial vaginosis, but also during and after metronidazole treatment, which is used to treat BV. [19] Researchers have predicted that *L. iners*' role may aid in recovering unstable states to eubiosis with more *Lactobacillus* species. [126] *L. iners* can also survive in an elevated or neutral pH as opposed to *L. crispatus*, which is usually more prevalent in environments with a low pH of 4. During menses, the pH is also raised. *L. iners* has also demonstrated resilience during menses and coitus. Although *L. iners* has been shown to help transition community states to a more normal vaginal microflora with more lactobacilli, its role as a pathogen is also considered. Its production of inerolysin, as well as its requirement for iron, is characteristic of pathogens such as *G. vaginalis*. It has also been speculated that pathogenesis may occur on the strain level, which is similar to strains of *G. vaginalis* that are associated with BV, clades 1-2, intermediate vaginal microflora, clade 3, and no correlation with BV, clade 4. [41, 51]

Conversely, the role of *L. crispatus* has been understood to be associated with health. Reasons include its production of both D and L-lactic acid, its ability to lower pH to 4, its ability to slow the infection rate of HIV-1, and its ability to break down putrescine, a malodorous amino acid that is mostly found during instances of BV, but in small amounts in asymptomatic women.

Investigating competitive interactions using a chemostat or serial dilutions have the advantage of having continuous culture of microorganisms. [127–129] For instance, Abreu *et al.* examined the effect of dilution on two-species co-culture and used the two-species co-culture outcomes to understand how community composition changes with respect to increased dilution. [129] They found that a tradeoff exists between

growth rate and competitive ability which favors slow growers in high-density and low-dilution milieus. [129] In another study, Smith constructed a mathematical model to determine what factors most determined an organisms' fitness and competitive ability in serial transfer culture. Their models suggest that more than two species may coexist when competing for a single nutrient. [128] Smith *et al.* in another study conducted a competition experiment between an obligate and a facultative chemolithotrophic *Thiobacillus* species in a chemostat where they reported competitive and commensal interactions between them, which appeared to be sometimes controlled by pH. [127] Very few competition experiments that involve a chemostat and/or serial dilutions have been used for the HVM, which would be useful for further elucidating competitive interactions in the HVM.

The purpose of the work in this chapter is (1) to determine the effects of iron as a resource on the growth of *L. iners* ATCC 55195 and *L. crispatus* ATCC 33820 pT1-aFP, and (2) to characterize pairwise interactions between three vaginal *Lactobacillus* species in a continuous competition experiment in synthetic laboratory medium.

3.3 Results and discussion

3.3.1 Overview

In this chapter, we investigate the type of interactions that took place between several *Lactobacillus* species in three ways. First, we demonstrate the effects of iron as a limiting resource to empirically show how varying concentrations can affect the growth of *L. crispatus* ATCC 33820 pT1-aFP and *L. iners* ATCC 55195. Second, we conduct pairwise competition experiments between *L. iners* and *L. crispatus*, and *L. crispatus* and *L. gasseri* JV-V03 to characterize their interactions in MNC broth

and MRS broth, respectively. Third, we examine whether inhibiting effects can be detected between each pair of *Lactobacillus* species, otherwise known as interference competition. Collectively, our approach demonstrates that by reducing the availability of iron, the fitness of *L. iners* and not *L. crispatus* is affected determines whether resource or interference competition occurs between *L. iners* and *L. crispatus*, and *L. crispatus* and *L. gasseri, L. crispatus* and *L. iners* were selected because they are the most dominant species in the HVM and most transitions have occurred between them, which is indicative of competitive interactions. *L. gasseri* was of particular interest because no transitions occurred between it and *L. crispatus* according to Gajer's study, yet it is one of the most dominant lactobacilli in the HVM. [34]

3.3.2 Effects of 2,2'-bipyridyl and Fe(II)SO_4

Although many lactobacilli do not require iron to grow, *L. iners* has been shown to require iron for survival. [121, 124, 125] Despite this fact, it is still unknown as to whether *L. crispatus* requires iron; however, a complete iron transport system that is unique to *L. iners* was identified in core genomes of *L. crispatus* strains, and may give *L. crispatus* a competitive advantage in the presence of iron. [19, 68, 121, 125] In this study, we tested whether *L. iners* and *L. crispatus* growth would positively correlate with increased concentrations of free iron.

We supplemented MNC broth, a blend MRS and NYC III medium, with horse serum. The horse serum contained 2.21 mg/L iron according to the HyClone™ biochemical assay. We also supplemented MNC with Fe(II)SO_4 and 2,2'-bipyridyl to increase the concentration of iron and to decrease the availability of free iron, respectively. 2,2'-bipyridyl is a chelator of Fe^{+2}; it forms complexes with iron. The following conditions of MNC broth contained different concentrations of 2,2'-bipyridyl and Fe(II)SO_4. A

50mM 2,2'-bipyridyl stock solution dissolved in 95% ethanol was used to prepare 0.1mM, 0.5mM, 1mM, and 1.5mM 2,2'-bipyridyl in MNC broth. A 1M $Fe(II)SO_4$ stock solution dissolved in ddH_2O was used to prepare 0.1mM, 1mM, 10mM, and 100mM $Fe(II)SO_4$ in MNC broth. The concentrations of 2,2'-bipyridyl and $Fe(II)SO_4$ were selected based on experimental results from previous experiments (data not shown). The two following conditions excluded both 2,2'-bipyridyl and $Fe(II)SO_4$: MNC and MNC with 3% ethanol. The condition with 3% ethanol in MNC broth tested the inhibiting effects of ethanol on cell growth, which did not inhibit either species.

Inoculum cultures of *L. crispatus* and *L. iners* were prepared using pure seed cultures in exponential phase that were cultured in MNC broth at pH 7 overnight. We measured the optical densities (OD) of each pure culture and normalized them by diluting the culture with the highest cell density to equal the cell density of the other pure culture using MNC broth. Once the cell densities were equal, we proceeded to prepare pure inoculum cultures each with a 100-fold dilution by aliquoting 6.6 μL of seed culture into 659.4 μL MNC for each condition.

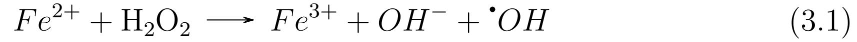
Inoculum pure cultures of *L. crispatus* and *L. iners* were grown in 200 μL in triplicate in a 96-well plate. The OD was recorded at 0 and 24 hours for each of the cultures. We calculated the net change in OD by subtracting the OD at 0 hours from the OD at 24 hours. Net changes in OD were compared for each condition as shown in Figure 3.1. *L. iners*' highest net change, 0.95 ± 0.046, was in 0.1mM 2,2'-bipyridyl, which showed no statistical difference from the net changes in MNC, 0.77 ± 0.084, and MNC + 3% ethanol, 0.79 ± 0.11. As hypothesized, a higher concentration of 2,2'-bipyridyl was inversely proportional to the net change of *L. iners*, which was

0.094 ± .0049 at 1mM and 0.076 ± 0.019 at 1.5mM, P = 0.0003 for both conditions. Conversely, the growth of *L. crispatus* was unaffected by higher concentrations of 2,2'-bipyridyl. However, the growth of both *L. crispatus* and *L. iners* decreased after the concentration of Fe(II)SO_4 was 10 mM Fe(II)SO_4; P = 0.012 and P = 0.0065, respectively. Conditions are shown in Figure 3.1.

L. iners' largely reduced growth under high levels of 2,2'-bipyridyl indicates that concentrations ≥ 1 mM 2,2'-bipyridyl caused toxicity for *L. iners* or that there was an insufficient supply of iron. However, stoichiometry reveals 0.1 mM 2,2'-bipyridyl is in excess, as three molecules of 2,2'bipyridyl form a complex with one iron atom. Interestingly, there were no changes in *L. crispatus* growth when any concentration of 2,2'-bipyridyl was added, which suggests iron is not required and/or that higher concentrations of 2,2'-bipyridyl did not cause toxicity for *L. crispatus*. Since *L. crispatus* can survive in 1 mM and 1.5 mM 2,2'-bipyridyl, high concentrations of 2,2'-bipyridyl may not be toxic to *L. iners*. We speculate that the growth of *L. iners* is reduced by the restriction of by the presence of free iron.

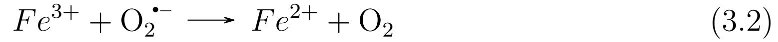
Both *L. crispatus* and *L. iners* growth was reduced in 10 mM Fe(II)SO_4 and they did not grow at 100 mM Fe(II)SO_4. One may speculate that both species were inhibited by high concentrations of Fe(II)SO_4 because of the iron-catalyzed Haber-Weiss reaction. [130] This reaction uses Fenton chemistry as shown in Equation 3.1. [130] Reactions from Fenton chemistry produce reactive oxygen species (ROS) like hydroxyl radicals ($^{\bullet}OH$). [130] This process is understood to occur in biological processes, one of which was discovered in the white-rot fungi in 1982 by Forney *et al.* [131] Haber and Weiss illustrated that an additional reaction (Eq. 3.2) to the

Fenton reaction, can create a net reaction (Eq. 3.3) that produces $^{\bullet}$OH. [132]

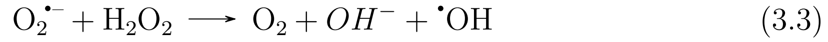


$$Fe^{2+} + \mathrm{H_2O_2} \longrightarrow Fe^{3+} + OH^{-} + {}^{\bullet}OH \quad (3.1)$$

(Fenton reaction)



$$Fe^{3+} + \mathrm{O_2^{\bullet -}} \longrightarrow Fe^{2+} + \mathrm{O_2} \quad (3.2)$$



$$\mathrm{O_2^{\bullet -}} + \mathrm{H_2O_2} \longrightarrow \mathrm{O_2} + OH^{-} + {}^{\bullet}\mathrm{OH} \quad (3.3)$$

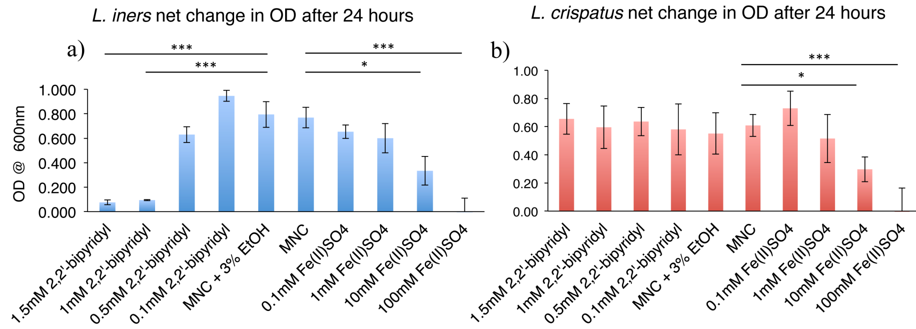


Figure 3.1: Net change in OD after 24 hours of (a) *L. iners* and (b) *L. crispatus* in MNC with varying concentrations of Fe(II)SO_4 and 2,2'-bipyridyl. Error bars are standard deviations three biological replicates. 1.5 mM and 1.0 mM, P = 0.0003. *L. crispatus* and *L. iners* at 10mM Fe(II)SO_4, P = 0.0107 and P = 0.0065, respectively. *L. crispatus* and *L. iners* at 100mM Fe(II)SO_4, P = 0.0051 and P = 0.0009, respectively.

L. iners and not *L. crispatus* was influenced by higher concentrations of 2,2'-bipyridyl, and *L. iners* growth was inversely proportional to increasing concentrations of 2,2'-bipyridyl as shown in Figure 3.2. To further demonstrate the effect of 2,2'-bipyridyl

on the growth *L. iners*, we conducted a 30-hour culture experiment with eight conditions: 1.5 mM, 1.0 mM, 0.7 mM, 0.4 mM, and 0.1 mM 2,2'-bipyridyl in MNC broth, MNC broth with no 2,2'-bipyridyl, and 0.01 mM and 0.1 mM Fe(II)SO_4 in MNC broth. MNC with 3% ethanol was excluded as it did not affect the net change in OD after 24 hours. As concentration decreased, so did the carrying capacity of *L. iners* as shown in Figure 3.2(a). Addition of Fe(II)SO_4 did not enhance the growth of *L. iners*, which suggests the concentration of iron in MNC broth does not require additional amounts of iron to achieve optimal growth.

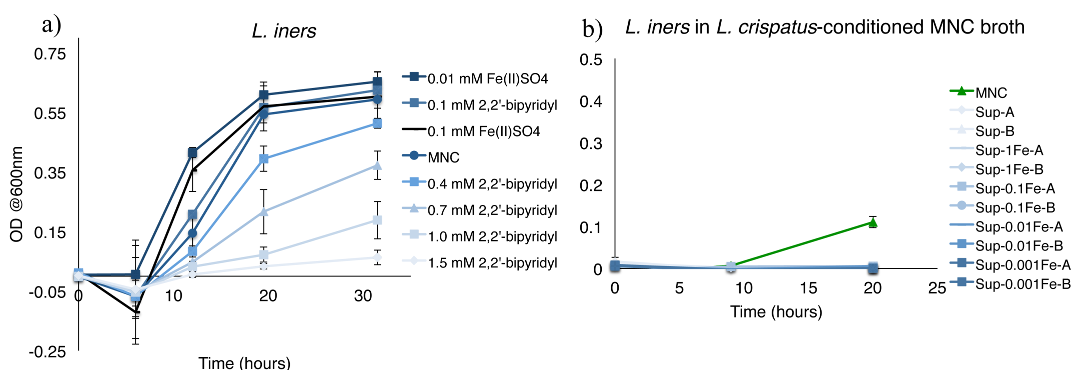


Figure 3.2: Effects of Fe(II)SO_4 and 2,2'-bipyridyl on *L. iners*. (a) *L. iners* growth with respect to 2,2'-bipyridyl and Fe(II)SO_4. Error bars are the standard deviations of ODs from three biological replicates. (b) *L. iners* in *L. crispatus*-spent MNC broth. Error bars are the standard deviation of two biological replicates.

We also wanted to determine whether iron in *L. crispatus*-spent media would stimulate the growth of *L. iners*. Two tubes of *L. crispatus* were cultured for 24 hours enabling them to reach exponential phase. The cell cultures were harvested, the cells were pelleted, sterile filtered with a 0.22 μ filter, and removed which left two tubes of cell-free *L. crispatus* (LC)-spent media. Each replicate of LC-spent media was used to create five conditions: no Fe(II)SO_4, 0.001 mM Fe(II)SO_4, 0.01 mM Fe(II)SO_4,

0.1 mM Fe(II)SO_4, and 1 mM Fe(II)SO_4. Glucose was added in the amount of 1g/L because it was a shared carbon source for both species. MNC broth was used as a positive control and the pH of all conditions was set to 7.0.

As shown by the green line in Figure 3.2(b), *L. iners* only grew in MNC broth. No growth in the other conditions indicates that resources other than iron and glucose were limited in those conditions.

3.3.3 Competition experiments between *L. crispatus* and *L. iners*

Very few publications have empirically demonstrated competitive interactions between *L. crispatus* and *L. iners*, the most widely detected lactobacilli in the HVM. [1] Gajer *et al.* observed that vaginal communities dominated by *L. crispatus* can transform to CSTs that are dominated by *L. iners* or a community that is characterized by modest proportions of either *L. crispatus* or *L. iners*. [34] In this work, we conducted serial dilution co-culture experiments to determine whether an interaction can be observed in MNC broth at pH 7. The purpose of the serial dilution was to allow the cells to grow for an extended period to reach a stable composition. For each dilution, a small fraction of the cell population was inoculated into fresh medium and the pH was maintained at 7.

Co-cultures were started when *L. crispatus* and *L. iners* were in exponential phase although they have different growth rates. Initial seed cultures of *L. crispatus* and *L. iners* were grown in 4 ml MNC at pH 7 for 16 hours and 24 hours, respectively. To ensure cells were in exponential phase, a 20-fold dilution was prepared by aliquoting both seed cultures into fresh MNC broth, which were checked periodically until they reached exponential phase in approximately 4 hours. Afterward, the initial dilution

was prepared as a 100-fold dilution by aliquoting 20 μL of the dilution's preceding seed culture into 1,998 μL of fresh media. Later serial dilutions followed a similar dilution protocol except for 20 μL of the most recent co-culture was transferred into fresh broth. Each serial dilution was incubated for 7 to 11 hours which enabled sufficient time for *L. iners* to grow which has a longer doubling time that *L. crispatus*.

Three conditions of co-cultures were prepared: 1 to 1 (1:1), 10 times more *L. crispatus* (10x LC) from the initial seed cultures, and 10 times more *L. iners* from the initial cell culture (10x LI). Based on the work of Abreu which claimed different dilution rates to influence species fitness. [129] By preparing three co-cultures with different ratios, we tested the effects of initial co-culture composition on competitive interaction. Each condition had three biological replicates that were averaged in Figure 3.3.

Immediately before each serial dilution, ODs from diluted co-cultures and their initial co-cultures were measured. Then co-cultures underwent a serial dilution, followed by plating onto agar medium, and after one to two days, colony-forming units (CFUs) were enumerated. Figure 3.3(a-c) shows the growth of *L. crispatus* and *L. iners* in three different co-culture conditions that underwent five serial dilutions. The OD of three biological replicates in each condition were averaged and connected by a dashed line. Each vertical dashed line indicated a serial dilution. Cultures were diluted 100-fold once the OD reached approximately 0.6 and cells had not yet reached stationary phase. ODs after the first dilution in Figure 3.3(a), 3.3(b), and 3.3(c) ranged between 0.0087 ± 0.0049 to 0.75 ± 0.032, 0.0033 ± 0.00091 to 0.60 ± 0.034, and 0.0031 ± 0.00057 to 0.65 ± 0.10, respectively. The range that is two orders of magnitude in OD is representative of the 100-fold dilution.

Turquoise data points in Figure 3.3(a-c) indicate the average pH value before and immediately after each serial dilution. Low pH values that were directly beneath a pH value of 7 indicates the initial pH before the culture was diluted 100-fold in MNC broth. The pH was returned to 7.0 when the pH was below 6.0 and was monitored every few hours. Average pH values < 6.0 after the first dilution were 4.5, 4.6, 5.8, 5.3, 5.53 ± 0.58, and 5.8 ± 0.58 from 21 to 56.5 hours for the 1:1 co-culture, 4.5, 5.0, 5.7, 5.67 ± 0.58, 5.87 ± 0.29, and 5.53 ± 0.58 from 21 to 56.5 hours for the 10x LC co-culture, and 4.5, 4.9 ± 0.53, and 5.6 ± 0.66 from 21 to 37 hours for the 10x LI co-culture, respectively.

The microbial composition of each co-culture condition was graphed in Figure 3.3(a-c) and their relative abundances are in Figure 3.3(d-f). The composition of the initial seed culture is reflected by the CFUs enumerated at time zero in Figure 3.3(a-c). Figure 3.3(d-f) more easily depicts the relative proportions of communities. The average proportions of *L. crispatus* and *L. iners* at time zero were 0.838 and 0.162 for the 1:1 ratio condition, 0.988 and 0.012 for the 10x LC condition, and 0.340 and 0.660 for the 10x LI condition, respectively. As is the case for the composition in CFU/ml, for the 1:1 ratio of *L. iners* to *L. crispatus*, there were $(2.1 \pm 0.5) \times 10^5$ CFU/ml to $(1 \pm 0.2) \times 10^6$ CFU/ml, respectively. The initial composition of the 10x LC co-culture was $(2 \pm 0.5) \times 10^4$ CFU/ml and $(1 \pm 0.4) \times 10^6$ CFU/ml for *L. iners* and *L. crispatus*, respectively. The starting composition of the 10x LI co-culture was $(2 \pm 0.4) \times 10^5$ CFU/ml and $(1 \pm 0.4) \times 10^5$ CFUs/ml for *L. iners* and *L. crispatus*, respectively.

After the initial dilution, *L. crispatus* consistently became the most dominant species despite the initial seeding ratio and the proportions have remained relatively consis-

tent overtime. After the first dilution, *L. crispatus* ranged between $(2.8 \pm 0.2) \times 10^8$ to $(6.2 \pm 0.9) \times 10^8$, $(2.6 \pm 0.5) \times 10^8$ to $(5.4 \pm 0.9) \times 10^8$, and $(1.4 \pm 0.7) \times 10^8$ to $(4.8 \pm 2) \times 10^8$ CFU/ml for the 1:1, 10x LC and 10x LI co-cultures, respectively. However, *L. iners* ranged between $(3.8 \pm 1.0) \times 10^4$ to $(1.7 \pm 2) \times 10^6$, $(2.9 \pm 0.9) \times 10^4$ to $(1.5 \pm 0.8) \times 10^6$, and $(5.2 \pm 2) \times 10^4$ to $(7.1 \pm 3) \times 10^5$ CFU/ml for the 1:1, 10x LC and 10x LI co-cultures, respectively.

There was little change in abundance in either species after each dilution. *L. iners* grew slower than *L. crispatus*, and therefore maintained a lower cell density. The growth rate of *L. crispatus* is faster than *L. iners*, which helped it to maintain abundance. However, *L. iners* sometimes outcompetes *L. crispatus* in the HVM. This could be due to the absence of a factor potentially from the host that hinders *L. crispatus* and stimulates *L. iners* growth. *L. crispatus* had the competitive advantage likely because of its faster growth rate.

3.3.4 Spent-medium experiments between *L. crispatus* and *L. iners*

Interference competition is caused when inhibiting molecules intoxicate other species. We hypothesized that interference competition may be the reason why there was less growth between either species by conducting spent-medium experiments. By carrying out spent-medium experiments, we can rule out inhibition as a mechanism for lower abundances of *L. iners* in Figure 3.3 and can determine whether interference competition is detected *in vitro*; no literature has mentioned interference competition taking place between other vaginal *Lactobacillus* species except *Lactobacillus* species are known to preclude bacterial vaginosis-associated bacteria by producing lactic acid.

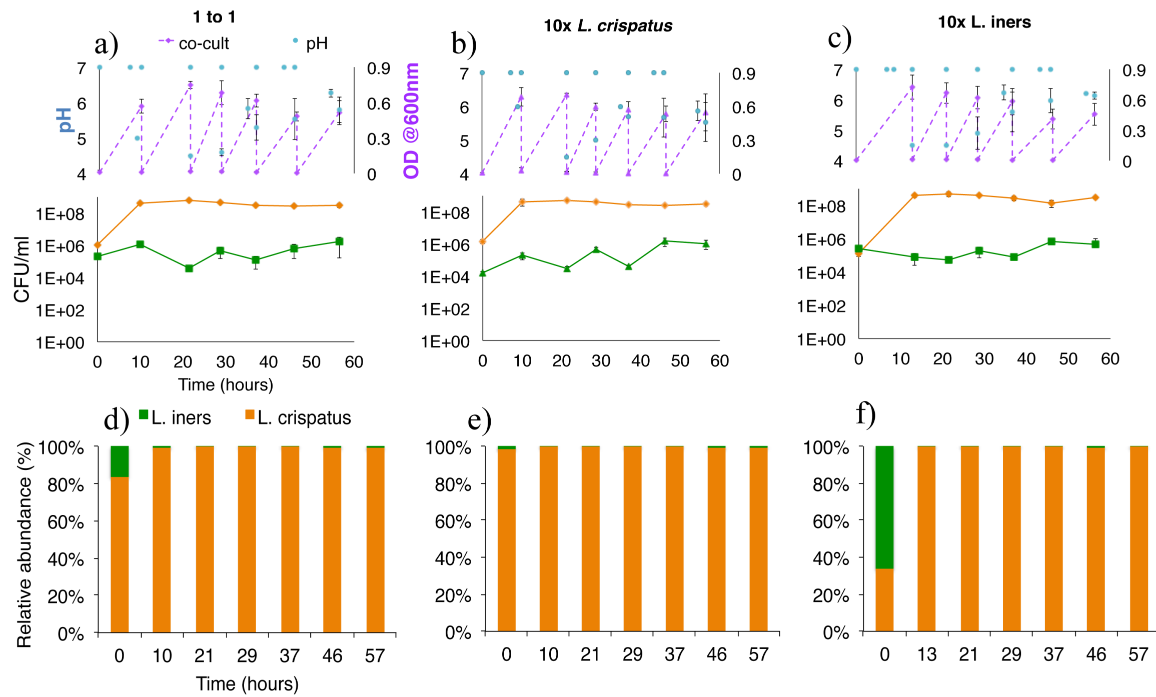


Figure 3.3: Serial dilution co-cultures and relative abundances of *L. crispatus* ATCC 33820 pT1-aFP and *L. iners* ATCC 55195 at different initial seed densities. (a-c) pH values, OD of co-cultures, microbial compositions of co-cultures and (d-f) relative abundances for the 1:1, 10xLC, and a 10xLI conditions. Vertical dashed lines are the number of serial dilutions. Turquoise dots represent the pH value on the upper primary axis. OD data is shown in purple for the co-culture on the secondary axis. The orange line is *L. crispatus* (LC) and the green line is *L. iners* (LI). Error bars for pH values are the standard deviations of three biological replicates. Error bars for co-cultures are standard deviations of three biological replicates and technical replicates of single plate-serial dilution spots. Green bars represent *L. iners* and orange bars represent *L. crispatus*. Bar charts are the average values of three biological replicates.

To determine whether interference interactions could be detected, we first prepared two sets of pure culture inoculums of *L. crispatus* and *L. iners* that were cultured until stationary phase. The cell cultures were harvested, the cells were pelleted, sterile filtered with a 0.22 μ filter, and removed which left two tubes of cell-free *L. crispatus* (LC) and two tubes of cell-free *L. iners* (LI)-spent media. Each replicate of spent media was used to create two separate conditions: spent media and spent media with supplemented ingredients from MNC. The controls were 1x MNC, 2x MNC incase >1x concentration of any particular nutrient(s) became inhibiting and phosphate buffer saline (PBS), an isotonic saline solution that is free of nutrients. All conditions are shown in Figure 3.4.

L. iners was cultured in LC-spent media with and without supplemented MNC broth as shown in Figure 3.4(a). After 24 hours, $(4 \pm 6) \times 10^2$ CFU/ml and no CFUs were detected for both biological replicates of *L. iners* in LC-spent media. The later replicate had a similar trend to the PBS condition, which also had no CFUs after 24 hours. There were many more CFUs of *L. iners* in LC-spent media that was supplemented with MNC; both replicates were $(6.8 \pm 5.7) \times 10^7$ CFUs/ml and $(4.9 \pm 0.95) \times 10^4$ CFUs/ml after 24 hours. In addition, *L. iners* CFUs were 1.5 logs higher in MNC 1x compared to MNC 2x at 0 hours and 6 hours, $P = 0.0002$ and $P = 0.0440$, respectively, which suggested a higher concentration of one or more molecules inhibits *L. iners*. Figure 3.4(b) shows *L. iners* in LI-spent media. After 24 hours, CFUs of *L. iners* in both biological replicates of LI-spent media and LI-spent media with supplemented MNC ranged between CFU/ml and $(3.1 \pm 1.0) \times 104$ and $(5.0 \pm 0.14) \times 10^6$ CFU/ml.

Results in Figure 3.4 demonstrate no interference competition between *L. crispatus*

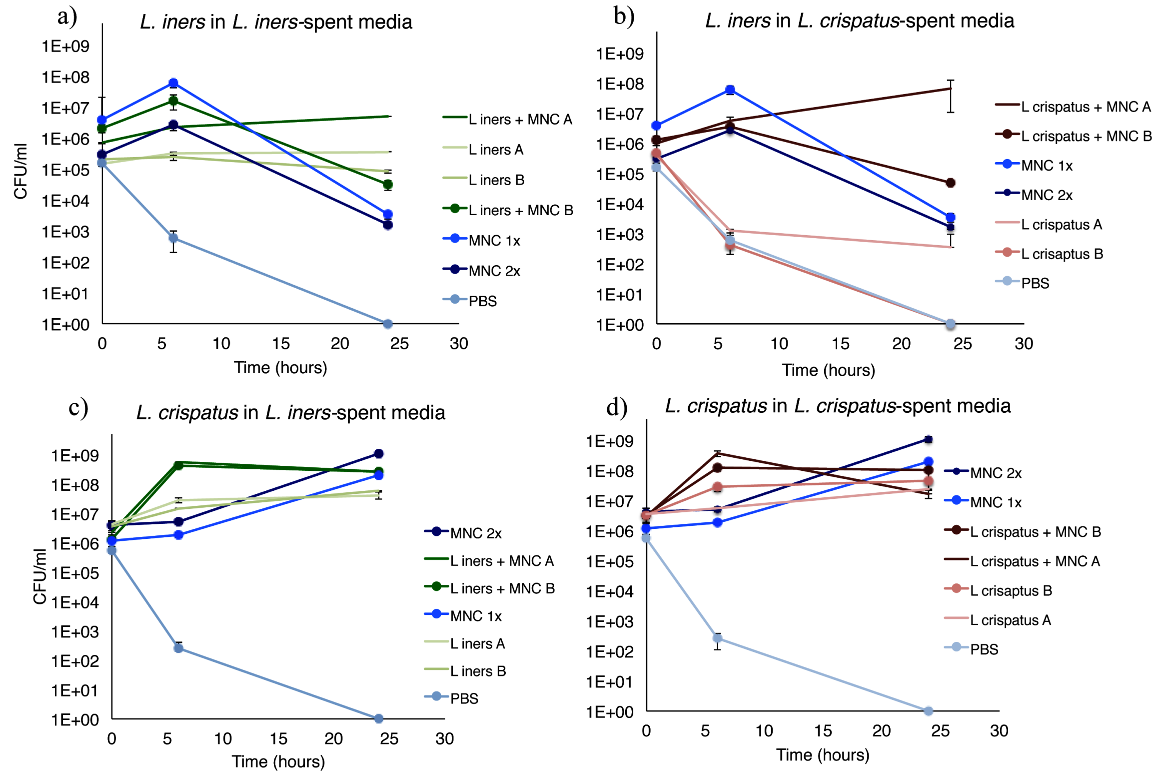


Figure 3.4: Spent-medium experiments for (a) *L. iners* in *L. iners* (LI)-spent MNC media, (b) *L. iners* in *L. crispatus* (LC)-spent MNC media, (c) *L. crispatus* in LI-spent MNC media, and (d) *L. crispatus* in LC-spent MNC media. Dark green lines are LI-spent media with supplemented MNC. Light green lines are LI-spent media. Magenta lines are LC-spent media with supplemented MNC. Pink lines are LC-spent media. Darkest blue line ins MNC 2x, the blue line is MNC 1x, and the light blue line is PBS.

and *L. iners*. As shown in Figure 3.4(a), after 24 hours, there were more CFUs in LC-spent media after MNC was added which indicates additional nutrients were needed for *L. iners* to grow in LC-spent media, which suggests key nutrients were depleted that are shared by both *L. iners* and *L. crispatus*. *L. iners* in spent media with no additional nutrients showed a similar pattern of decline in PBS. Additional nutrients were needed to sustain the viability of *L. iners*. Interestingly, *L. iners* in 1x MNC media had the highest number of CFUs at 0 and 6 hours, but it was less than the condition with LC-spent media with MNC. Improved resilience of *L. iners* in spent

media with MNC compared to MNC broth is speculated to be due to a growth advantage in stationary phase (GASP) phenotype that has been demonstrated in several models using *Escherichia coli*. [133–136] GASP strains carry a growth advantage in stationary phase and we speculate that byproducts and/or signaling molecules produced by initial seed culture that remained in spent media were detected by *L. iners*. GASP could explain two things; why one of the biological replicates, LC-MNC A was higher than MNC after 24 hours. It could also explain why CFUs in LI-spent media with and without MNC had little variation compared to MNC broth. The drop in CFUs in the MNC broth conditions could be from rapid cell growth. Phenotypic differences between each biological replicate could have influenced variation between replicates.

L. crispatus showed a different trend in LI-spent media as shown in Figure 3.4(c & d). Between LI-spent media and LI-spent media with MNC after 6 hours, there were more CFUs in the condition with supplemented MNC for replicates A and B, $P = 0.0139$ and $P = 0.0067$, and after 24 hours, $P = 0.0105$ and $P = 0.0067$. Results showed that *L. crispatus* did benefit from additional nutrients added to *L. iners*. After 24 hours, MNC 1x and 2x grew to $(1.9 \pm 0.31) \times 10^8$ CFU/ml and $(1.1 \pm 0.21) \times 10^9$ CFU/ml, which is much higher than *L. iners*. However, all conditions were statistically higher than in PBS. Increased growth is due to the increased robustness of *L. crispatus* compared to the fastidiousness of *L. iners*. A similar pattern was observed in Figure 3.4(d). After 6 hours, *L. crispatus* in LC-spent media with MNC was higher than in LC-spent media for biological replicates A and B, $P = 0.0244$ and $P = 0.0471$, respectively. No interference or exploitation competition was detected between *L. iners* and *L. crispatus*.

3.3.5 Competition experiments between *L. crispatus* and *L. gasseri*

L. gasseri did not transition to the *L. crispatus* CST in the longitudinal study that Gajer *et al.* conducted in 2012. However, *L. gasseri*remains one of the most dominant species in the HVM. We aimed to investigate competitive interactions between *L. crispatus* and *L. gasseri* using synthetic laboratory media. [1] In this work, we conducted serial dilution co-culture experiments to determine whether an interaction could be observed in MRS broth at pH 4.

We began co-culture experiments when *L. crispatus* and *L. gasseri* were in exponential phase. Initial seed cultures of *L. crispatus* and *L. iners* were grown in 4ml MRS overnight. To ensure that cells were in exponential phase, a 20-fold dilution was prepared by aliquoting both seed cultures into fresh MRS broth and were used for co-culture experiments approximately three hours later. Afterward, the initial dilution was prepared as a 100-fold dilution by aliquoting 20 μL of the dilution's preceding seed culture into 1,980 μL of fresh MRS. The following serial dilutions followed a similar dilution protocol except 20 μL were used as inoculum from its preceding co-culture. Each serial dilution was incubated for 6 to 8 hours, the time it took for cultures to reach an OD of 0.6. The three co-culture conditions that were prepared were a 1 to 1 (1:1), 10 times more *L. crispatus* that *L. gasseri* cells (10x LC), and 10 times more *L. gasseri* than *L. crispatus* cells (10x LG). This way, we tested the effects of initial co-culture composition on competitive interaction. [129] Each condition had three biological replicates that were averaged in Figure 3.5.

Immediately before each serial dilution, ODs from diluted co-cultures and their initial co-cultures were measured. Then, co-cultures underwent a serial dilution, followed

by plating onto agar medium, and after two days, CFUs were enumerated. Figure 3.5(a-c) shows the growth of *L. crispatus* and *L. gasseri* in three different co-culture conditions that underwent five serial dilutions. The OD of three biological replicates in each condition were averaged and connected by a dashed line. Each vertical dashed line indicated a serial dilution. Cultures were diluted 100-fold once the OD reached approximately 1.0 and cells had not yet reached stationary phase. ODs after the first dilution in Figure 3.5(a-c) ranged between 0.0394 ± 0.00868 to 1.40 ± 0.0205, 0.0314 ± 0.00533 to 1.38 ± 0.0148, and 0.0426 ± 0.00436 to 1.36 ± 0.123, respectively. The range that is two orders of magnitude in OD is representative of the 100-fold dilution.

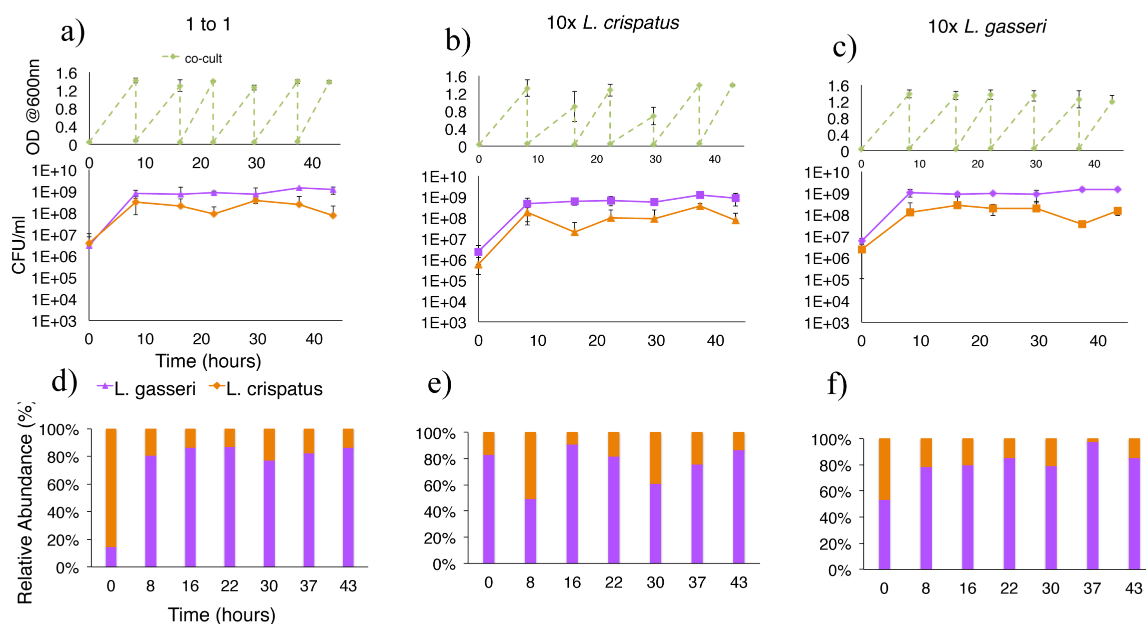


Figure 3.5: Serial dilution co-cultures and relative abundances of *L. crispatus* ATCC 33820 pT1-aFP and *L. gasseri* JV-V03 at different initial seed densities. (a-c) OD of co-cultures, microbial compositions of co-cultures and (d-f) relative abundances for the 1:1, 10xLC, and a 10xLG conditions. Vertical dashed lines are the number of serial dilutions. OD data is shown in green for the co-culture on the upper axis. The orange line is *L. crispatus* (LC) and the purple line is *L. gasseri* (LG). Error bars for co-cultures are standard deviations of three biological replicates and technical replicates of single plate-serial dilution spots. Bar charts are the average values of three biological replicates.

Production of lactic acid by *L. crispatus* and *L. gasseri* maintains a pH value of

4.0. The microbial composition of each co-culture condition was graphed in Figure 3.5(a-c) and their relative abundances are in Figure 3.5(d-f). The composition of the initial seed culture is reflected by the CFUs enumerated at time zero in Figure 3.5(a-c). Figure 3.5(d-f) depicts the relative proportions of communities. The average proportions of *L. crispatus* and *L. gasseri* at time zero were 0.355 and 0.645 for the 1:1 ratio condition, 0.204 and 0.796 for the 10x LC condition, and 0.317 and 0.683 for the 10x LG condition, respectively. The ratios at time zero did not agree with our experimental design, likely due to the method used to enumerate *L. crispatus* CFUs. *L. gasseri* grows well on Tryptic Soy Agar (TSA) and MRS agar with similar viability; *L. gasseri* on MRS agar is $(3.2 \pm 0.51) \times 10^8$ CFU/ml and on TSA is $(3.3 \pm 0.63) \times 10^8$ CFU/ml. When *L. crispatus* was washed in PBS before plating, it only grew on MRS agar and not on TSA; concentrated dilutions, ie. 10^{-1} dilution, when cells were not washed likely because of residual broth from the seed culture. The method of enumerating CFUs was different from the *L. crispatus* and *L. iners* model, which used differential plates. Consequently, *L. gasseri* was enumerated on TSA and these colonies were subtracted from the colonies on the MRS agar to enumerate *L. crispatus*; this method introduced some variability because the number of estimated *L. crispatus* CFUs was sometimes negative. Negative values were set to zero and included in the average as zero.

As is the case for the composition in CFU/ml at 0 hours, for the 1:1 ratio of *L. gasseri* to *L. crispatus*, there were $(7.1 \pm 5) \times 10^6$ CFU/ml to $(3.9 \pm 7) \times 10^6$ CFU/ml, respectively. The initial composition of the 10x LC co-culture was $(2.4 \pm 1.1) \times 10^6$ CFU/ml and $(6.1 \pm 6) \times 10^5$ CFU/ml for *L. iners* and *L. crispatus*, respectively, and the starting composition of the 10x LG co-culture was $(5.3 \pm 2) \times 10^6$ CFU/ml and $(2.4 \pm 2) \times 10^6$ CFUs/ml for *L. gasseri* and *L. crispatus*, respectively.

After the initial dilution, *L. gasseri* became the most dominant species in each condition. After the first dilution, *L. gasseri* ranged between $(9.6 \pm 2) \times 10^8$ to $(1.6 \pm 0.05) \times 10^9$, $(5.4 \pm 2) \times 10^8$ to $(1.2 \pm 0.1) \times 10^9$, and $(9.2 \pm 2) \times 10^8$ to $(1.5 \pm 0.2) \times 10^9$ CFU/ml for the 1:1, 10x LC and 10x LG co-cultures, respectively. However, we estimate that *L. crispatus* ranges between $(1.2 \pm 0.8) \times 10^8$ to $(3.7 \pm 1.1) \times 10^8$, $(3.4 \pm 5) \times 10^7$ to $(3.7 \pm 1.1) \times 10^8$, and $(1.2 \pm 1.0) \times 10^8$ to $(2.7 \pm 4) \times 10^8$ CFU/ml for the 1:1, 10x LC and 10x LG co-cultures, respectively.

Similar to *L. iners* and *L. gasseri*, no competitive interactions were detected between *L. crispatus* and *L. gasseri*. There was little change in abundance in either species after each dilution. *L. gasseri* also grows faster than *L. crispatus*, which is the speculated reason why *L. gasseri* has maintained dominance. In nature, *L. gasseri* is detected less frequently among women than *L. crispatus*, but in our competitive experiment, *L. gasseri* remained the most dominant species. This difference could be due to the presence or absence of factors more so from the host and other bacteria that were not present in MRS. Our method of subtracting CFUs introduced some error it did not account for the exact number of *L. crispatus*-colonies, and those that were negative values were set to zero.

3.3.6 Spent-medium experiments between *L. crispatus* and *L. gasseri*

To test whether interference competition may be present between *L. crispatus* and *L. gasseri*, we conducted spent-medium experiments. By carrying out spent-medium experiments, we may rule out inhibition as a mechanism that takes place in MRS broth. Currently, no literature has cited interference competition between them.

To determine whether interference interactions could be detected, we prepared two

sets of pure culture inoculums of *L. crispatus* and *L. gasseri* that were cultured until they reached stationary phase. The cell cultures were harvested, the cells were pelleted, sterile filtered with a 0.22 μ sterile filter, and removed, which left two tubes of cell-free *L. crispatus* (LC) and two tubes of cell-free *L. gasseri* (LG)-spent media. Each replicate of spent media was used to create two separate conditions: spent media and spent media with supplemented ingredients from MRS. The controls were 1x MRS notated as MRS, and phosphate buffer saline (PBS). Previous experiments showed 2x MRS did not have a toxic effect on *L. crispatus* or *L. gasseri*.

L. gasseri was cultured in LC-spent media with and without supplemented MRS broth as shown in Figure 3.6(a). After 24 hours, $(9.7 \pm 0.8) \times 10^8$ CFU/ml and $(1.1 \pm 0.13) \times 10^9$ CFU/ml were detected for both biological replicates of *L. gasseri* in LC-spent media. After 24 hours, the number of CFUs of both biological replicates of *L. gasseri* in LC-spent media that was respectively. This condition was also higher than the MRS condition, $P < 0.0001$ for both conditions. Differences between these conditions were found to be significant for biological replicates A and B, both were $P < 0.0001$. Figure 3.6(b) showed *L. gasseri* in LG-spent media. After 24 hours, CFUs of *L. gasseri* in both biological replicates of LG-spent media were $(3.5 \pm 0.3) \times 10^8$ and $(3.8 \pm 0.6) \times 10^8$ CFU/ml; in LG-spent media with MRS, there were $(2.0 \pm 0.3) \times 10^9$ and $(1.7 \pm 0.13) \times 10^9$ CFU/ml. *L. gasseri* CFUs were higher in LG-spent media with MRS after 24 hours compared to the MRS condition. Similarly, perhaps byproducts and signaling molecules from GASP could have increased the viability of *L. gasseri*. It's also worth noting that *L. gasseri* CFU is much higher, in some cases, 5 log greater than *L. iners*, which is likely due to the robustness of *L. gasseri* compared to the fastidiousness of *L. iners*. After 24 hours, there were $(1.9 \pm 0.4) \times 10^8$ CFU/ml of *L. gasseri*.

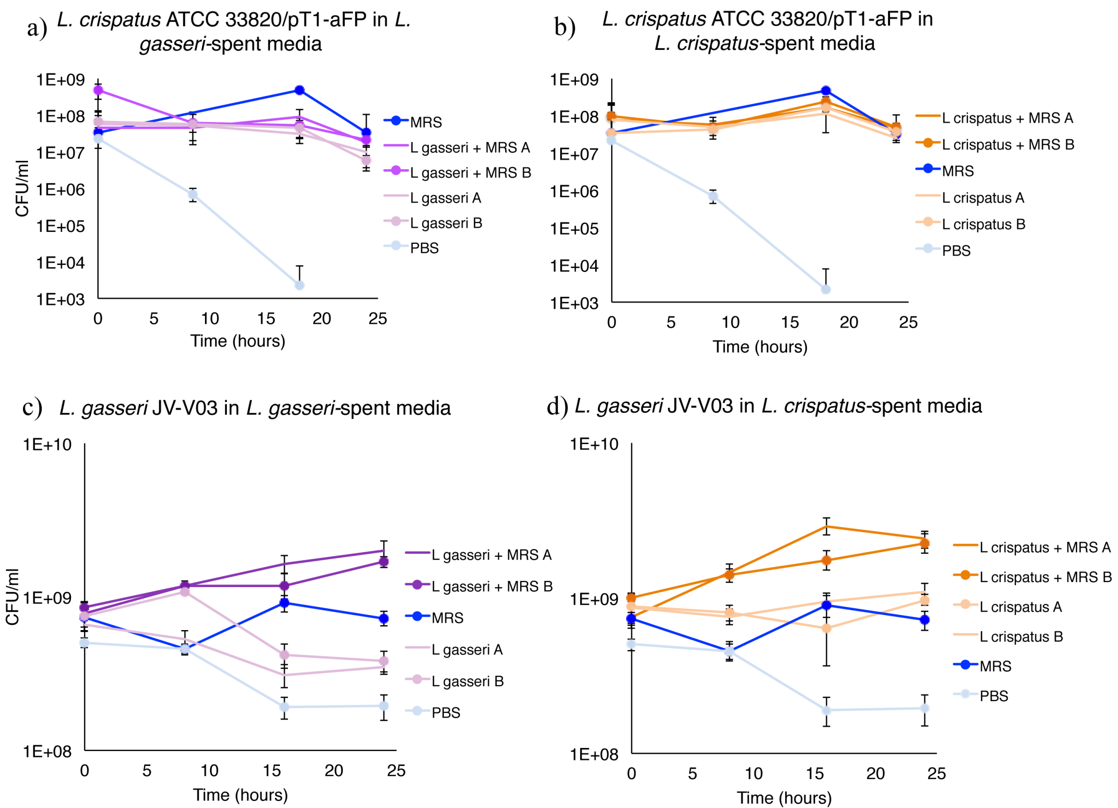


Figure 3.6: Spent-medium experiment for (a) *L. gasseri* in *L. gasseri* (LG)-spent MRS media, (b) *L. gasseri* in *L. crispatus* (LC)-spent MRS media, (c) *L. crispatus* in LG-spent MRS media, and (d) *L. crispatus* in LC-spent MNC media. Purple lines are LG-spent media with supplemented MRS. Pink lines are LG-spent media. Orange lines are LC-spent media with supplemented MNC. Yellow lines are LC-spent media. Royal blue line in MRS, and the light blue line is PBS.

Results in Figure 3.6 demonstrate no interference competition from *L. crispatus* to *L. gasseri* and vice versa. As shown by Figure 3.6(a), after 24 hours, there were more CFUs in LC-spent media after MRS was added which indicates additional nutrients improved growth of *L. gasseri* in both conditions; this suggests shared nutrients were depleted by *L. crispatus* in LC-spent media, that could have also been metabolized by *L. gasseri*. To date, no study explicitly compares the core and accessory genes of *L. gasseri* with *L. crispatus*. *L. gasseri* in spent media with no additional nutrients grew more than in PBS, though in PBS, enumerated CFUs were

$(1.9 \pm 0.4) \times 10^8$ CFU/ml, significantly higher than those of *L. iners*, which also attests to the robustness of *L. gasseri*. Ironically, *L. gasseri* did not grow as much in MRS. This is speculated to be due to byproducts and signaling molecules from the GASP phenotype that were absent in MRS broth. [133–136] Though there was a difference between spent media with and without supplemented, the number of CFUs present was not as distinct. *L. crispatus* trend in LG and LC-spent media was closer together although there were differences between both LC biological replicates of A and B and LG A; P = 0.0058, 0.0148, and 0.0029, respectively. However, all conditions were statistically higher than in PBS.

3.4 Conclusion

In conclusion, we have found that increasing concentrations of 2,2'-dipyridyl, a Fe^{2+} chelator, are inversely proportional to the growth of *L. iners* ATCC 55195, but have a neutral effect on the growth of *L. crispatus* ATCC 33820 pT1-aFP. The former is unsurprising as *L. iners* has been reported to grow in MRS upon the addition of 1-5% sheep or human blood, which contain iron. This is in support of other literature that lactobacilli usually require manganese as opposed to iron, another reason why *L. iners* is unique. [64] We have also found that concentrations of iron $\geq$ 10 mM $Fe(II)SO_4$ inhibits growth; reasons for this are unclear but we have speculated results to be linked to the Haber-Weiss reaction which involves Fenton chemistry, when H_2O_2 reacts with iron, a catalyst, to produce toxic hydroxyl radicals *in vivo*; this theory has not been tested, but has been shown in other biological systems. No competitive or interference interactions were detected between species in synthetic laboratory media. Synthetic laboratory media such as MRS and MNC have many ingredients that are indistinct, and some are in higher concentrations than in the vagina. The complex media introduces additional challenges when identifying the

nutrients and mechanisms for resource competition. [69] The biotic and abiotic factors in the vagina were not the same in the co-culture experiments in MRS and MNC broth, which would hinder the phenomena of conditional differentiation, and even temporal fluctuations. Also, in the vagina, nutrients are replenished. We mimicked this by conducting a serial dilution co-culture experiment. More work would need to be done to create a cultivation medium closer to the natural condition, and to control the influx of nutrients to diligently regulate the kinds and availability of nutrients to recapitulate competitive interactions like those in the HVM between *Lactobacillus* species.

3.5 Material and methods

3.5.1 Bacterial strains and cultivation protocols

L. crispatus ATCC 33820 pT1-aFP was obtained from the Forney lab and was originally from ATCC in Rockville, MD. *L. gasseri* JV-V03 was obtained from the Forney lab and was originally isolated from the vagina. [76] *L. iners* ATCC 55195 was isolated from the human vagina and was isolated from the American Type Culture Collection (ATCC). [76] To start a seed culture, a cryostock was streaked onto agar medium. *L. iners* was streaked onto ATCC medium 260 (Tryptic Soy agar) with 5% sheep blood and incubated for 36 to 48 hours. *L. crispatus* and *L. gasseri* were streaked on MRS agar and incubated for 18 hours. Then, a single colony was inoculated into pre-warmed media. Initial seed cultures of *Lactobacillus crispatus* ATCC 33820 pT1-aFP and *L. gasseri* JV-V03 were grown in ATCC medium 416 (MRS: 20 g/liter glucose, 10 g/liter casein peptone, 10 g/liter meat extract, 5 g/liter yeast extract, 2 g/liter K_2HPO, 0.2 g/liter $MgSO_4$, 0.05 g/liter $MnSO_4$, 5 g/liter Na-acetate, 2 g/liter $(NH_4)_2$ citrate, 1 g/liter Tween 80) broth at pH 4. Initial seed cultures of

Lactobacillus iners ATCC 55195 were grown in MNC (10g/liter proteose peptone, 10 g/liter beef extract, 5 g/liter yeast extract, 5 g/liter, NaCl, 0.1 g/liter $MgSO_4$ 0.05 g/liter $MnSO_4$, 2 g/liter K_2HPO_4, 20 g/liter glucose, 100 ml/liter horse serum) broth, a compromise medium of MRS broth and ATCC medium 1685 (NYC III) broth. All species were grown in an anaerobic chamber or BD GasPak™ EZ Anaerobic System at 37∘C with no agitation. The pT1-aFP (evoglow) plasmid constitutively expresses fluorescence in aerobic and anaerobic environments; no antibiotic was used during cultivation. [137]

3.5.2 Methods for determining the effects of 2,2'-bipyridyl and Fe(II)SO_4 at 0 and 24 hours

A 100 mM stock solution of Fe(II)SO_4 was prepared in 10 ml MNC broth; 100 mM Fe(II)SO_4 was prepared in MNC broth. A 2,2'-bipyridyl stock solution was prepared by dissolving 50 mM 2,2'-bipyridyl in 10 ml 95% (v/v) ethanol. We prepared 1.5 mM 2,2'-bipyridyl by aliquoting 300 μL 50 mM 2,2'-bipyridyl in 9.7 ml MNC; 1 mM, 0.5 mM, and 0.1 mM 2,2'-bipyridyl were prepared by diluting 1.5 mM 2,2'-bipyridyl in MNC broth. A 300 μL aliquot of ethanol was added to 9.7 ml MNC. Two 3 ml pure seed cultures of *L. crispatus* ATCC 33820 pT1-aFP and *L. iners* ATCC 55195 were cultured in a 12 ml culture tube with a blue snap cap from Fisherbrand (Waltham, MA, US) overnight; densities of both seed cultures were normalized using a VersaMax™ Microplate Reader from Molecular Devices (San Jose, CA, US). An inoculum volume of 6.6 μL of *L. crispatus* and *L. iners* pure seed cultures was pipetted into separate 1.5 ml Eppendorf tubes with 653.4 μL of the following: 2,2'-bipyridyl (1.5 mM, 1 mM, 0.5 mM, 0.1 mM), Fe(II)SO4 (100 mM, 10 mM, 1 mM, 0.1 mM), MNC broth, and MNC + 3% ethanol. Three 200 μL aliquots of each of those conditions were pipetted into a 96-well microplate. Cultures were agitated with a

pipette before their ODs were measured at 0 and 24 hours at 600 nm.

3.5.3 Methods for examining effects of 2,2'-bipyridyl and Fe(II)SO_4 on *L. iners*

A 3 ml seed culture of *L. iners* was cultured in a 12 ml culture tube overnight. *L. iners* seed culture was diluted 100-fold and the following conditions were prepared: MNC broth, MNC broth supplemented with 2,2'-bipyridyl (1.5 mM, 1 mM, 0.7 mM, 0.4 mM, 0.1 mM) and Fe(II)SO_4 (0.1 mM, 0.01 mM). Three 200 μL aliquots of each condition were pipetted into a 96-well microplate. Cultures were agitated with a pipette before their ODs were measured at 0, 6, 12, 20, and 32 hours at 600 nm.

3.5.4 Methods for culturing *L. iners* in *L. crispatus*-spent MNC broth

Two biological replicates of *L. crispatus* ATCC 33820 pT1-aFP were cultured in MNC broth for 24 hours in 12 ml culture tubes. Cells were removed by centrifuging cultures in an Eppendorf Centrifuge 5810 R from Fisher Scientific (Waltham, MA, US), and using a 0.22 μ sterile filter. Both replicates of cell-free *L. crispatus* (LC)-spent-media were supplemented with 1 g/liter glucose and different concentrations of Fe(II)SO_4. The conditions were the following: MNC broth, LC-spent media with 1 g/liter glucose (0 g/liter Fe(II)SO_4, 0.001 g/liter Fe(II)SO_4, 0.01 g/liter Fe(II)SO_4, 0.1 g/liter Fe(II)SO_4, and 1 g/liter Fe(II)SO_4); the pH was adjusted to 7 using 5M NaOH. A 100-fold dilution was prepared. Each culture was grown in 2 mL in a 15 ml falcon tube. Cultures were agitated with a pipette before their ODs were measured in a 96-well microplate at 0, 9, and 20 hours at 600 nm; there were two biological replicates for each condition.

3.5.5 Methods for co-culturing *L. crispatus* and *L. iners*

A single colony of *L. iners* was inoculated into a 12 ml culture tube with 2 ml pre-warmed MNC broth and incubated overnight. A single colony of *L. crispatus* was inoculated into a 12 ml culture tube with 5 ml pre-warmed MNC broth and incubated overnight. Then, both cultures were suspended in a 20-fold dilution and incubated for 6 hours to ensure they will be in exponential phase. Afterward, the ODs of *L. iners* and *L. crispatus* seed culture densities were equalized. Then, 1,980 μL MNC broth was added to 16 12-ml glass culture tubes from Kimble Chase (Austin, TX, US): three tubes were labeled '1:1'; three more tubes were labeled '10xLC'; three more tubes were labeled '10xLI' and the remaining six tubes were used to culture *L. iners* and *L. crispatus* as monocultures in triplicate. Both 100-fold and 1,000-fold dilutions were prepared from *L. crispatus* and *L. iners* monocultures. The '1:1' was prepared by adding 10 μL of the 100-fold dilution of each species to 1,980 μl MNC tubes labeled '1:1'. The condition with '10xLC' was prepared by adding 10 μL of the 100-fold dilution of *L. crispatus* and 10 μL of the 1,000-fold dilution of *L. iners* to 1,980 μl MNC tubes labeled '10xLC'. The condition with '10xLI' was prepared by adding 10 μL of the 100-fold dilution of *L. iners* and 10 μL of the 1,000-fold dilution of *L. crispatus* to 1,980 μl MNC tubes labeled '10xLI'. After 1:1 and 10xLC conditions incubated for 10 hours and 10xLI incubated for 13 hours, the cultures underwent the following steps: (1) agitation with a vortex, (2) all co-cultures were diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} and plated using the single plate-serial dilution spotting (SP-SDS) technique, [117] (3) the OD was measured for all cultures, (4) aliquots of 20 μL of each co-culture were inoculated into 1,980 μl fresh MNC broth, co-cultures were diluted 100-fold and their ODs were measured, and (5) co-cultures and pure cultures were incubated without agitation for 8 to 11 hours. Afterward, steps 1-5 were repeated four times until five dilutions were achieved.

3.5.6 Methods spent-medium experiments between *L. crispatus* and *L. iners*

A single colony of *L. iners* and *L. crispatus* was inoculated into MNC media at pH 7; two biological replicates were prepared from each and they were incubated until they reached stationary phase. Cells of *L. iners* and *L. crispatus* were removed from their cultures by centrifugation and using a 0.22 μ sterile filter. Remaining cells of *L. iners* and *L. crispatus* were washed three times in PBS and suspended in the following conditions: LI and LC-spent media, LI and LC-spent media with 1x MNC powder, LI and LC-spent media with 2x MNC powder, MNC broth, and PBS. Cultures underwent SP-SDS and were plated at 0, 6, and 24 hours.

3.5.7 Methods for co-culturing *L. crispatus* and *L. gasseri*

A single colony of *L. gasseri* was inoculated into a 12 ml culture tube with 6 ml pre-warmed MRS broth and incubated overnight. A single colony of *L. crispatus* was inoculated into a 12 ml culture tube with 5 ml pre-warmed MRS broth and incubated overnight. Then, both cultures were suspended in a 20-fold dilution and incubated for 4 hours to ensure they will be in exponential phase. Afterward, the ODs of *L. gasseri*and *L. crispatus* seed culture densities were equalized. Then, 1,980 μL MRS broth was added to 16 12-ml glass culture tubes: three tubes were labeled '1:1'; three more tubes were labeled '10xLC'; three more tubes were labeled '10xLG' and the remaining six tubes were used to culture *L. gasseri* and *L. crispatus* as monocultures in triplicate. Both 100-fold and 1,000-fold dilutions were prepared from *L. crispatus* and *L. gasseri* monocultures. The '1:1' was prepared by adding 10 μL of the 100-fold dilution of each species to 1,980 μL MRS tubes labeled '1:1'. The condition with '10xLC' was prepared by adding 10 μL of the 100-fold dilution of *L. crispatus* and 10 μL of the 1,000-fold dilution of *L. gasseri* to 1,980 μL MRS tubes labeled

'10xLC'. The condition with '10xLG' was prepared by adding 10 μL of the 100-fold dilution of *L. gasseri* and 10 μL of the 1,000-fold dilution of *L. crispatus* to 1,980 μL MRS tubes labeled '10xLG'. After 1:1 and 10xLC conditions incubated for 10 hours and 10xLG incubated for 13 hours, the cultures underwent the following steps: (1) agitation with a vortex, (2) all co-cultures were diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} and plated using the single plate-serial dilution spotting (SP-SDS) technique,40 (3) the OD was measured for all cultures, (4) aliquots of 20 μL of each co-culture were inoculated into 1,980 μL fresh MRS broth, co-cultures were diluted 100-fold and their ODs were measured, and (5) co-cultures and pure cultures were incubated without agitation for 6 to 8 hours. Afterward, steps one through five were repeated four times until five dilutions were achieved.

3.5.8 Methods for spent-medium experiments between *L. crispatus* and *L. gasseri*

A single colony of *L. gasseri* and *L. crispatus* was inoculated into MRS media at pH 4; two biological replicates were prepared from each and they were incubated until they reached stationary phase. Cells of *L. gasseri* and *L. crispatus* were removed from their cultures by centrifugation and using a 0.22 μ sterile filter. Remaining cells of *L. gasseri* and *L. crispatus* were washed three times in PBS and suspended in the following conditions: LG and LC-spent media, LG and LC-spent media with 1x MRS powder, MRS broth, and PBS. Cultures underwent SP-SDS and were plated at 0, 8.5, 18, and 24 hours.

Chapter 4

Technology development for cultivating vaginal bacteria in microdroplets using human cervicovaginal fluid

4.1 Summary

In Chapter 4, the objective is to extend our technology to enable the cultivation of vaginal bacteria in microdroplets using cervicovaginal fluid (CVF) collected from female donors. We collected 49 samples of CVF from nonpregnant, healthy women of reproductive age. We pooled these samples based on similarities in the most abundant taxa, and we developed a protocol to utilize the pooled human CVF to culture lactobacilli in microdroplets, which are advantageous when handling small fluid volumes. We report that most of our women subjects had a high prevalence of *L. crispatus* (LC). Pooled LC-dominated CVF was prepared and used to culture *L. crispatus* ATCC 33820, *L. gasseri* JV-V03, and *L. iners* ATCC 55195. In LC-CVF at pH 4, *L. crispatus* survived and *L. gasseri* decreased in viability after 24 hours, but *L. iners* was killed within just 12 hours. However, in LC-CVF that was neutralized to pH 7, *L. iners* grew. Such results raise questions as to the effect of pH on microbial fitness and competitive ability in the HVM. Advances we have made in Chapter 4 provide a foundation for future work towards elucidating interspecies

interactions relevant to the *in vivo* environment using a microdroplet co-cultivation technology platform.

All of the work presented in this chapter was previously unpublished.

4.2 Introduction

4.2.1 Culture-media simulating cervicovaginal fluid

Culturing bacteria in vaginal fluid offers the advantage of studying bacteria and interactions between them, while simulating a vaginal environment. However, this concept has seldom been applied in published studies due to the infeasibility of using such limited volumes of CVF. Most often, lactobacilli are cultured in MRS broth with the exception of *Lactobacillus iners*, that is cultured in NYC III broth. However, both media contain a myriad of unspecified peptides that projects challenges when comparing the presence and quantity of molecules in MRS and NYC III broth with those in CVF, not to mention, their differences in molarity and osmolarity. [24, 69] Consequently, researchers have designed several media that simulate the vaginal environment based on the chemical composition and pH of CVF, and some have supplemented growth factors such as a vitamin mix (Table 4.1) to promote the grow of nutrient exigent species that are endogenous to the vagina. [138–140]

In recent years, three different liquid media have been developed with osmolarities, molarities, and pH values that are comparable to vaginal fluid as shown in Table 4.1. [78, 138, 140] In 1992, Geshnizgani and Onderdonk created a chemically defined medium (CDM), which was used to grow *Lactobacillus acidophilus, Bacteroides fragilis*, and others species. [138] In 1999, Owen *et al.* created a vaginal fluid stimulant

Table 1. Comparative composition of different culture media simulating vaginal fluid.

Component	Vaginal fluid [a] (g/l)	CDM [b] (g/l)	VFS [c] (g/l)	MSVF [d] (g/l)
Glucose	6.2-10	10.80	5.00	10.00
Glycogen	15	1.00	-	10.00
Glycerol	0.16	-	0.16	-
Lactic acid	0.9-4.0	-	2.00	2.00
Acetic acid	0.52	-	1.00	1.00
Albumin	0.018-3.75	2.00	0.018	2.00
Mucin	0.25	0.25	-	0.25
Urea	0.49	0.50	0.40	0.50
NaCl	Na^+: 1.00-1.95; Cl^-: 2.20-2.94; K^+: 0.55-1.17; Ca^{2+}: 0.12	3.50 (Na^+: 1.38; Cl^-: 2.12)	3.51(Na^+: 1.38; Cl^-: 2.13)	3.50 (Na^+: 1.38; Cl^-: 2.12)
KCl		1.50 (K^+: 0.79; Cl^-: 0.71)	-	1.50 (K^+: 0.79; Cl^-: 0.71)
KOH		-	1.40 (K^+: 0.97)	-
$Ca(OH)_2$		-	0.222 (Ca^{2+}: 0.12)	-
K_2HPO_4	-	1.74	-	-
KH_2PO_4	-	1.36	-	-
$MgSO_4$	-	0.30	-	-
$NaHCO_3$	-	0.04	-	-
Tween 80	-	0.20	-	1.064
Cystein-HCl	-	0.50	-	0.50
Hemin	-	0.50	-	-
Vitamin K	-	0.10	-	-
Vitamin mix [e]	-	5 ml of 100X solution	-	-
pH	Normal: < 4.5	7.2	4.2	4.25 ± 0.05

[a] The vaginal fluid contains other components non detailed in this Table: others short chain aliphatic acids (formic, succinic, propionic, butyric acids), immunoglobulins, antimicrobial peptides and proteins, free amino acids, etc. [6, 7]

Table 4.1: Comparison of the chemical compositions of vaginal fluid vs. recipes of chemically defined medium (CDM), vaginal fluid simulant (VFS), and medium-simulating vaginal fluid (MSVF). Reproduced from [140].

(VFS) to emulate a similar physicochemical environment for implications of investigating how drugs are released in the vagina. [139] In 2007, Tomás *et al.* developed a medium-simulating vaginal fluid (MSVF) to investigate its effects on the growth of potentially probiotic microorganisms. [140]

Although the aforementioned medium-simulated vaginal media are comparable to vaginal fluid, they have many differences and do not adequately reflect the vaginal environment. For example, over 600 proteins have been identified in CVF that were not included in the medium-simulating vaginal fluid. [141] In addition, other growth factors that were not detected in CVF were added to the media such as Tween 80 and vitamin mix. [138,140] The presence and absence of abiotic and biotic factors and nutrients can influence the phenotype, metabolism, microbial interactions, and overall expression of microorganisms. [17, 142] Further work and investigation is needed for culturing bacteria and investigating their interactions in an environment reflecting their vaginal environment.

4.2.2 Vaginal metabolome and microbial composition

Cervicovaginal fluid (CVF) is a mixture of liquids and many compounds originating from the vagina, cervix, endometrium and oviduct. [141] Some of these compounds have gained recognition for their role in the innate immune defenses. [141] CVF is often composed of exfoliated epithelial cells, menstrual material, residual urine, and semen. [139] Classes of compounds that have been detected in CVF are salts, carbohydrates, low molecular weight organic compounds such as lactic acid, acetic acid, glycerol, urea, glucose, proteins, and other components. [139] CVF usually contains cationic peptides, lysozyme, lactoferrin, secretory leukocyte protease inhibitors, human neutrophil peptides, and human â-defensins that have been identified as an-

timicrobial substances in CVF. [141] Despite the complexity of CVF, metabolomic approaches have been helpful for identifying certain molecules as potential biomarkers for pathological conditions such as bacterial vaginosis (BV). [139, 143, 144]

Both targeted and untargeted metabolomics have been employed to examine human vaginal metabolomes during pregnancy and the onset of BV. [12, 38, 145] Although McMillan *et al.* detected no biomarkers distinguishing pregnant women from nonpregnant women, McMillan *et al.* and several other researchers have detected strong correlations between specific metabolites, microbial diversity and clinical BV. [145] Metabolites that were linked to BV included 2-hydroxyisovalerate and γ-hydroxybutyrate. [145] According to another study by Srinivasan *et al.*, succinate along with other amino acid catabolites, polyamines and eicosanoid 12-hydroxyeicosatetraenoic (12-HETE), a biomarker for inflammation, positively correlated with clinical BV. [12] In a study by Vitali *et al.*, 17 molecules were identified that were previously undetected, which amounted to 32 molecules with significantly different concentrations in healthy women compared to those with BV; malonate, and acetate were associated with BV, while maltose, kynurenine, and NAD^+ were not. [38] Metabolomics have been essential for identifying molecules in vaginal fluid and have also linked certain metabolites to women's risk for BV and vaginal microbial composition.

Metabolomic profiles from vaginal fluid may be indicative of vaginal microbial composition. [1,12,38,145] In Srinivasan *et al.*'s study, of the 279 biochemicals identified, 62% of them were detected from women with BV, and the difference was detected in healthy women who were colonized by lactobacilli and characterized with more amino acids and dipeptides. [12] Women with a high prevalence of *L. crispatus* and

L. jensenii also had similar metabolomic profiles that were distinct from women with BV. [12] Consequently, vaginal fluid from women with a high prevalence in the same kinds of bacteria may have similarities in their metabolic profiles.

4.2.3 Human cervicovaginal fluid to cultivate lactobacilli in microdroplets

Few studies have used vaginal fluid to culture vaginal bacteria or study their interactions. In 2002, Valore *et al.* grew vaginal bacteria in vaginal fluid to examine its antimicrobial activity against bacterial species. [78] Valore *et al.* reported that vaginal fluid from five of five female donors promoted the growth of *L. crispatus, L. vaginalis*, and *C. albicans*, but not for *Streptococcus, E. coli*, and *L. jensenii* in three of five donors as shown in Figure 4.1. [78] This finding indicates differences in antimicrobial activity across donors. Valore *et al.* is one of the few researchers who have incubated vaginal bacteria in vaginal fluid.

Growing bacteria in CVF offers the advantages of confining small volumes of fluid to stochastically confine cells, and closely simulating the vaginal environment while conducting high-throughput experiments that reduce cost, time, and labor. [81] However, challenges on the optimal method(s) for collecting vaginal fluid, and how to increase the throughput can enhance or hamper growth outcomes. Vaginal fluid is typically collected in minute amounts. Consequently, many methods that collect vaginal fluid involve diluting the CVF by as much as 50 ml, and in some cases, lyophilizing CVF to return the volume closer to its original volume. [78] A cervicovaginal lavage (CVL) is a common technique that uses aspiration of a solution to retrieve compounds and fluid from the vagina and cervix. For instance, Van Raemdonck *et al.* collected CVL samples that were rinsed with 25-40 ml of 5% acetic acid, which was then lyophilized to 200 μL. [146] Conversely, Softdiscs™ , a nonabsorbent, flexible, over-the-counter

product similar to a diaphragm that is advertised to collect menstrual fluid, can also collect CVF; no dilution would be needed. Nunn *et al.* and other scientists have used the Instead® Softdisc™, to collect CVF and cervicovaginal mucus (CVM), but little to no published data demonstrates the ability to incubate vaginal bacteria in CVF from a Softdisc™ due to the small amount of CVF and intrinsically low number of replicates. [28, 73, 147, 148]17-20

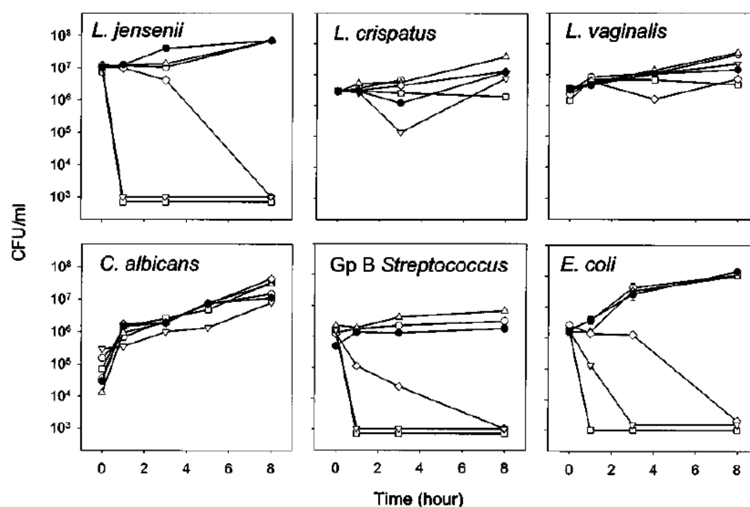


Figure 4.1: Antimicrobial activity of vaginal fluid. Microbes added were *L. jensenii, L. crispatus, L. vaginalis, Candida albicans*, group B *Streptococcus*, and *Escherichia coli* incubated at 37°C. Samples included genital tract buffer control (closed circles), donor 1 (open circles), donor 2 (open squares), donor 3 (open triangles), donor 4 (open diamonds), donor 5 (open inverted triangles). Reproduced from [78].

Microdroplets offer a unique advantage of performing high-throughput experiments while using minimal volume of chemical reagent or biological sample. [96, 149–151] Microdroplets stochastically confine a range of cells or even microbial communities at the nanoliter scale. For instance, a single microdroplet is approximately 6.14×10^{-4} μL whereas a single well on a 384-well plate requires 40 μL, approximately 65,000 microdroplets. Using microdroplets to culture CVF proposes a novel way to cultivate vaginal bacteria in a more natural environment.

4.3 Results and discussion

4.3.1 Overview

In this research, we investigated whether pooled CVF could be used to grow *Lactobacillus crispatus* ATCC 33820 pT1-aFP, *L. gasseri* JV-V03, and/or *L. iners* ATCC 55195. *L. crispatus* and *L. iners* were selected because they are the most prevalent species in the HVM, and transitions between them occur often. [33, 34] Frequent transitions are indicative of substantial fundamental niche overlap. [68] *L. gasseri* is 1 of 4 vaginal lactobacilli, but is less prevalent than *L. crispatus* and *L. iners*. A small N may have been the reason for no transitions being reported between it and *L. crispatus* according to Gajer's study, yet it is one of the most dominant lactobacilli in the HVM. [34] Collectively, our approach suggests an alternate, well-defined medium from the natural environment to more carefully examine how species expression and metabolism is influenced by vaginal fluid.

We aimed to cultivate *Lactobacillus* species in pooled CVF to understand how bacteria behave in a more natural environment without diluting the CVF using microdroplets. Samples were collected from healthy non-pregnant college students of reproductive age. Only 30 μL vaginal fluid was used to generate each microdroplet experiment. Vaginal samples were processed and analyzed with 16S rRNA gene sequencing. The most widely occurring taxa that highly colonized CVF samples were combined with other CVF samples with the most dominant species to increase the volume of CVF.

4.3.2 Collecting human vaginal bacteria and CVF

Donors used Instead® Softdiscs™ to self-collect CVF and dual-headed rayon swabs to self-collect vaginal bacteria for this study from April 2017 to June 2019. A dual-headed swab was selected because one swab was to be submitted for 16S rRNA gene sequencing and the other was for culturing bacteria. Vaginal swabs were submitted to the Microbial Community Analysis Core at the University of Michigan for genomic DNA extraction, standard polymerase chain reaction (PCR), and 16S rRNA gene sequencing. [152, 153] Once the vaginal swabs underwent 16S DNA sequencing, their corresponding CVF samples were sterilized with a 0.22 μ filter and pooled to use as a culture medium. We measured the volume and pH of the aqueous phase by withdrawing the fluid into a pipette until all of the volume was removed.

An open-source platform-independent community-supplied software, mothur, was used to process the 16S rRNA gene sequences from the vaginal specimens. [152, 154] The Dual indexing strategy offers the advantage of processing many samples in a single run. A total of 49 samples were processed using mothur. The SILVA database [155] was used as our reference, which has a resolution to the genus level. To achieve this, we customized the SILVA database to include species-specific sequences of 15 *Lactobacillus* bacteria: *Lactobacillus crispatus, L. gasseri, L. jensenii, L. iners, L. acidophilus, L. brevis, L. casei, L. reuteri, L. fermentum, L. ruminis, L. vaginalis, L. salivarius, L. plantarum, L. oris,* and *L. delbruecki.* Original *Lactobacillus* sequences in the SILVA file were only at the genus level and were all subsequently replaced by 15 species-specific sequences and names. The 16S rRNA gene sequences of several strains for each of the 15 *Lactobacillus* species were obtained from the National Center for Biotechnology Institute (NCBI).

We made a consensus sequence from different strains in the V4 region of the 16S rRNA gene. The V4 region started just before the 500th nucleotide and ended just before the 800th nucleotide, which corroborates with the variable regions of the 16S ribosomal RNA in a review by Yarza *et al.*. [156] We then aligned sequences to preserve positional homology across each sequence. We also used a cutoff of 99% similarity to increase the resolution when clustering sequences into OTUs.

4.3.3 Analyzing vaginal samples

Vaginal microbial composition has shown concordance with the microflora of the same woman's cervix. [144] We tested for concordance between four vaginal swabs (VS) and CVF or cervicovaginal secretions (CVS) samples from four different subjects as shown in Figure 4.2. Results indicated concordance between CVF and swab samples for each donor. The percentage of *L. crispatus* in vaginal swabs (VS) and CVF in donor 16 was 98% and 95%, respectively. The percentages of *L. crispatus* and *L. gasseri* in VS were 53% and 42%, respectively, and in CVF were 42% and 52%, respectively in donor 38. The percentage of *L. iners* in VS and CVF in donor 46 was almost 100% for both strains, and 94% and 95% *L. crispatus* for donor 8.

Operational taxonomic units (OTUs) were categorized into groups of *L. jensenii, L. iners, L. gasseri, L. crispatus*, other *Lactobacillus*, and others as shown in Figure 4.3(a). Taxa were considered dominant in a subject if its relative abundance was $\geq$ 85%. A subject that is mostly colonized by a taxa refers to having $\geq$ 85% threshold. Contrary to other studies that use a threshold of $>$ 50%, an 85% threshold may be more indicative of supporting the growth of the most dominant taxa at the time of sample collection. No subjects had a high prevalence of *L. jensenii*, which was synonymous with subjects 43 and 46 that were mostly colonized by *L. gasseri*; which

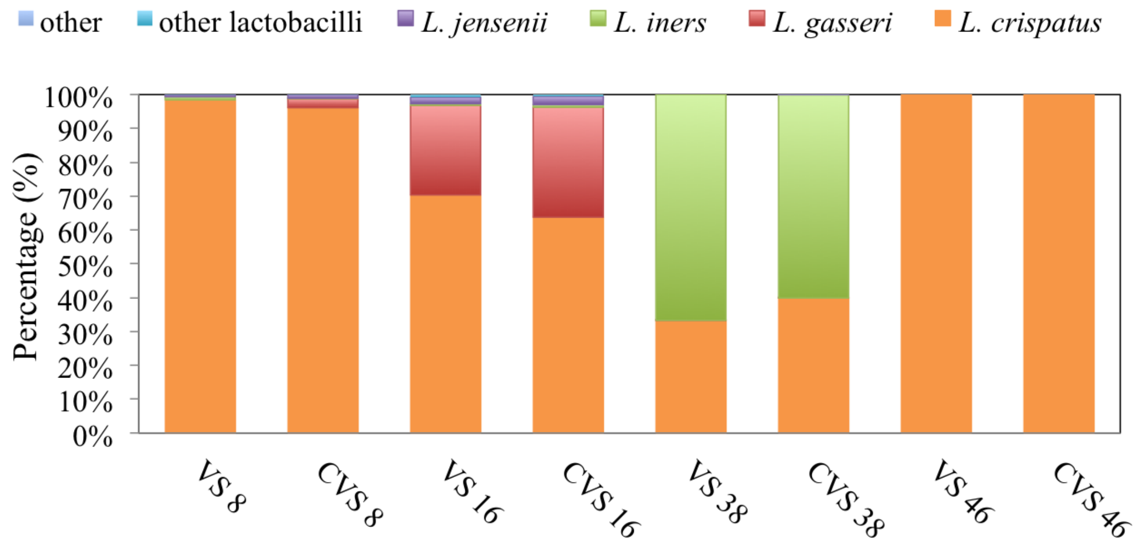


Figure 4.2: Relative abundances of different taxa in the vaginal microbiome of four donors who donated vaginal swabs (VS) and cervicovaginal secretions (CVS). Donors are shown by numbers 8, 16, 38, and 46.

is also synonymous with the low number of women who had a high prevalence of *L. gasseri* in previous studies. [1] In our study, two women were dominated by *L. iners*. However, 25 women were mostly colonized by *L. crispatus* as shown in Figure 4.3(b).

4.3.4 Creating pooled CVF

Not all CVF samples included in the pooled CVF that was used in this study. First, we combined CVF from the most abundant taxa across all subjects, *L. crispatus*. Thus, the term pooled *L. crispatus* (LC)-dominated CVF comes from combining CVF from donors who were mostly colonized by LC. Subjects who were mostly colonized by different taxa other than *L. crispatus* were not included in the pool of CVF in this study. Second, CVF that was collected earlier in the study was

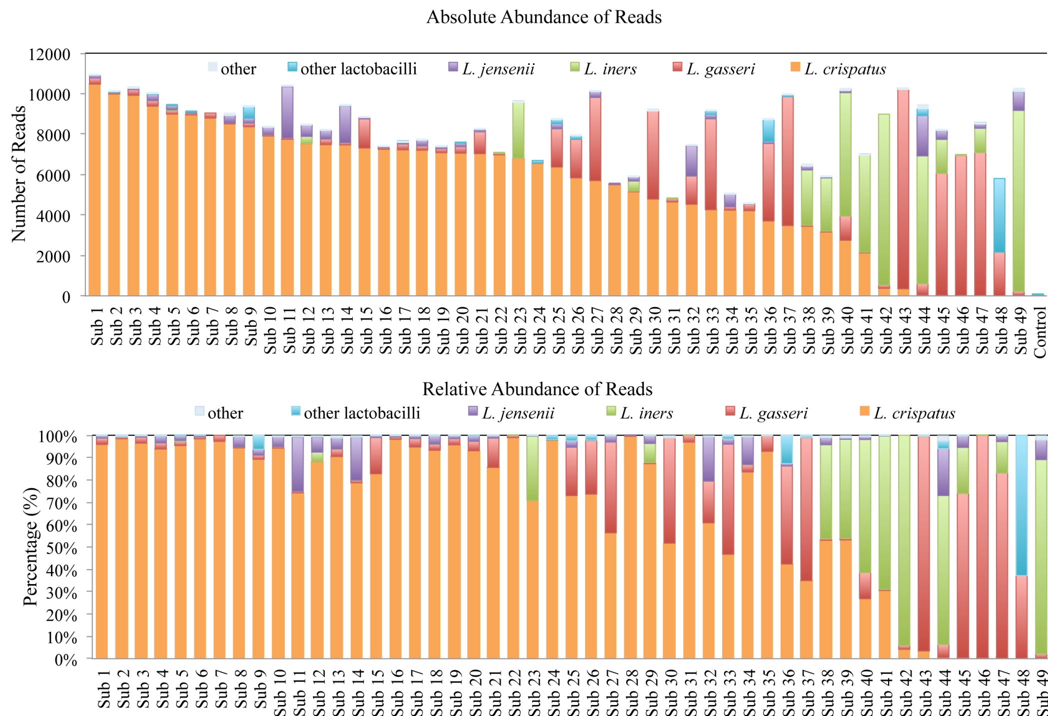


Figure 4.3: Absolute (a) and relative (b) abundances of reads from vaginal samples of donors. There were 49 samples in total. TE50 is a control made of TE buffer.

used to conduct preliminary experiments whereas additional CVF samples that were collected later in the study contributed to a different pool of CVF. Consequently, the samples considered for pooled LC-CVF used for this study were from subjects 1, 2, 5, 8, 10, 12, 13, 15, 16, 18, 19, 28, 31, 34, and 35. Third, we normalized the volumes of CVF for each subject. Of the subjects with a high prevalence of LC, volumes of CVF ranged between 5.5 μL to 872 μL with a median of 183 μL and an average of 218 ± 234 μL; by combining samples of varying volumes, biases may be introduced to the pooled CVF. To surmount any difficulties with generating droplets with a small amount of CVF for a single droplet experiment, approximately 30 μL, we decided that subjects with ≥ 40 μL CVF would be included in the pool. Consequently, 15

of 25 LC-CVF samples were included in the pooled LC-CVF.

The pH of all of the LC-CVF samples was measured and averaged as 4.0 ± 0.18 using pH indicator strips; Riedewald *et al.* reported no significant difference between measuring vaginal pH with pH indicator strips or a pH electrode. [157]

4.3.5 Culturing in pooled CVF

Lactobacillus crispatus ATCC 33820 pT1-aFP was grown in LC-CVF microdroplets as shown in Figure 4.4. Figure 4.4(a-b) are *L. crispatus* at 0 hours and Figure 4.4(c-d) are *L. crispatus* after 24 hours. Figure 4.4(a & c) shows fluorescence of *L. crispatus*; the pT1-aFP evoglow plasmid constitutively expresses neon green fluorescence under anaerobic and aerobic conditions and was provided to us by the Forney Lab. [137] Figure 4.4(a & c) show overlay images of *L. crispatus* in microdroplets; an overlay image is when the fluorescent and bright-field images are superimposed to form one image.

L. iners at pH 7 was the only condition that supported growth of a *Lactobacillus* species in LC-CVF after 12 hours. Results from a previous preliminary experiment showed growth of *L. crispatus* ATCC 33820 and *L. gasseri* JV-V03 in pooled CVF at pH 4, but *L. iners* at pH 4 did not grown (data not shown). Using 5M NaOH and a different and most recent batch of pooled CVF, the pH was ionized to 7 to determine whether an increase in pH could potentially stimulate *L. iners* growth. PH 7 was chosen because it is thought that *L. iners* prefers a neutral pH compared to other vaginal lactobacilli. In the latter experiment, *L. iners* at pH 7 did grow after 12 hours as shown in Figure 4.5(h), but *L. crispatus, L. gasseri*, and *L. iners* at pH 4 did not grow after 24 hours. Figure 4.5 shows the growth of each condition at

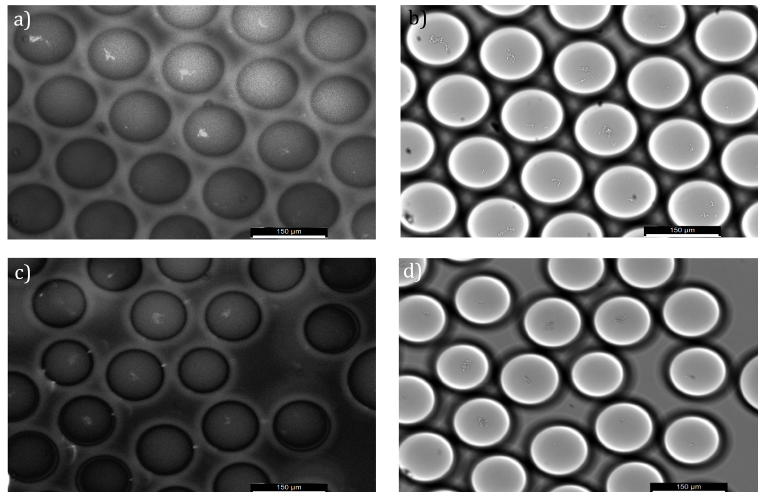


Figure 4.4: *Lactobacillus crispatus* ATCC 33820/pT1-aFP in pooled *L. crispatus*-dominated CVS: an overlay image at 0 hours (a), a bright-field image at 0 hours (b), an overlay image at 24 hours (c), and a bright-field image at 24 hours (d).

0 and 12 hours: Figure 4.5(a-d) shows each condition at 0 hours and Figure 4.5(e-h) shows each condition in the same order after 12 hours. Empty droplets were also generated and incubated, and showed no growth after 12 hours. [19, 56, 60, 123]

The number of colony-forming units (CFU) of each *Lactobacillus* species that grew in LC-CVF droplets as shown in Figure 4.6 were recorded at 0, 12, and 24 hours. One microliter of LC-CVF microdroplets was withdrawn from the 1.5 ml microcentrifuge tube, plated onto agar plates at 0, 12, and 24 hours, and enumerated after one to two days of incubation. *L. crispatus* showed no change in the number of CFUs between 0 and 24 hours. However, *L. gasseri* decreased from $(6.6 \pm 0.2) \times 10^6$ CFU/ml to $(3.3 \pm 0.3) \times 10^5$ CFU/ml after 24 hours, $P < 0.0001$. Previous results showed *L. crispatus* and *L. gasseri* grew in CVF that was pooled randomly (data not shown). *L. iners* at pH 7 increased from $(1.1 \pm 0.05) \times 10^7$ CFU/ml to $(4.7 \pm 3) \times 10^9$ CFU/ml after 12 hours; however this was not quite statistically significant, $P = 0.0537$. *L.*

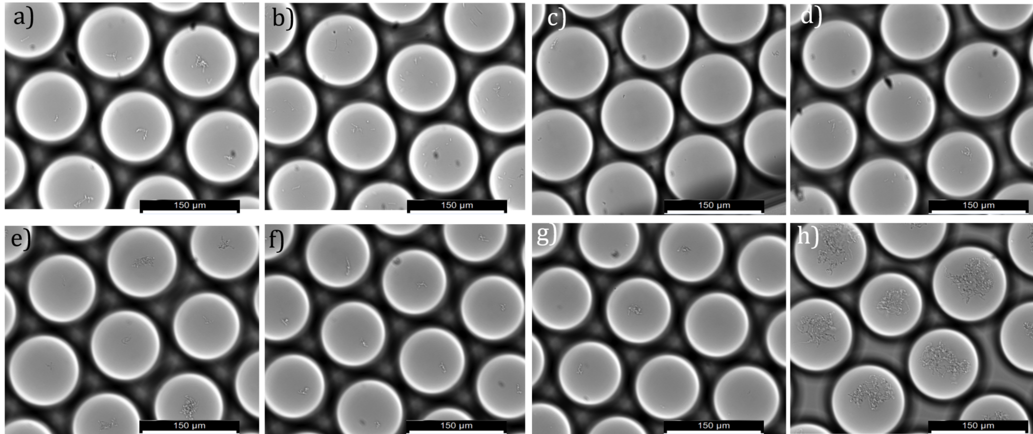


Figure 4.5: Pure cultures of *Lactobacillus* species in pooled LC-CVS at 0 hours (a-d) and 12 hours (e-f). *L. crispatus* ATCC 33820/pT1-aFP at 0 h (a,e) *L. gasseri* JV-V03 (b,f), *L. iners* ATCC 55195 at pH 4 (c,g), and pH 7 (d,h). Microdroplet from the 24 hour time point are not shown.

iners at pH 7 decreased to $(2.2 \times 4) \times 10^4$ CFU/ml after 24 hours. The increase and decrease in CFU/ml is likely due to the fast growth after 12 hours and depletion of nutrients by 24 hours. The initial seed densities of *L. crispatus* and *L. gasseri* at pH 4, and *L. iners* at pH 7 was between $(0.34 \text{ and } 1.1) \times 10^7$ CFU/ml. However, the initial seed density of *L. iners* at pH 4 was over three orders of magnitude lower at 1.7×10^4 CFU/ml at 0 hour, and became undetectable after 12 and 24 hours. We reason that pH 7 stimulated *L. iners* growth and pH 4 was indicative of stress for *L. iners*. In addition, Chapter 3 described inerolysin, a cholesterol-dependent cytolysin that has maximum activity at pH 4.5 and is inactive at pH 7.4. [60] Perhaps there is a relationship between stress at low pH, and maximum inerolysin activity, which

creates pores in *L. iners*. [60] It is tempting to speculate that *L. iners*' creation of pores in vaginal epithelial cells at low pH may free glycogen, a potential carbon source that could temporarily improve host fitness.

The CFUs of *L. crispatus, L. iners,* and *L. gasseri* were enumerated for 1 μL of CVF microdroplets after 0, 12, and 24 hours. Our results show that *L. crispatus* survived in LC-CVF, whereas *L. gasseri* viability decreased after 24 hours. However, *L. iners* grew in LC-dominated CVF. Panel 4.5(b) in Chapter 3 showed a decrease in *L. iners* colonies after being incubated in spent LC-MNC broth, but an increase in spent LC-MNC broth after nutrients were added. We postulate *L. crispatus* did not have enough nutrients to grow but to survive in LC-CVF, but enough nutrients and kind of nutrients to stimulate *L. iners* growth. As shown in Figure 4.6 the CFUs of *L. crispatus* hardly changed which shows it was not inhibited in LC-CVF as was the case for *L. iners* at pH 4. Figure 4.6 may also suggest that *L. iners* may prefer a neutral pH in pooled LC-CVF compared to pH 4. Although the concentration of glucose in pooled LC-CVF was 3.5 $\pm$ 0.2 mM (0.63 $\pm$ 0.036 g/L), an additional 20 g/L glucose was added to the pooled LC-CVF to remove any speculation that glucose was a limiting factor. The supplemented and pooled LC-CVF was sterile filtered and used to generate microdroplets that enclosed *L. crispatus* and *L. gasseri* with a lambda value or average number of cells per microdroplet of 10.

Neither *L. crispatus* nor *L. gasseri* grew in the supplemented pooled LC-CVF, which suggests at least one other nutrient was limiting. *L. iners* did grow in LC-CVF. Although *L. iners* is fastidious, *L. iners* grew in LC-CVF when *L. crispatus* could not. In the laboratory, additional and unspecified nutrients are often added to media for *L. iners.* Scientists have made medium-simulating vaginal fluids. [138–140] However,

scientists have yet to publish a simulated vaginal fluid that has similar osmolarity to vaginal fluid and demonstrates its ability to support the growth of *L. iners*, the most widely detected species in the human vaginal microbiome (HVM). [1,33,34] The small genome of *L. iners* is comparable to parasites and symbionts and has a low GC content, which suggests its dependence on the host and neighboring microbiota. [22]

4.4 Conclusion

Work in this chapter has demonstrated our ability to achieve high-throughput experiments using pooled CVF. Employing microdroplets enables us to conserve limited material while conducting high-throughput experiments. We collected 49 vaginal swabs and CVF samples. Among those, we demonstrated that the microbial compositions of four vaginal was very similar to that of their corresponding cervix samples, as reported by Smith *et al.*. [144] 16S rRNA gene given that sequencing data revealed that many donors were colonized with a high prevalence of *L. crispatus*, which is not surprising given that *L. crispatus* is one of two most common species. No donors were mostly colonized by *L. jensenii*, which was also the case of the longitudinal study by Gajer in 2012 that included 32 donors. [34] By increasing our sample pool, we could have increased the number of donors with a high prevalence of *L. jensenii* or *L. gasseri*. However, *L. iners* is the most widely detected vaginal species, but was $\geq 85\%$ of the community, in 2 of 49 samples; sampling from a more ethnically diverse pool of women may increase the number of *L. iners*-dominated samples. [22,34] Furthermore, after culturing lactobacilli in pooled CVF, *L. crispatus* survived but did not grow in LC-dominated CVF. We hypothesize that the pooled LC-CVF was depleted of nutrients to stimulate the growth of *L. crispatus*. Surprisingly, *L. iners* grew in the LC-CVF that was ionized to pH 7 with 5M NaOH, but was killed at pH 4 after 12 hours. We postulate *L. iners* growth is stimulated in a neutral pH in the

vaginal environment but is stressed at pH 4. Advances made in this chapter provides a foundation for future work towards elucidating interspecies interactions relevant to the *in vivo* environment using the microdroplet co-cultivation technology platform.

4.5 Material and methods

4.5.1 Bacterial strains and cultivation protocols

Bacterial strains and cultivation protocols for seed cultures are identical to that of Chapter 3.

4.5.2 Methods for recruiting donors

The University of Michigan Institutional Review Board-Health Science and Behavioral Sciences approved recruiting materials, which include pre-screening questions, flyers, oral scripts, and email messages. All methods relating to recruitment and participation of donors, and use, handling, and storage of vaginal samples are documented in study ID: HUM00111306, entitled Pilot study for microdroplet co-cultivation and analysis of bacterial interactions in the vaginal microbiome.

4.5.3 Criteria for donors

Subjects completed a questionnaire to provide their information. Women were excluded for reasons that were speculated to change the composition of the vaginal microbiota. Healthy female college students, faculty or staff who were between the ages of 21 to 45 years old, are of reproductive age could participate in the study if they did the following: were not pregnant; were regularly menstruating between 25 to 35 days; were not currently menstruating; had not taken antibiotics within the past 30 days; have not douched, used vaginal medication, feminine sprays, genital wipes, or contraceptive, no sexual activity or vaginal discharge in the last 48 hours

before the study; had no gynecological surgery such as loop electrical excision procedure (LEEP), laser, a cone biopsy, or a hysterectomy; were not wearing a copper or hormonal intrauterine uterine device (IUD) as advised by Instead Softdisc™; has never had toxic shock syndrome. Written informed consent was obtained from all women.

4.5.4 Methods for collecting vaginal samples

Two vaginal samples were self-collected in the women's restroom on the ground floor of building 28 in the NCRC. Bacterial samples were collected using sterile rayon dual-headed swabs from the Fisherfinest® Dry Transport Swab (Hampton, NH, USA). CVS was collected using an Instead Softdisc™ (Venice, CA, USA). Women were provided with a brown paper bag filled with a sterile rayon-tipped dual-headed vaginal swab, an Instead Softdisc™, and a 50 ml Falcon tube, instructions, a receipt from the Human Subject Incentive Program (HSIP). The vaginal swab collection tube and the Falcon 50 ml conical tube had the same identification number on it to identify both samples that were taken from the sample donor. Then, participants washed their hands, entered a bathroom stall, and self-collected samples.

First, women unpeeled the swab package, removed the swab without touching the soft tip or laying it down; if the swab came in contact with a substance other than the vagina, subjects were instructed to collect a different swab. Participants followed instructions that were provided to properly insert the Softdisc™. The instructions were similar to this website: http://softdisc.com/how-it-works/. The swab was removed and carefully inserted into its transport tube and placed in a brown paper bag.

To collect CVF, women washed their hands, re-entered the bathroom stall, and

prepared to insert the Softdisc™. Women inserted the Softdisc™ as recommended by their website. Donors washed their hands after insertion and either set a timer for 15 minutes or returned with the Softdisc™ in less than 12 hours as instructed by Softdisc™. After, both the enclosed Softdisc™ and vaginal swabs were temporarily placed on dry ice in Styrofoam cooler located in the locker room of the women's bathroom. Vaginal samples were placed in a -80°C freezer until they were processed.

All donors were de-identified. Women were allowed to donated samples no more than once a month. Donors were compensated with a $20 Visa card for donating both samples and a $10 Visa card if only one sample was donated. Permission to collect vaginal samples in the women's restroom on the ground floor of building 28 in NCRC was granted from NCRC, the Department of Chemical Engineering, the College of Engineering, and the Department of Occupation Safety Environmental Health, now known as the Department of Environmental Health and Safety.

4.5.5 Methods for processing Softdiscs™

Each Softdisc™ was processed using the procedure that follows. Frozen Instead Softdiscs™ in Falcon 50ml conical tubes were thawed on ice for 15 minutes. Condensation on the outside of thawed conical tubes was removed by using a Kimtech® Wipe. Conical tubes with Softdiscs™ were centrifuged at 6,000 rpm for two minutes in an Eppendorf Centrifuge 5810 R from Fisher Scientific (Waltham, MA, US) that was set to 4°C. Each sample was pushed into the ice, cap-up, to maintain the integrity of the loose pellet of CVF inside of the tube. CVF samples, a Wiretrol® I pipette from Drummond Scientific (Broomall, PA, US) Kimtech® Wipes, sterilized scissors, and sterile 1.5 ml microcentrifuge tubes were placed inside of the biological safety cabinet. Each Softdisc™ was removed from the conical tube. Using a Wiretrol®

pipette, residual amounts of CVF and cervicovaginal mucus (CVM) was removed from the Softdisc™ and the 50 ml conical tube. CVF and CVM were transferred to a sterile 1.5 ml microcentrifuge tube and were placed on ice. The Softdisc™ and 50 ml conical tubes were disposed of.

4.5.6 Methods for separating CVF and CVM

CVF in 1.5 ml microcentrifuge tubes were weighted on a digital gram scale, paired with samples of similar weight, and centrifuged in an accuSpin Micro 17 microcentrifuge from Fisher Scientific at 6,000 rpm for two minutes. After the CVM was pelleted at the bottom and the CVF was at the top, the CVF was measured, with a 0.22 μ sterile filter, and transferred into a separate sterile 1.5 ml microcentrifuge tube using a pipette. The pH was measured using pH indicator strips by HYDRION® Lab 325 Tape pH Paper Strip Range 3.0-5.5. pH and volume (μL) were recorded. CVM remained in the 1.5 ml microcentrifuge tube. Several aliquots of 140 μL of pooled and sterile filtered LC-CVF were prepared and frozen at -80°C in addition to CVM.

4.5.7 Methods for processing and submitting vaginal swabs for 16S rRNA gene sequencing

Vaginal swabs were placed in a cooler with dry ice and transported to the Medical Research Science building I. Vaginal swabs were placed into the biological safety cabinet along with a Glass Bead Sterilizer, three pet nail trimmers, two sterile glass Petri dishes to prop the trimmers, and a 96-well PowerMag Glass Bead plate (Qiagen, Hilden, Germany). The Glass Bead Sterilizer was turned on. When it was ready, we sterilized the trimmers by placing them in the hot glass beads. A soft-tip from each vaginal swab was clipped and dispensed into a separate well in the 96-well

plate. When all swabs were inserted, the plate was sealed, packaged, and submitted according to the instructions of the University of Michigan Microbial Community Analysis Core.

Samples were submitted for gDNA extraction, standard polymerase chain reaction (PCR) using a Dual indexing sequencing strategy, and 16S rRNA gene sequencing. DNA extraction used the Eppendorf EpMotion liquid handling system followed by the Qiagen MagAttract PowerMicrobiome kit (previously MoBio PowerMag Microbiome). Samples underwent Illumina 16S rRNA gene sequencing of the V4 region. The Dual-index sequencing strategy was developed by Kozich *et al.*. [152] Details about the primers can be found in a study by Kozich *et al.*. [152] The standard PCR protocol was as follows: Stage 1, 1 cycle of 95°C (2 min); Stage 2, 30 cycles of 95°C (20 s), 55°C (15 s) and 72°C (5 s); and Stage 3, 1 cycle of 72°C (10 min); Stage 4, 1 cycle of 4°C (forever). A 20 μL volume of PCR master mix was composed of 2 μL 10x AccuPrime PCR Buffer II, 11.85 μL water, 0.15 μL AccuPrime HiFi Polymerase, 1 μL DNA, and 5 μL primer set (4 μM).

4.5.8 Methods for analyzing 16S rRNA gene sequencing data

An open-source platform-independent community-supported software called mothur was used to process the contigs from 16S rRNA gene sequences. Files were decompressed as fastq format. The files were uploaded into mothur and commands were based on the MiSeq standard operating procedure that was uploaded to the mothur website which was accessed on April 19, 2019. [152, 154] The code was as follows:

```
make.file(inputdir=., type=fastq, prefix=phd)
```

```
make.contigs(file=phd.files, processors=8)

summary.seqs(fasta=current)

screen.seqs(fasta=current, group=current, summary=current, maxambig=0, maxlength=275)

get.current()

summary.seqs()

unique.seqs(fasta=current)

count.seqs(name=current, group=current)

summary.seqs(count=current)

align.seqs(fasta=phd.trim.contigs.good.unique.fasta, reference=silva.v4.fasta)

summary.seqs(fasta=current, count=current)

screen.seqs(fasta=current, count=current, summary=current, start=1968, end=11550, maxhomop=8)
```

```
summary.seqs(fasta=current, count=current)

filter.seqs(fasta=current, vertical=T, trump=.)

unique.seqs(fasta=current, count=current)

pre.cluster(fasta=current, count=current, diffs=2)

chimera.vsearch(fasta=current, count=current, dereplicate=t)

remove.seqs(fasta=current, accnos=current)

summary.seqs(fasta=current, count=current)

classify.seqs(fasta=phd.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,
count=phd.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.
count_table, reference=silva.seed.lb.pick.ng.fasta, taxonomy=silva.seed123.pick.tax,
cutoff=80)

remove.lineage(fasta=phd.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,
count=phd.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.pick.
count_table, taxonomy=phd.trim.contigs.good.unique.good.filter.unique.precluster.pick.
pick.wang.taxonomy,taxon=Chloroplast-Mitochondria-unknown-Archaea- Eukary-
ota)
```

```
summary.tax(taxonomy=current, count=current)

remove.groups(count=current, fasta=current, taxonomy=current)

dist.seqs(fasta=current, cutoff=0.01)

cluster(column=current, count=current)

make.shared(list=current, count=current, label=0.01)

classify.otu(list=current, count=current, taxonomy=current, label=0.01)
```

The resulting OTUs were categorized into five groups: *Lactobacillus crispatus, L. jensenii, L. iners, L. gasseri, Lactobacillus* other, and others that are within the genus of *Aerococcus, Atopobium, Bifidobacteriaceae, Clostridia, Corynebacteriume, Dialister, Finegoldia, Megasphaera, Mobiluncus, Peptoniphilus, Prevotella,* and *Veillonella.* This was carried out by labeling each OTU with its respective taxa, and summing the number of reads for each of the five categories. These genera were detected in the vagina in previous studies. [1, 32] Sequences were aligned by following instructions on the mothur blog entitled, Customize your reference alignment for your favorite region. http://blog.mothur.org/2016/07/07/Customization-for-your-region/

4.5.9 Methods for pooling and characterizing pooled CVF

85% was selected to determine the most dominant species for each sample. Forty μL was the volume collected from each sample that was equal to or greater than 40 μL. Pooled *L. crispatus*-dominated CVF was vortexed and 30 μL was transferred to a sterile 1.5 ml microcentrifuge and given to Matt Sorensen who is a Ph.D. candidate in the Kennedy lab in the Department of Chemistry at the University of Michigan to measure the concentration of glucose using Mass Spectrometry.

4.5.10 Method for generating CVF-microdroplets with pure cultures

Seed cultures of *L. iners, L. crispatus*, and *L. gasseri* were washed three times in PBS, an isotonic saline solution. Cells were counted to achieve a lambda value of 10; λ is the average number of cells per microdroplet. The diameter of the microdroplets was 125 μm and the height of the microfluidic device was 50 μm. Microdroplets were generated from a device made of polydimethylsiloxane with a flow-focusing device geometry. [158] Thirty microliters of *L. crispatus* (LC)-dominated CVF was used as a culture medium for species. Microdroplets were transferred to a 1.5 ml microcentrifuge tube that was covered with mineral oil. Specific instructions about droplet generation can be found in Jackman *et al.*. [99] We generated empty LC-CVF droplets at pH 7, LC-CVF droplets with *L. gasseri* at pH 4, with *L. crispatus* at pH 4, with *L. iners* at pH 4, and with *L. iners* at pH 7.

4.5.11 Methods for observing fluorescence of *L. crispatus* ATCC 33820 pT1-aFP

Fluorescence was observed on the Nikon Eclipse Ti microscope by setting the exposure time between 2 to 3 seconds and increasing the gain to 36.

4.5.12 Method for enumerating pure cultures from CVF-microdroplets

At 0, 12, and 24 hours, 1 μL of microdroplets from each condition was transferred into a fresh 1.5 ml microcentrifuge tube that was destabilized with four μL 1H, 1H, 2H, 2H-perfluoro-1-octanol from Sigma-Aldrich (St. Louis, MO, USA). The destabilized microdroplets were at room temperature for 10 minutes until the droplets were destabilized. Afterward, 500 μL PBS was added to each culture. *L. crispatus* and *L. gasseri* were spot-plated onto MRS agar and both conditions of *L. iners* were plated onto Tryptic Soy Agar with 5% defibrinated sheep blood. [117] Serial dilutions were 50×10^{-1}, 50×10^{-2}, 50×10^{-3}, 50×10^{-4}, and 50×10^{-5}. Three 7.5 μL spots were pipetted onto each agar plate for each condition. Spots dried on agar in the biological safety cabinet for approximately 30 minutes. Then, they were incubated in the anaerobic chamber for one to two days. No colonies were present on agar with empty droplets.

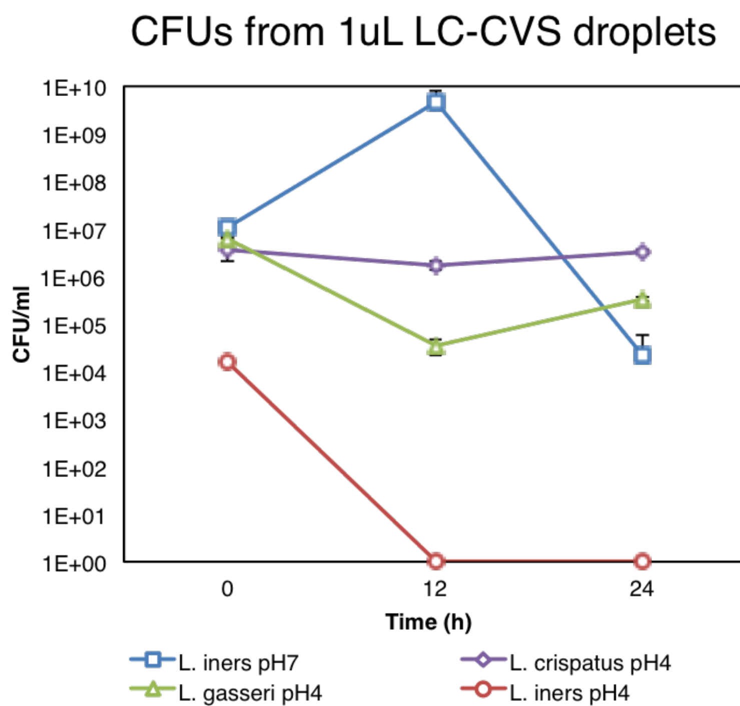


Figure 4.6: Colony forming units (CFUs) of *Lactobacillus* species enumerated on agar after being cultured in LC-CVS in microdroplets. *L. iners* at pH 7 (blue square), *L. gasseri* at pH 4 (green triangle), *L. crispatus* at pH 4 (purple diamond), and *L. iners* at pH 4 (red circle). One microliter microdroplets were eluted into buffer and plated.

Chapter 5

Concluding Remarks and Future Directions

5.1 Concluding remarks

As the role of the human vaginal microbiome (HVM) in women's reproductive health becomes increasingly salient, so do the questions regarding the ecological roles that each species has with each other and their hosts. New approaches and technologies are required for elucidating microbial interactions and applying newfound knowledge to advance women's health. In this dissertation, we addressed this need by employing microdroplets and dissecting interspecies interactions in three objectives. First, we evaluated the efficacy of microdroplets to recapitulate interactions that were previously reported in bench-scale studies. Second, we examined the effects of iron on the growth of *Lactobacillus* species and determined whether competition was observed when using synthetic laboratory media. Third, we collected 49 vaginal samples and developed methods for cultivating *Lactobacillus* in microdroplets containing cervicovaginal fluid (CVF) that partially simulate the *in vivo* condition with the exclusion of vaginal epithelial cells, transudate, additional compounds from the cervix, and indigenous bacteria. In conclusion, this dissertation has demonstrated the general applicability and advantages of the microfluidic co-cultivation technology platform for elucidating microbial interactions in the HVM.

In our first objective, we adapted a microdroplet co-cultivation technology platform to demonstrate an interaction that had been previously reported by Atassi *et al.* in 2006. Atassi *et al.* cultured *Gardnerella vaginalis* in the spent media of several vaginal lactobacilli strains and evaluated their killing effect on *G. vaginalis* and another putative pathogen. [18] Based on this literature, we selected *Gardnerella vaginalis* ATCC 49145 and *L. jensenii* JV-V16 to demonstrate the killing effect of *L. jensenii* on *G. vaginalis* using microdroplets. [99] Although colonies of *G. vaginalis* were absent after culture with *L. jensenii* in individual microdroplets but were present in axenic culture, this observation was not statistically substantiated due to the wide range of colony-forming units from each microdroplet. We detected reduced growth of *L. jensenii* in co-cultures with *G. vaginalis.* However, we acknowledge that qPCR is a highly sensitive assay and when used with pooled microdroplets, creates a high signal-to-noise ratio based on DNA content rather than cell viability. Further experiments with a second model system in microdroplets showed amensalism as *L. jensenii* JV-V16 inhibited *Enterococcus faecalis* ATCC 19433, a urogenital pathogen associated with aerobic vaginitis. [99, 159]

In our second objective, we investigated the effect of iron as a potentially limiting resource for *L. iners* ATCC 55195 and *L. crispatus* ATCC 33820. We chose iron because *L. crispatus* was reported to have ABC transporters to help acquire iron [68] and *L. iners* was reported to grow in MRS after the addition of 5% horse or sheep blood, which contains iron. [55] However, removing iron from the culture media created an additional challenge because supplements such as serum and blood are used to culture *L. iners* and contain iron among other compounds. To reduce the concentration of free iron in MNC broth, which contained horse serum, we added varying amounts between 0 to 1.5 mM 2,2'-bipyridyl. This chelator reduced the

availability of free iron and 2,2'-bipyridyl was added to both *L. iners* and *L. crispatus*. We demonstrated that increasing concentrations of 2,2'-bipyridyl resulted in reduced growth of *L. iners* ATCC 55195 while that of *L. crispatus* ATCC 33820 pT1-aFP was unaffected. The reduced growth of *L. iners* may be due to its requirement for iron during electron transport as opposed to cobalt and manganese, which is used by other lactobacilli. [64] We speculate that *L. crispatus* was unaffected by 2,2'-bipyridyl because it may use manganese instead of iron [62,64] and/or it sequestered iron from the chelator. Iron was also added to spent media of *L. crispatus*, but no growth of *L. iners* was detected. As a result, we have speculated that other nutrients were limiting.

In the second objective, we investigated whether there was evidence for direct competition of resources or inhibiting byproducts from either species, using synthetic laboratory media in two model systems: *L. iners* ATCC 55195 and *L. crispatus* ATCC 33820 in MRS broth, and *L. crispatus* ATCC 33820 and *L. gasseri* JV-V03 in MNC broth. We reported that the mechanism for direct competition was undetected and further investigation is needed to identify which nutrient(s) become limiting. As observed in our direct competition experiments, *L. crispatus* was consistently more abundant than *L. iners*, which is likely due to the faster growth rate of *L. crispatus* compared to that of *L. iners*. In the second model, *L. crispatus* and *L. gasseri* both grew in co-culture to similar densities. No evidence of inhibiting byproducts from either species was detected in either model system, suggesting interference competition was not present. These results suggest that dynamics between vaginal lactobacilli may not be governed by inhibiting molecules, but likely due in part to direct competition for resources from the host and between neighboring bacteria. The presence of signaling molecules may also influence the abundances of vaginal

lactobacilli, but very little research has covered this topic and further investigation is needed to understand the dynamics between vaginal lactobacilli.

Our third objective aimed to leverage the small-volume characteristics of micro-droplets to exploit cervicovaginal fluid (CVF) for cultivation. Vaginal samples were collected from donors and were subsequently used to culture *L. crispatus* ATCC 33820 and *L. gasseri* JV-V03 at pH 4, and *L. iners* ATCC 55195 at pH 4 and 7. Most women in our cohort had a high prevalence of *L. crispatus*, one of the two most widely detected vaginal species in the HVM. [1, 34] However, *L. iners*, which has been reported as the most widely detected vaginal species, [22, 123] was detected in lower amounts, which is possibly due to the demographics of our recruiting site. Using *L. crispatus* (LC)-dominated CVF, we observed that at pH 7, *L. iners* grew after 12 hours, but at pH 4, no colonies were detected after 12 hours. We also report that *L. crispatus* did not grow in LC-CVF possibly due to limited resources in the LC-CVF that were required for *L. crispatus* growth. The CFU count of *L. gasseri* JV-V03 decreased after 12 hours. This observation is in contrast to the robust and resilient phenotype of *L. gasseri* JV-V03 in MRS broth. The reason for this observation is unknown, but may be due to inherent differences between the vaginal environment and synthetic media. As *L. iners* is arguably the most fastidious and nutrient exigent species of the four vaginal lactobacilli [56], its ability to grow in LC-CVF indicates the different requirements for nutrients between *L. iners* and *L. crispatus*. However, after adding glucose and iron (data not shown), as an attempt to stimulate the growth of *L. crispatus*, we conclude that other compounds are limiting in the LC-CVF. This result provides a foundation for future research towards understanding how the abundances of different *Lactobacillus* species are regulated in the vagina.

In conclusion, this dissertation demonstrates several methods for investigating interactions in the human vaginal microbiome through co-cultivation in microdroplets in a high-throughput manner. We have also shown the utilization of small volumes of human samples while simulating the natural condition of the vagina. Further extension of this approach and its future applications hold potential for elucidating how microbial interactions shape the ecology of various ecosystems.

5.2 Future directions

5.2.1 Iron requirements for *Lactobacillus iners*

Iron is an important nutrient that enables electron transfer in most organisms excluding some lactobacilli. [62,64] Of the *Lactobacillus* species, which has been reported to utilize manganese and cobalt instead of iron,*L. iners* is considered nutrient exigent even when compared to other vaginal lactobacilli. However, *L. iners*' dependency on iron has not yet been validated. [55, 123] Our work in Chapter 3 has found that by restricting iron with 2,2'-bipyridyl, the growth of *L. iners* is reduced while that of *L. crispatus* remained unaffected, indicating an interaction between *L. iners* and 2,2'-bipyridyl that is absent in *L. crispatus*. Further investigation into how iron influences *L. iners* can shed light as to how *L. iners* interacts with iron *in vivo*.

An alternative solution for investigating the effect of iron on the growth of *L. iners* is by adding Fe(II)SO_4 to 1M 2,2'-bipyridyl MNC broth. It is understood that a 2,2 '-bipyridyl complex with iron(II) is formed when one mole of Fe(II)SO_4 reacts with three moles of 2,2'-bipyridyl. [160,161] Once 2,2'-bipyridyl is limited, additional iron(II) will be in excess, which may stimulate the growth of *L. iners*. Chapter 3 has shown that 1M 2,2'-bipyridyl reduces *L. iners* growth. However, if the growth

of *L. iners* increases after Fe(II)SO_4 is in excess, this can provide evidence that the restriction of iron prevents growth of *L. iners*. It would be advantageous to add Fe(II)SO_4 in small increments as too much iron(II) has also been shown to reduce the growth of *L. iners* (Chapter 3).

Another method for investigating the effects of iron on *L. iners* growth is by removing iron from MNC broth. One potential method for removing iron ions can involve magnetic beads in microdroplets. [150, 162] By applying a greater magnetic field to droplets that contain magnetic beads, a magnetic bead could move towards the magnet. By splitting the droplet, we can create a droplet with no magnetic beads and potentially free of iron ions too. Droplets can be coalesced to increase total volume, and the concentration of iron can be measured in the merged droplets.

5.2.2 Competition experiments in synthetic media

Competition experiments in current media that are used to grow vaginal lactobacilli have limitations. Currently, there exists no media that can grow all four dominant lactobacilli without the inclusion of serum. Substituting carbon sources in serum-containing media presents challenges when substituting nutrients such as glucose. However, MRS broth, used to grow lactobacilli, does not contain serum. By manually adding each ingredient of MRS broth, glucose can be replaced with other carbon sources and can be used to conduct competition experiments between vaginal lactobacilli other than *L. iners*.

5.2.3 Optimizing chemically defined medium for vaginal bacteria

Chemically defined medium is ideal for conducting competition experiments. However, it is challenging to use chemically defined medium for vaginal lactobacilli be-

cause they are nutrient exigent and have not been shown to grow well in the current chemically defined medium. Data from Tomás *et al.* showed that *L. gasseri* did not grow well in medium simulated vaginal fluid (MSVF), which is composed of some compounds that were detect in vaginal fluid. [140] To optimize the MSVF to grow vaginal lactobacilli, we added twenty amino acids as shown in Figure 5.1, which only stimulated the growth of *L. jensenii* and *G. vaginalis* to an OD_{620nm} of 0.4 and 0.1, respectively. Additional nutrients is needed to grow vaginal bacteria to higher densities. Other compounds that are absent from MSVF that may stimulate growth are manganese, Tween 80 and potentially iron for *G. vaginalis* and possibly *L. iners*. [125] Supplementing these nutrients to MSVF with twenty amino acids may improve the growth of lactobacilli with future applications for competitive experiments between vaginal bacteria.

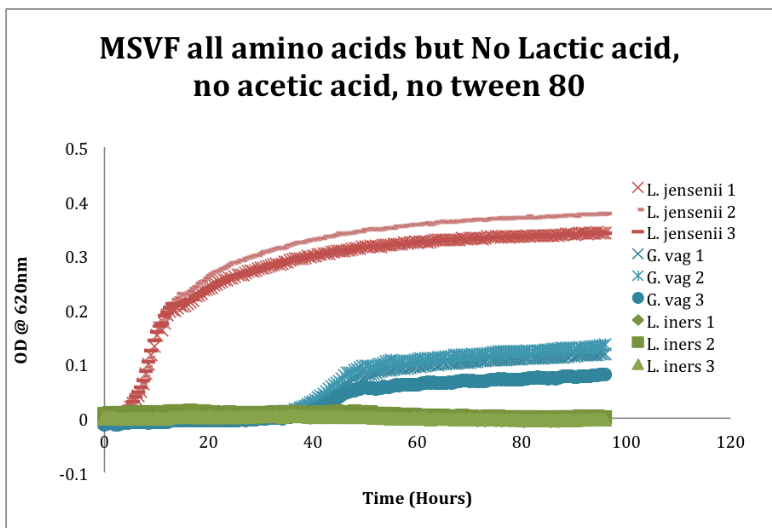


Figure 5.1: *L. jensenii* JV-V16, *G. vaginalis* ATCC 49145, and *L. iners* ATCC 55195 in medium simulating vaginal fluid (MSVF) with twenty amino acids added, no tween 80, and no acetic acid or lactic acid.

5.2.4 Cervicovaginal fluid microdroplets with vaginal epithelial cells

Our results in Chapter 4 showed us that *L. crispatus* did not grow in *L. crispatus*-dominated CVF. We hypothesized this to be due to a limited availability of nutrients

in the CVF. Nutrients that were constitutively expressed by vaginal epithelial cells (VECs) were not present in CVF-microdroplets. However, combining VECs with vaginal fluid, would add an additional factor that was not present in microdroplets. By growing VECs and using a transwell plate, we can grow VECs on a microfluidic device and flow vaginal fluid through the device. If we can image cells that are growing in their microhabitats as described by Keymer *et al.* [136, 163] this experimental setup may provide more insight when culturing bacteria and conducting competition experiments.

5.2.5 Comparing metabolomic profiles of *Lactobacillus crispatus* and *L. iners*-dominated communities from the human vagina

The metabolomic profiles of human vaginal fluid differ between those that are dominated by lactobacilli verses BV-associated bacteria. [38, 145] However, the number of metabolomic studies on the HVM are limited. So far, no significant differences have been reported between *Lactobacillus* species. Due to the unique characteristics of *L. iners*, including its ability to grow at a neutral pH, its inability to grow in MRS, and its relatively small genome, it is likely that its profile may reflect these differences when compared to other lactobacilli. Intensive investigation can be conducted by collecting samples from women with a high prevalence of *L. iners* and other lactobacilli. By comparing the metabolomes of multiple women with a high enough N value, differences may become observable. However, if we are seeking to collect samples that have a high prevalence of *L. iners* more diverse communities may need to be considered. [1, 45] Locations other than the University of Michigan could include Eastern University in Ypsilanti, M.I., Wayne Community College, Wayne County Hospital or the Mercy Hospital in Detroit, which are all in Detroit, Michigan.

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