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Optimizing Clean Catch Urine Collection and Its Applications in Urinary Microbiome Studies

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LOYOLA UNIVERSITY CHICAGO

OPTIMIZING CLEAN CATCH URINE COLLECTION

AND ITS APPLICATIONS IN URINARY MICROBIOME STUDIES

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE & IMMUNOLOGY

BY

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LIST OF ABBREVIATIONS

ADH   Alcohol Dehydrogenase

ALDH   Aldehyde Dehydrogenase

BAP   Blood Agar Plate

CFU   Colony Forming Units

CNA   Colistin and Nalidixic Acid

ENS   Effective Number of Species

GIT   Gastrointestinal Tract

GUM   Genitourinary Microbiome

IRB   Institutional Review Board

LUEREC   Loyola Urinary Education & Research Collaborative

LUTS   Lower Urinary Tract Symptoms

MALDI-TOF   Matrix Assisted Laser Desorption/Ionization Time of Flight

MEQUC   Modified Enhanced Quantitative Urine Culture

MIC   Minimum Inhibitory Concentration

ML   Milliliter

MRS   Man, Rogosa, Sharpe

SUC   Standard Urine Culture

UUI   Urgency Urinary Incontinence

ABSTRACT

The clean catch urine collection method was originally developed in the 1950's, during a time when urine was considered sterile (Thomas-White *et al.*, 2016). Its development replaced the invasive catheter urine collection method and allowed for what was thought to be a urine sample without genital contamination. However, since its development over half a century ago (Smythe *et al.*, 1960; Thomas-White *et al.*, 2016), more sensitive assays have been advanced for culturing urinary bacteria (Hilt *et al.*, 2014), whereas the clean catch method itself has remained unchanged. These new assays, which culture a higher percentage of bacteria present, have shown clean catch is not as clean as once speculated and actually contains high levels of vulvo-vaginal contamination. In light of these more sensitive assays, I reassessed the clean catch method, endeavoring to optimize this method while recording various lifestyle factors. Periurethral swabs and voided urines were routinely collected, while analyzing different aspects of the clean catch method, such as type of void, time of void, and use of antiseptic wipes prior to void. I determined that midstream urine contained the least amount of vulvo-vaginal contamination and that the time of collection did not impact the results. I also determined that the use of antiseptic wipes prior to void increased the level of vulvo-vaginal contamination.

Since a urine collection method that could be used daily did not exist, the stability of the genitourinary tract in healthy females had remained unknown. In contrast, others had studied the microbial stability of other body sites, such as the gastrointestinal tract (GIT) and the vagina, and found that the microbiota varied very little over short time intervals (Aagaard *et al.*, 2013; Faith

et al., 2013; "The Human Microbiome Project Consortium", 2012). To begin to fill this knowledge gap, I performed a longitudinal study, using a modified version of the enhanced quantitative culture technique (MEQUC) to follow the microbiota of the lower urinary tract (LUT) and periurethra (the genitourinary microbiome or GUM) of one female over the course of 7 months. I noted that both the voided urine and periurethra contained very distinct microbial niches and that they remained relatively stable over the course of the 7-month period. Using the recorded life style factors data, I noticed that alcohol may have an effect on the LUT microbiome of one female and that the LUT microbiome appeared to be resilient, returning to baseline levels within a few days. I recruited 8 female participants and assessed the stability of the GUM pre- and post-alcohol consumption. These females collected daily periurethral swabs and midstream voids over a 17-day period and kept a strict alcohol diary including amount of alcohol consumed, types of alcohol consumed, and length of alcohol consumption. The microbial and chemical composition of these specimens were compared and showed an acute drop in urinary *Lactobacillus* following binge drinking. It was hypothesized that this drop in *Lactobacillus* was due to an increase in alcohol metabolites in the urine. In order to determine whether alcohol metabolites played a role, I measured ethanol, acetaldehyde, and acetate in urine pre- and post- alcohol consumption. I then subjected urinary isolates collected pre- and post-alcohol consumption to sensitivity assays containing these alcohol by-products at the levels detected. I found that the concentration of alcohol metabolites found in the urine did not inhibit bacterial growth and was not a direct factor for the acute drop in urinary *Lactobacillus*. During this study, one participant developed a urinary tract infection (UTI), which I was able to detect prior to patient's self-reported symptoms. *E. coli* was determined to be the causative agent of infection and the levels of acetaldehyde found in the

urine during the final stages of the UTI were high enough to inhibit growth of *Lactobacillus*. This

may suggest a potential mechanism of virulence associated with *E. coli* during UTI.

CHAPTER I

INTRODUCTION

Every Ecosystem on Earth Contains Living Microbes – Why Not the Bladder?

Microbial diversity is quite unique and microbes thrive in all parts of the planet. These microbial populations are vital to all ecosystems of the earth, and the human body should not be overlooked. The human microbiome project, started in 2008, sought to map the microbial diversity found within the human body and began by mapping the microbial communities present in the oral cavity, nares, skin, gastrointestinal tract, and vagina ("The Human Microbiome Project Consortium", 2012). However, due to the clinical dogma that urine is sterile, the bladder was overlooked. Consequently, there is little knowledge about this unique niche and the microbiota that reside there.

In standard clinical practice, 1uL of urine is spread onto both blood agar plates (BAP) and MacConkey agar plates, and incubated aerobically for 24 hours. Often, following this procedure, the plates do not contain bacterial colonies or contain colonies that resemble vulvo-vaginal organisms that are thought to be contaminants. Thus, the clinical microbiologist reports "no growth." The use of the term "no growth" has fueled the clinical dogma that urine is sterile. First, "no growth" does not mean "no bacteria," but instead "no uropathogens." Second, many microbes do not thrive under the conditions of the standard urine culture test. Therefore, the Loyola Urinary Education and Research Collaborative (LUEREC) predicted that bacteria may

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thrive in the bladder, but are not detected by the standard culture method. Using the polymerase chain reaction and deep 16S rRNA gene sequencing, LUEREC identified bacterial DNA from catheterized urine samples which were deemed "no growth" by standard urine culture (Wolfe *et al.,* 2012). This raised the question: why were the standard cultures negative? A survey of the sequenced genera revealed bacteria that could not grow under the standard conditions. It was determined that if growth conditions were modified, then the sequenced bacteria would be able to grow. Thus, the Enhanced Quantitative Urine Culture (EQUC) protocol was developed. This new technique revealed live cultivable bacteria in urine collected by transurethral catheterization from women with symptoms of urinary urgency incontinence and from asymptomatic controls (Hilt *et al.*, 2014). The discovery of live bacteria in the bladders of women with negative (no growth) standard urine cultures raises many questions, but especially which bacteria contribute to good health and which contribute to disease. Understanding the microbial diversity in healthy individuals and the conditions that allow the proper diversity to thrive may assist us in visualizing and interpreting microbial dysbiosis of diseased states.

Human Microbiome Stability and Disease

Several research groups have investigated the longitudinal stability of various body sites of both symptomatic and asymptomatic individuals, and dysbiosis of these communities has been associated with various disease states (Engen *et al.*, 2015; Gajer *et al.*, 2012*;* Galloway-Peña *et al.*, 2017; Mutlu *et al.*, 2012). In one study, an individual's gastrointestinal tract (GIT) and salivary microbiomes were followed over the course of one year. The researchers found that these microbial communities were relatively stable and that various factors could alter the stability for short time periods, but that they were resilient and returned to their baseline states (David *et al.*, 2016).

Another study, following hospitalized cancer patients with acute myeloid leukemia, found longitudinal instability of both the GIT and oral microbiome (Galloway-Peña *et* al., 2017), which suggests there may be interplay between bacterial communities and human health.

Research has revealed distinct differences in the microbiota of the female urinary bladder (termed the Female Urinary Microbiota or FUM) between populations of individuals with lower urinary tract symptoms (LUTS), such as those associated with urinary tract infection (UTI) or urgency urinary incontinence (UUI), and individuals without LUTS (Pearce *et al.*, 2014; Pearce *et al.*, 2016). However, these seminal studies were performed at a single time point and there are no studies investigating the longitudinal FUM stability, in part due to the clinical dogma that urine is sterile. In order to better understand urinary health, it is imperative that we fill this gap. Associations between healthy and diseased states may be the missing links to improving overall treatment and outcomes of women and men with urinary tract disorders.

Measuring Species Diversity of Microbial Communities

One fundamental feature of microbial communities is diversity. Species diversity of a community is the variety of organisms that make up the community and it is composed of two components: richness, which is the number of different species in the community, and evenness, a measure of distribution that takes into account the proportion of each species within the community. **Figure 1** provides an example. In this figure, there are two trees. The question is: which tree has greater biodiversity or a greater variety of life forms? In tree 1, there are 4 different kinds of birds and in tree 2 there are also 4 different kinds of birds. This makes their species richness the same. However, tree 1 is more diverse because each species is represented an equal amount of times, whereas in tree 2, one species of bird is much more common than the others making tree 2 less diverse. This

difference in diversity is due to species evenness. To determine species evenness, the number of individuals in each species is counted to determine relative abundance or how common each species is. In tree 1, there are 4 species (A, B, C, and D). If we count the number of individual species, we find that each species represents 25% of the total number of individuals in the community. Since all of the species are represented equally, this community has high evenness. In contrast, tree 2 has one species that is much more common than all the rest. Since species "A" represents 75% of individuals, tree B has low evenness.

Community A
A: 25% **B**: 25% **C**: 25% **D**:25%

Community B
A: 75% **B**: 8.3% **C**: 8.3% **D**: 8.3%

Figure 1. Avian Species Diversity of a Tree

Several different indices have been developed that measure richness and/or evenness, providing the researcher with a single number that can be used to compare diversity among communities. The Shannon diversity index measures species richness or how many unique species are found in a community. However, the values of this index are non-linear and are merely the

value of the index, not the real diversity and thus it contains no units. One way to obtain a measure of true diversity is to convert the index to the effective numbers of species (ENS). This gives the values a common property and is always measured as number of species. The Simpson's Index is a measure of the chance that two randomly selected microorganisms are of the same species and thus it is a measure of evenness. Again, these values are non-linear and are merely the value of the index, but these values can be used to compare communities to each other.

Principal Component Analysis

PCA is a method for condensing an abundance of data in a way that captures the essence of the original data, while allowing one to interpret a dataset with a lot of dimensions (i.e., multiple sample types with varying microbial contents). PCA graphs will flatten complex data into a 2 or 3-dimensional graph to allow the researcher to visualize differences between the sample sets.

Creating PCA plots is a very complex statistical method and it may be overwhelming to imagine how the total colony forming units from a variety of bacteria cultured from a single specimen are compressed to a single dot on a graph. To better understand this process, I have provided a simplified PCA analysis using two periurethral swab specimens (**Table 1**) plotted as a 2-dimensional plot (**Figure 2**). In **Figure 2A**, it is obvious that most of the dots are spread out along a diagonal line and the maximum amount of variation is found between the two endpoints of Line 1. Additionally, the dots will be spread out a little above and below the first line, forming Line 2. If we rotate the graph (**Figure 2**), it allows us to visualize left/right and above/below variation more easily. What we can conclude is that the data varies a lot on the x-axis from left to right (Principal Component 1) and on the y-axis going up and down (Principal Component 2). Principal Component 1 is the axis that spans the most variation in the data, while Principal

Component 2 is the axis that spans the second most variation in the data. Now, for example, if we had 3 different samples, we would have an additional Principal Component 3 and it would span the direction of the third most variation. Therefore, there is a principal component for each sample and if we collected 200 samples, we would have 200 Principal Components.

	Swab 1	Swab 2	Influence on PCA1	Influence on PCA2
Corynebacterium	19000	19000	12	0.3
Escherichia	6000	10000	0.3	12
Lactobacillus	4000	5000	-8	-2
Staphylococcus	15500	16400	6	10
Enterococcus	20000	8000	9	-0.1
Propionibacterium	0	3200	-14	-0.2

Table 1. Total CFU and Influence Scores of Bacteria Cultured from Periurethral Swabs

A)
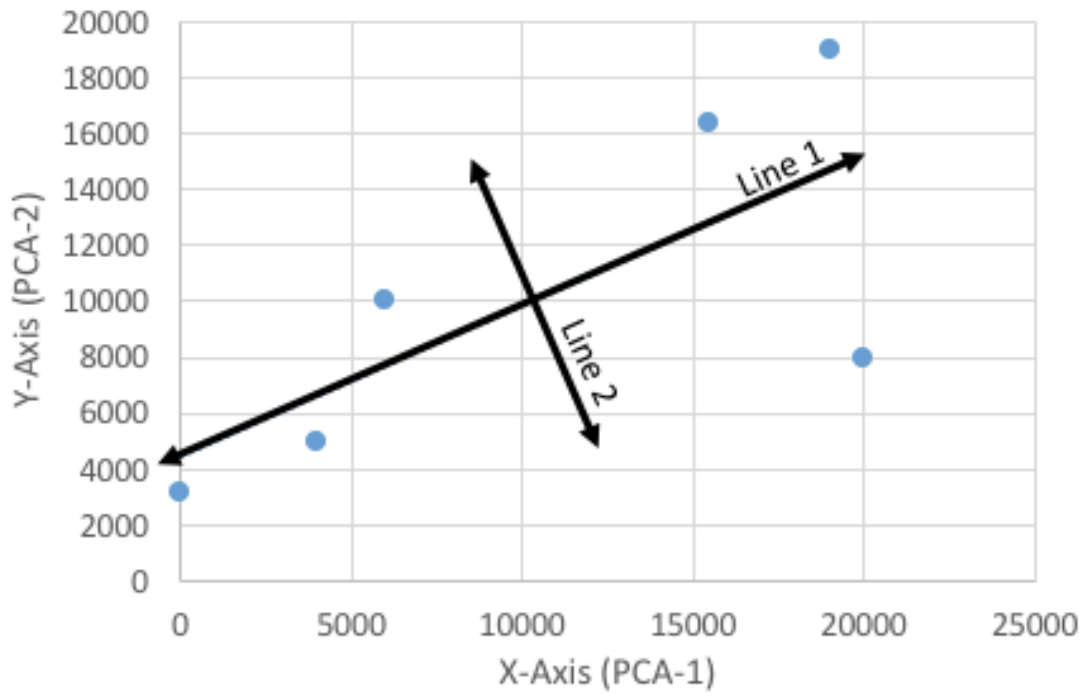
B)
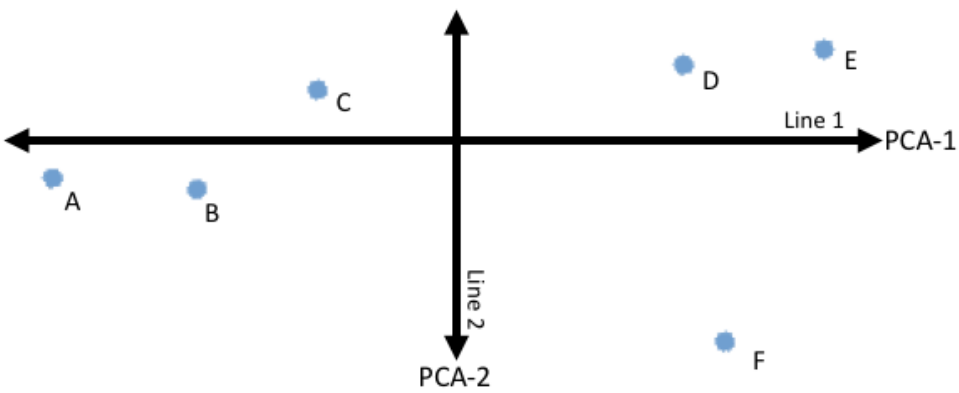
Figure 2. Principal Component Analysis of Two Periurethral Swabs

It is important to note that each dot represents one single sample and does not directly plot the bacterial CFU. Due to this, it is important to understand how bacterial CFU is incorporated into each plotted specimen. The length and direction of Principal Component 1 is determined by the extreme values at each end of the line (i.e., Points A and B, Points D and E). These points are then scored on how much they influenced Principal Component 1 using a statistical equation (**Figure S1**). Points close to the ends will have scores further from 0, either negative or positive values, because they highly influence PCA 1. Points at opposite sides of the line will be scored with similar values, but with opposite signs. Points in the middle will have values closer to zero because they have less influence on PCA 1. Similarly, to PCA 1, the bacterial CFU are also ranked on how they influence Principle Component 2 and this is repeated until all Principle Components are calculated. Now that the influences of PCA 1 and PCA 2 are calculated (**Figure S1**), we can use them to plot the individual samples. For PCA 1 and PCA 2, a coordinate of (470500, 222700) and (316600, 278260) were calculated respectively and plotted using a scatter plot (**Figure 3**). Each coordinate pair represents each individual sample and combines the total CFU of every unique microbe found in the sample into a single value. It is important to note that these 2 data points plot closely together, or cluster, because they are very similar to each other both in terms of bacterial diversity and abundance. If we were to use a third sample from a different site with much different bacterial diversity and abundance, such as from the GIT, we would expect to see 2 clusters. One cluster would contain the two periurethral swabs, while the other cluster would contain the single GIT sample.
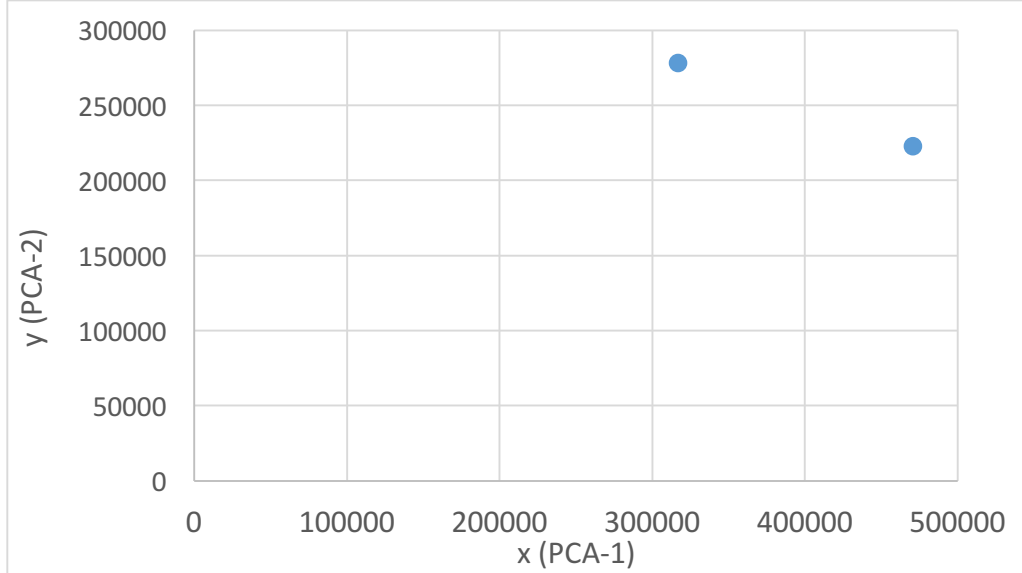
Figure 3. Scatter Plot Displaying Principal Component Analysis Data

A Brief History of Urine Collection Techniques

Two common methods of urine collection include transurethral catheter and clean catch collection. Catheter, from the ancient Greek word *kathiénai*, plainly means "to thrust into" or "to send down" and has been used since the time of Egyptian pharaohs, dating back to 1500 BC (Feneley *et al.*, 2015). This collection technique was largely used for individuals experiencing urinary retention and often included the insertion of bronze tubes, reeds, straws and curled-up palm leaves (Feneley *et al.*, 2015). Since its development, this procedure has been modified to incorporate aseptic technique but unfortunately still remains relatively invasive. It was not until the mid 1950's that physicians started collecting urine via a non-invasive "clean catch" method which was thought to provide a urine sample with the least amount of genital contamination, while allowing physicians to treat based on the bacterial findings. Using this method, it was determined that 10^5 colony forming units (CFU) per milliliter (mL) represented the threshold between genital

contamination and evidence of pyelonephritis (Kass E. 1956; Kass E.H., 1957). Subsequently, this method was adopted for diagnosing bladder infections despite scant evidence that this was appropriate (Thomas-White *et al.*, 2016). Furthermore, the 10^5 CFU/mL is controversial, as several researchers have recommended lower thresholds (Lipsky *et al.*, 1987; Stamm *et al.*, 1982; Stark and Maki, 1984; Price *et al.*, in preparation). Finally, it is important to consider that the "clean catch" protocol was developed before the advent of more sensitive tools, such as deep sequencing and EQUC. Relative to EQUC, the standard urine culture method has a 90% false negative detection rate for all bacteria and a 50% false negative detection rate for uropathogens. Thus, the "clean catch" protocol must be reassessed. First, it must be determined whether a "clean catch" is actually possible and, if so, then it is imperative that it be optimized. Combining EQUC with a modified "clean catch" collection method would give physicians and researchers a more accurate look at the lower urinary tract (LUT) microbiota (i.e., those of the bladder and urethra), while allowing collection of urine in a non-invasive and healthy manner.

How Soap Works

The current "clean catch" protocol includes a wipe with Castile soap. Soap molecules are composed of a hydrophobic tail and a hydrophilic head. When soap is added to the surface of the skin the hydrophobic tails attach themselves to bacteria. Once attached, the soap molecules begin to change the bacterium's shape, causing it to lift away from the surface. Eventually, this exposes new areas of the bacterium, which allows more soap molecules to attach to these newly exposed areas. Eventually, it will form a micelle, which is a spherical structure of soap molecules containing the bacterium at the center. Once the micelle is formed, it remains suspended on the surface of the skin. It is important to note that micelles are coated with hydrophilic heads that are attracted to

water. Therefore, with the addition of water, such as when your washing your hands, the hydrophilic heads attract to the water and are washed away taking the encapsulated bacteria with them and reducing the amount of bacteria present on the surface of the skin. The same would be true of a urine stream.

Alcohol Consumption and the Human Microbiome

Alcohol consumption is a normal part of life for many people. Research has shown alcohol consumption in moderation can be considered healthy. For example, by drinking just one glass of red wine a day (272mL/day), it is possible to modulate your GIT microbiome and attain select beneficial microbiota, suggesting a possible prebiotic effect of red wine polyphenols on the GIT (Queipo-Ortuno, 2012). In contrast, chronic alcohol consumption can negatively impact the GIT (Engen *et al.*, 2015; Mutlu *et al.*, 2012; Purohit *et al,*. 2008; Queipo-Ortuno *et al.*, 2012) and can cause issues such as small intestine bacterial overgrowth, increased intestinal permeability to endotoxins, and subsequent liver and organ injury (Bode *et al.*, 1984; Purohit *et al,*. 2008). With as many as 25% of the population regularly engaging in binge drinking every month (Women 3+/day, Men 4+/day), it is obvious that alcohol may be a contributing factor to various human disease states (Center for Behavioral Health Statistics and Quality, 2015). While alcohol consumption has been linked to various disorders of the brain, heart, liver, pancreas, and the immune system (Center for Behavioral Health Statistics and Quality, 2015), relatively few studies have examined the effects on microbial communities outside of the GIT. It is possible that if alcohol is able to alter the homeostasis of the GIT microbiome, that it may also influence changes to the microbiota of other sites, such as the LUT, and could potentially be an additional factor in genitourinary disease.

Alcohol is an organic compound. It refers to ethyl alcohol (i.e., ethanol), which is the predominant form of alcohol found in alcoholic beverages (International Alliance for Responsible Drinking). The chemical breakdown of alcohol involves the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (U.S. Department of Health and Human Services, 2007). ADH breaks down alcohol into acetaldehyde, a known carcinogen, and ALDH can rapidly metabolize acetaldehyde further into a less harmful by-product, acetate. On average, acetaldehyde exposure from alcoholic beverages is estimated to be around 0.112mg/kg body weight/day and significantly increases one's risk of developing cancer (Lachenmeier *et al.*, 2009). Therefore, individuals who participate in episodes of binge drinking (Women 3+/day; Men 4+/day) or chronic alcohol abuse (Women 7+/week; Men 14+/week) are exposed to increased levels of acetaldehyde and are at greater risk for developing disease (Center for Behavioral Health Statistics and Quality, 2015). Ethanol and the by-products associated with alcohol consumption (acetaldehyde and acetate) have subsequently been measured in the blood and urine of individuals who consumed alcohol (Kitazawa *et al*., 1994; Tsukamoto *et al.*, 1988; Tsukamoto *et al.*, 1993), indicating that the body excretes these metabolites into the blood circulation, which is then filtered by the kidneys and excreted into the urinary tract. Remarkably, these metabolites remain detectable in the urine for at least 8 hours post-alcohol consumption (Tsukamoto *et al.*, 1988; Tsukamoto *et al.*, 1993). Interestingly, the work done by Tsukamoto *et al.* only had participants consume beer, a low alcohol content malt beverage, for 20 minutes with a dose between 8-16mL/kg. Even at these very low levels of alcohol consumption, they were able to see substantial increases in ethanol, acetaldehyde, and acetate. There are no documented studies on the effects of hard liquor on metabolite

concentrations in urine. Furthermore, there are no studies that queried the effects of these alcohol metabolites on the microbial communities of the urinary tract.

Alcohol Consumption Effects Bacterial Diversity

As stated previously, drinking alcoholic beverages has the ability to modulate your GIT microbiome. However, the types of bacteria that thrive depends on the type and amount of alcoholic beverages consumed. Therefore, it is possible to modulate one's GIT microbiome and attain select beneficial or non-beneficial microbiota strictly by controlling the type or amount of alcoholic beverages consumed (Bode *et al*., 1984; Miki *et al*., 2012; Yan *et al*., 2011; Queipo-Ortuno *et al.*, 2012).

In one study, jejunal aspirates from chronic alcoholics and control patients were collected, cultured, and then compared. The researchers found that coliform microorganisms, commonly including the genera *Citrobacter, Enterobacter, Hafnia, Klebsiella*, and *Escherichia*, were cultured 55.6% of the time in alcoholics and only 15.4% of the time in control participants. It was hypothesized that the fluctations in microorganisms may be attributed to functional and morphological abnormalities of the jejunum caused by chronic alcohol abuse (Bode *et al*., 1984).

Another study used a mouse model in which one group of mice was given intragastric feedings of alcohol and the other was fed an intragastric feeding of an isocaloric diet. Changes in microbial diversity was assessed using both conventional culturing techniques and 16s rRNA sequencing. The researchers found no changes in microbial diversity following 1 day or 1 week, which may be comparable to "binge drinking". However, intestinal bacteria overgrowth was observed in mice that were fed alcohol for 3 weeks compared to controls that received the isocaloric diet, which may be comparable to "chronic alcohol abuse". Alcohol fed mice had a

higher predominance of bacteria from the phyla Bacteroidetes and Verrucomicrobia bacteria, while control mice had a higher predominance of bacteria from the phylum Firmicutes. They also found that these changes in abundance and diversity were associated with the down regulation of gene and protein expression, particularly bactericidal c-type lectins including Reg3b and Reg3g (Yan *et al.*, 2011). This lectin family is able to shape the commensal and pathogenic bacteria present in the gut and it does this by binding lipopolysaccharides found in the outer membrane of Gram-negative bacteria (Miki *et al.*, 2012). Therefore, it is clear why the down regulation of these lectins would allow increased fluctuations in Gram-negative bacteria, such as Bacteroidetes.

Another study found that individuals could regulate gut bacteria by consuming alcohol. The following studies focused on wine. Wine is composed of various phenolic compounds classified into three distinct categories: non-flavonoids, flavonoids and tannins. Flavonoids have been found to have various antimicrobial capabilities. They are potent antioxidants that are capable of scavenging free radicals and are metal chelators. They also play a role in reducing the amount of inflammation, allergies, arthritis, high blood pressure and even the onset of cancer. One way they do this is by manipulating the growth and metabolism of bacteria. All of the wines tested showed antimicrobial properties that increased bacterial inhibition as polyphenol concentration increased. By testing various polyphenols, they found that multiple pure phenolic compounds and polyphenols were able to inhibit bacterial growth, including that of *Serratia marcescens*, *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumonia*, and *Staphylococcus* (Rodriguez Vaquero *et al.*, 2005), all of which have been known to cause various human disease states. Overall, they found that *Escherichia coli* was the most sensitive bacterium and that *Flavobacterium* species were

resistant to all phenolic compounds tested. This was further shown in a study that followed individuals longitudinal before and after the consumption of wine (**Table 2**).

Bacteria	Red Wine	De-Alcoholized Red Wine	Gin
Proteobacteria phylum	↑	-	↓
Firmicutes phylum	↑	-	↓
Bacteroidetes phylum	↑	-	↓
Fusobacteria phylum	↑	↑	↓
Enterococcus genus	↑	↑	↓
Prevotella genus	↑	↑	↓
Bacteroides genus	↑	↑	↓
Bifidobacterium genus	↑	↑	↓
Bacteroides uniformis species	↑	↑	↓
Eggerthella lenta species	↑	↑	↓
Blautia coccoides-Eubacterium rectale species	↑	↑	↓
Clostridium genus	↓	↓	↑
Clostridium histolyticum species	↓	↓	↑

Table 2. **Fluctuations in Gastrointestinal Tract Bacteria in Response to Alcohol**

Enterobacteriaceae Produce Enzymes That Catalyze the Formation of Acetaldehyde

A family of bacteria, known as Enterobacteriaceae, include a wide range of Gram-negative bacteria mainly found in the guts of humans and animals. Although many are harmless, this family of bacteria include genera that contain known pathogens, such as *Escherichia, Enterobacter*, and *Klebsiella*. These bacteria are facultative anaerobes, which means they are able to ferment sugars, such as glucose, to produce lactic acid and acetic acid along with other metabolites. In this group of bacteria, *E. coli* is one of the most heavily studied and is often found to be the causative agent of lower urinary tract disorders, such as UTI's.

To better understand its pathogenicity, it is important to assess how *E. coli* utilizes different carbon and nitrogen sources for survival. The preferred carbon source of *E. coli* is glucose, which

allows it to grow rapidly, while its preferred nitrogen source is ammonia (Bren *et al.*, 2016). When pyruvate (a derivative of glucose) is present, an enzyme produced by *E. coli*, pyruvate: formate-lyase can cleave the pyruvate into acetyl-CoA and formate. Subsequently, the acetyl-CoA is further cleaved into acetate and acetaldehyde. Acetaldehyde can now be reduced even further to ethanol (Elmar, 2006).
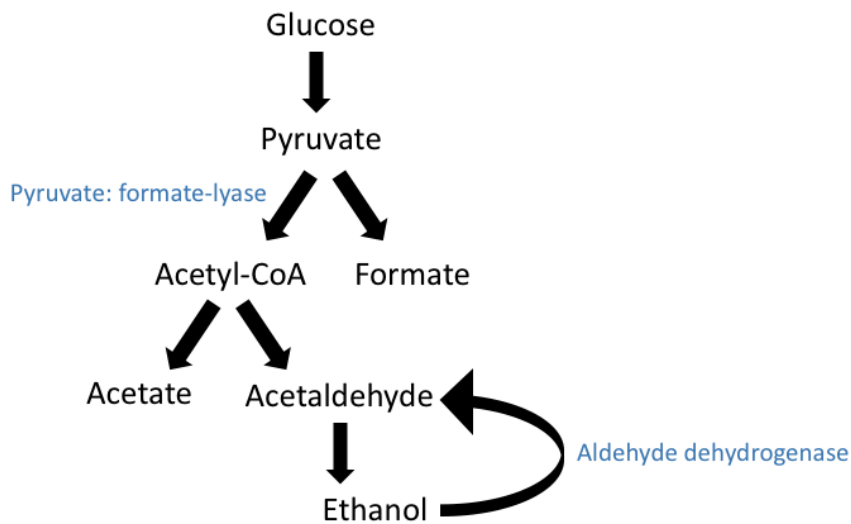
Figure 4. The Metabolism of Glucose by *E. coli*

Two Key Energy Sources for *E. coli* Can Be Found in the Bladder

As previously discussed, *E. coli* can use both glucose and ammonia as carbon and nitrogen sources. Ironically, both of these compounds can be found in urine and are found at higher concentrations in patients with different disease states.

One example of this is found in individuals with diabetes mellitus. These individuals tend to have fluctuations in the amount of glucose present in their blood. Under normal circumstances, the convoluted tubules are able to reabsorb glucose fluctuations. However, when blood sugar levels rise above a certain threshold (170-200/dL), the capacity of the convoluted tubules is reached and

the body rids itself of this excess sugar by excreting it into the urine (Andrianesis and Doupis, 2013).

Increased ammonia concentrations in the urine can be the result of a multitude of disorders. Many of these disorders include defective enzymes in the urea cycle, organic acidemias, congenital lactic acidosis, fatty acid oxidation defects, and dibasic amino acid transport defects (Chawla 2013). Similarly, as occurred with diabetes mellitus, when the blood is oversaturated with ammonia, it will be excreted into the urine.

Currently, diseases such as diabetes mellitus increase the risk for lower urinary tract disease. This is mainly thought to be caused by various structural defects along with defective host immune factors (Brusche, 2015). However, it is possible that these structural abnormalities combined with increased levels of glucose and ammonia in combination may make bladder conditions more favorable for pathogenic bacteria, such as *E. coli*. This may suggest why individuals with these disease states have increased risks for UTI's.

Roles of Acetaldehyde in Cancer

Acetaldehyde, a by-product of ethanol metabolism, is a known carcinogen and increases the risk of developing cancers (Lachenmeier *et al.*, 2009), such as those involved in alcoholic liver disease. Acetaldehyde is very reactive and combines chemically with cells of the ethanol consumer. These chemical combinations form acetaldehyde adducts, which have been shown to cause liver damage by triggering immune responses, such as inducing Kupffer cells, TNF- α, oxidative stress, and acetaldehyde adduct specific antibodies (IgM, IgG, IgA), which cause auto-immune attacks on hepatocytes leading to necrosis and fibrosis of the liver (Setshedi *et al.,* 2010). Additionally, acetaldehyde may directly interact with DNA, causing small point mutations or large

detrimental chromosomal damage, including induction of sister chromatid exchanges. In one study, these adducts were combined with cultured hepatic endothelial cells. Upon addition, the adducts stimulated the secretion of cytokines and chemokines including TNF- α, Monocyte chemo attractant protein-1, and macrophage inflammatory protein-2. Additionally, acetaldehyde adducts stimulated the activation of hepatic stellate cells, the cause of scar tissue and liver cirrhosis (Setshedi *et al.,* 2010). Overall, acetaldehyde has various mechanisms to cause liver disease.

Although acetaldehyde is known to be excreted into the bladder during times of alcohol consumption (Kitazawa *et al*., 1994; Tsukamoto *et al.*, 1988; Tsukamoto *et al.*, 1993), little research has been done to study its effects. However, in a case controlled study, they found the risk of bladder cancer increased in individuals who always drank compared to individuals who never drank (Zaitsu *et al*., 2016). Additionally, large doses of acetaldehyde were found to stop the urine flow and alter the smooth muscles of the bladder (Supniewski, 1927). Due to this, it is probable that excessive drinking, as found in individuals with alcohol induced liver diseases, may influence bladder disease and may impact the microbiota present.

CHAPTER II

MATERIALS & METHODS

Patient Recruitment

This project was approved by the Loyola University Chicago institutional review board

(IRB) (LU#208740, LU#209111). All participants gave verbal and written consent for the

collection and analysis of their periurethral swabs and urine samples for research purposes.

Participants were either the researchers themselves (***Chapter III***) or women who regularly

consumed alcoholic beverages (***Chapter III-V***). Participants were screened for eligibility with a

short questionnaire asking how much alcohol the participant consumed on a regular basis (***Chapter***

V).

Periurethral Swab Collection Protocol

Antiseptic wipes saturated in 2% castile soap, hydrogen peroxide, and water were used to cleanse

the genital area preceding periurethral swab collection for clinical clean-catch samples only. Wipe

packages consisted of two wipes. In women, one wipe was used to clean the labia in a front-to-

back wiping motion. The second wipe was used to clean over the urethra. Antiseptic wipes were

not used preceding modified clean catch protocols. A periurethral specimen was collected using

an Elution Swab (ESwab) Collection and Transport System. The periurethral swab was collected

prior to all urine collections to avoid contamination with urine. The ESwab system sustains the

viability of clinically important aerobes, anaerobes, and fastidious bacteria for up to 48 hours when

kept between 5-25°C.

<div align="center">**Voided Urine Collection Protocol**</div>

Initial Stream Urine Collection.

Initial stream urine collection involved collecting a voided urine sample into a sterile urine cup. The participant placed the sterile urine cup a few inches from the urethra and began urinating into the cup until it was half full, at which time the cup was removed. The urine was then transferred to a C&S Preservative Plus Urine Tube (Becton Dickinson and Co; Franklin Lakes, NJ) containing 2.63mg/mL boric acid, 2.08mg/mL sodium borate, and 1.65mg/mL sodium formate, a combination that preserves the urine specimen for up to 48 hours. 4 mL of the remaining urine collected in the sterile cup was combined with 10% AssayAssure (Thermo Scientific; Waltham, MA) and stored at -80°C for future DNA sequencing.

Midstream Urine Collection.

Midstream urine collection involved collecting a voided urine sample into a sterile urine specimen cup. The participant initially urinated into the toilet for about 3-5 seconds before placing a sterile urine cup a few inches from the urethra. The participant continued to urinate into the cup until it was about half full, at which time the cup was removed. The urine was transferred into a C&S Preservative Plus Urine tube (Becton Dickinson and Co; Franklin Lakes, NJ), which preserves the urine for up to 48 hours, as previously described. 4mL of the remaining urine collected in the sterile cup was combined with 10% AssayAssure (Thermo Scientific; Waltham, MA) and stored at -80°C for future sequencing.

<div align="center">**Periurethral Swab Cultivation Protocol**</div>

Periurethral swab specimens were cultivated using MEQUC (**Table 1**). MEQUC involved plating the periurethral swab specimen using a quantitative pinwheel streak onto Sheep BAP (BD

BBL™ Prepared Plated Media, Becton Dickinson and 94 Co; Sparks, MD), Columbia-Colistin and Nalidicix Acid (CNA) Agar (BD BBL™ Prepared Plated Media), and CDC-Anaerobic Blood Agar (BD BBL™ Prepared Plated Media). BAP and CNA Agar were incubated at 37°C in 5% CO_2 and the CDC-Anaerobic Blood Agar was incubated at 37°C in anaerobic conditions. All plates were incubated for 48-72 hours. Following incubation, visibly distinct bacterial colonies were counted and recorded. Due to the unpredicted high colony forming units of swab specimens, these specimens were initially plated using both 10 µL and 100µL of sample. The plated sample volume, either 10µL or 100µL, that could be read clearly without colony overgrowth was used for the remainder of that individual's samples.

Voided Urine Cultivation Protocol

All urine specimens, both initial and midstream, were cultivated using MEQUC (**Table 3**). 100µL of the voided urine specimens were plated using a quantitative pinwheel streak onto Sheep BAP (BD BBL™ Prepared Plated Media), CNA Agar (BD BBL™ Prepared Plated Media) and CDC Anaerobic Blood Agar (BD BBL™ Prepared Plated Media). BAP and CNA Agar were incubated at 37°C in 5% CO_2 and the CDC-Anaerobic Blood Agar was incubated at 37°C in anaerobic conditions. All plates were incubated for 48-72 hours. Following incubation, visibly distinct bacterial colonies were counted and recorded.

Sample Type (Volume)	Media	Conditions	Identification
Periurethral Swabs (10µL or 100µL)	BAP, CNA	5% CO_2 35° C	48 -72hr
	Anaerobic	Anaerobic 35° C	
Voided Urines (100µL)	BAP, CNA	5% CO_2 35° C	48 -72hr
	Anaerobic	Anaerobic 35° C	

Table 3. Periurethral Swab and Voided Urine MEQUC Protocol

Bacterial and Fungal Isolation, Identification, and Storage

For each plate, distinct bacterial and/or fungal colonies were counted, documented, sub-cultured to new plates and grown as pure cultures. The pure cultures were identified using Matrix-Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometry. MALDI-TOF is an ionization method used to detect, characterize, and quantify biomolecules. This analytical technique involves a laser striking a matrix of small organic molecules. The laser energy is absorbed by the matrix molecules, which are energetically ablated from the surface of the sample carrying the analyte molecules into the gas phase. These ions are then separated based on their mass-to-charge ratio to create a mass spectrum. The mass spectrum was compared to a database of reference spectra to identify the specific microbe to the species level. Once isolates were

identified by MALDI-TOF MS, they were stored in CryoSavers containing Brucella Broth with 10% glycerol using sterile swabs. Isolates were stored at -80°C until ready for use.

Recovering Stored Isolates

Bacterial and/or fungal isolates were removed from -80°C and inoculated onto BAP or CNA plates. Plates were incubated for 24-48 hours at 37°C in 5% CO_2 or anaerobic conditions depending on the isolates preferences. A single colony from the incubated pure agar culture was re-suspended into MRS broth, vortexed, then allowed to incubate an additional 24 hours at 37°C in CO_2 or anaerobic conditions depending on the isolates preferences.

Ethanol, Acetate, and Acetaldehyde Colorimetric Assays

Urine collected from participants pre- and post-alcohol consumption was assayed for alcohol metabolites (i.e, ethanol, acetaldehyde, and acetate). 100 μL of urine sample from the sterile cup was aliquoted into a cryovial and centrifuged for 10 minutes at 13500 rpm to remove any particulate.

Ethanol Assay.

To detect ethanol in the urine, I used an Ethanol Assay Kit (Sigma-Aldrich, MAK076). The ethanol kit standard dilution was decreased one order of magnitude to extend kit usage. This was achieved by diluting 5μL of the 17.15N ethanol standard with 80.87μL of the ethanol assay buffer (generating 1 umol/μL standard) followed by the dilution 1μl of the 1umole/μL standard solution with 99μL of ethanol assay buffer (generating a 10nmol/μL solution) and finally by diluting 10μL of the 10nmole/μL solution with 90μL of ethanol assay buffer to generate a 1nmole/μL ethanol standard. Urine supernatant (3.33μL) was added to a flat-bottom 96-well plate followed by the master reaction mix (46μL ethanol assay buffer, 2μL ethanol probe, 2μL ethanol

enzyme mix). Plates were mixed using a horizontal shaker. A standard curve ranging between 0.2-0.4 nmol/μL was generated using 0, 2, 4, 6, 8, and 10 μL of the 1nmole/μL ethanol standard solution in duplicate into a flat-bottom 96-well plate brought up to a final volume of 50μL using ethanol assay buffer. Plates were covered tightly and incubated for 60 minutes at room temperature. The absorbance was measured at 570nm (A_{570}). If the ethanol values of the urine did not fall within linear range of the standard curve, then the urine was diluted using ethanol assay buffer and re-tested. All urine samples and standards were run in duplicate.

Acetaldehyde Assay.

To detect acetaldehyde in the urine, an Acetaldehyde Assay Kit (EnzyChrom, EASCT-100) was used. The acetaldehyde kit standard dilution was lowered from 1.2mM-4mM to 10uM-60uM to obtain a more accurate standard curve vs sample comparison. The premix was changed from 1mL to 200μL working volume to extend kit usage. Briefly, 10μL of urine supernatant was added to a flat-bottom 96-well plate and 90μL working reagent (85μL Assay Buffer, 8μL NAD/MTT, and 1μL Enzyme A, and 1μL Enzyme B) was added to each urine sample. Sample blanks were prepared by combining 10μL pure H_2O and Blank Working Reagent (86μL assay buffer, 8μL NAD/MTT, and 1μL enzyme B). A standard curve was generated using 10μM, 30μM, and 60μM acetaldehyde standards. Plates were mixed using a horizontal shaker, covered tightly and incubated for 30 minutes at room temperature. The optical density was measured at 565nm and acetaldehyde concentrations were calculated using the obtained standard curve. If the urine acetaldehyde values did not fall within linear range of the standard curve, then the urine was diluted using pure H_20 and re-run. Resulting diluted acetaldehyde concentrations were multiplied by

dilution factor to obtain acetaldehyde concentrations. All urine samples, standards, and blanks were run in duplicate.

Acetate Assay.

To detect acetate in the urine samples, we used Acetate Assay Kit (Sigma-Aldrich, MAK086). The acetate kit standard dilution was proportionally decreased to extend kit usage. This was achieved by diluting 1μL of the 100mM acetate stock solution with 99μL of the ethanol assay buffer generating a 1mM standard solution. Briefly, 5μL urine supernatant was added to a flat-bottom 96-well plate and combined with reaction mix (42μL acetate assay buffer, 2μL acetate enzyme buffer, 2μL ATP, 2μL Acetate Substrate Mix, 2μL Probe). Plates were mixed using a horizontal shaker. A standard curve ranging between 0.04-0.2mM was generated using 0, 2, 4, 6, 8, and 10 μL of the 1 mM acetate standard solution in duplicate into a flat-bottom 96-well plate brought up to a final volume of 50μL using acetate assay buffer. Plates were covered tightly and incubated for 40 minutes at room temperature. The absorbance was measured at 450 nm (A_{450}). Urine was tested at several dilutions using acetate assay buffer to ensure that the ethanol values fell within the linear range of the standard curve. A sample blank (42μL acetate assay buffer, 2μL acetate enzyme buffer, 2μL ATP, 2μL Probe) was also set up to remove background signal from any ADP or NADH already present in the sample. All urine samples, standards, and blanks were run in duplicate.

2-Fold Broth Micro-Dilutions

The following dilutions were prepared using bacterial isolates collected pre- and post-binge drinking. The isolates subjected to alcohol metabolites were either *Lactobacillus crispatus* or *Lactobacillus jensenii*; these were the dominant species in the urine sample from which they were

isolated. All isolates were subjected to 2-Fold broth micro-dilutions following the same basic setup (**Figure 5**).

Ethanol Micro-Dilutions.

100µL of MRS broth was added to wells 2-12 and 200µL of 100% ethanol (17,126mM) was added to well 1. 100µL of ethanol was transferred from well 1 into well 2 and mixed by gently pipetting up and down to create an 8,563mM ethanol solution. The series of dilutions were continued until well 11 was reached and the remaining 100µL from well 11 was discarded. 100µL of the prepared bacteria-MRS broth solution was transferred into each well (1-12) and mixed by gently pipetting up and down to create ethanol dilutions ranging from 0-8,563mM. Well 12 contained no ethanol and was used as a control. 200µL of pure MRS broth was added to well 13 as a sterility control. The prepared 96-well plate was incubated for 24 hours. 1:10 serial dilutions were prepared from each well to quantify the viable CFU's at each metabolite concentration for 0 hour, 6 hour, and 24 hour time points. Growth in each of the wells is expressed relative to that of control well.

Acetaldehyde Micro-Dilutions.

99% acetaldehyde was first diluted to 24.75% (4,427mM) using MRS broth. 100µL of MRS broth was added to wells 2-12 and 200µL of the 24.75% Acetaldehyde solution was added to well 1. 100µL of the acetaldehyde dilution was transferred from well 1 into well 2 and mixed by gently pipetting up and down to create a 2,213mM acetaldehyde solution. The series of dilutions was continued until well 11 was reached and the remaining 100µL from well 11 was discarded. 100µL of the prepared bacteria-MRS solution was transferred into each well (1-12) and mixed by gently pipetting up and down to create acetaldehyde dilutions ranging from 0-2,213mM. Well 12 contains no acetaldehyde and was used as a control. 200µL of pure MRS broth was added to well 13 as a

sterility control. The prepared 96-well plate was incubated for 24 hours. 1:10 serial dilutions were prepared from each well to quantify the viable CFUs at each metabolite concentration for 0 hour, 3 hour, 6 hour, and 24 hours time points. Growth in each of the wells is expressed relative to that of control well.

Acetic Acid Micro-Dilutions.

100% acetic acid was first diluted to 0.0125% (218mM) using MRS broth. 100μL of MRS broth was added to wells 2-12 and 200μL of 0.0125% acetic acid solution was added to well 1. 100μL of the acetic acid dilution was transferred from well 1 into well 2 and mixed by gently pipetting up and down to create a 109mM acetic acid solution. This series of dilutions was continued until well 11 was reached and the remaining 100μL from well 11 was discarded. 100μL of the prepared bacteria-MRS solution was transferred into each well (1-12) and mixed by gently pipetting up and down to create acetic acid dilutions ranging from 0-109mM. Well 12 contained no acetic acid and was used as a control. 200μL of pure MRS broth was added to well 13 as a sterility control. The prepared 96-well plate was incubated for 24 hours. 1:10 serial dilutions were prepared from each well to quantify the viable CFUs at each metabolite concentration for 0 hour, 6 hour, and 24 hour time points. Growth in each of the wells is expressed relative to that of control well.

Step 1.
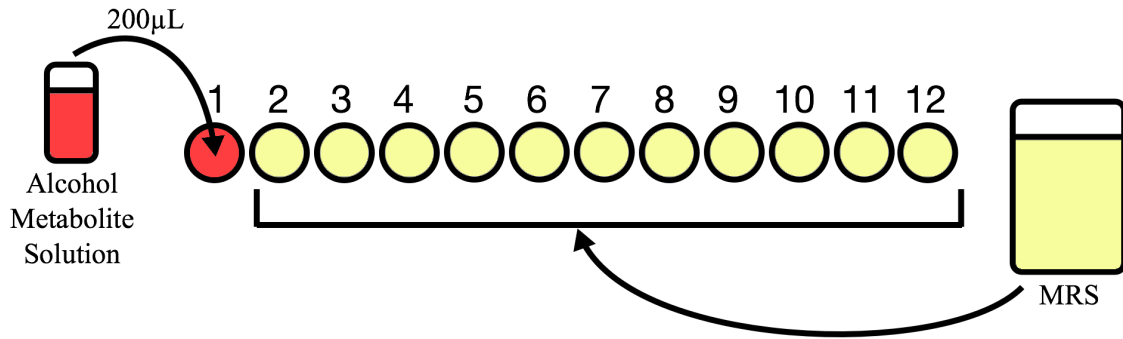
Step 2.
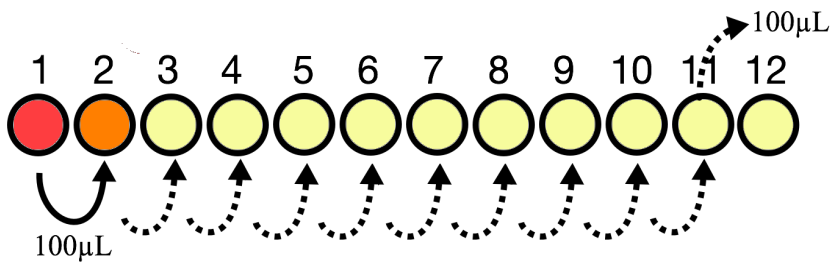
Step 3.
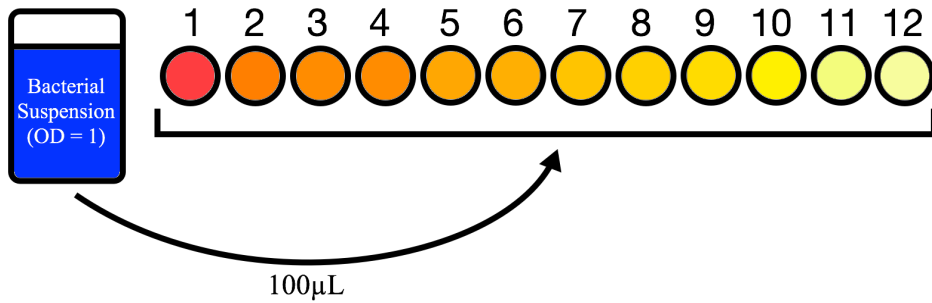
Figure 5. Protocol for 2-Fold Broth Micro-Dilution

Statistical Analysis

Statistical analysis was prepared using RStudio software version 3.3.2. The Shannon-Wiener Index and Simpson's Index were used to determine differences in biodiversity between the periurethral swabs and midstream urines (**Chapter III-IV**) and are a measure of species evenness and richness, respectively. These indices take into account the total number of all unique species present and the total CFU/mL of each species. When evenness (Shannon's) is high, it indicates that all species are equally relatively abundant. When evenness is low, it indicates one species dominates. The Simpson's Index measures the chance that two microorganisms drawn at random will be of the same species and is a measurement of richness. The ENS is the number of equally common species in a population and it is a measurement of the true diversity between communities. Graphs were created using Microsoft Excel for Mac 2016 version 15.19.1, BoxPlotR, and Plotly 2015.

CHAPTER III

ASSESSING THE LONGITUDINAL STABILITY OF THE GENITOURINARY

MICROBIOTA IN ONE FEMALE

Introduction

Microbial communities are longitudinally stable in many human body sites, such as the vagina and

GIT, and dysbiosis of these communities is a marker of disease (Aagaard *et al.*, 2013; Faith *et al.*,

2013; "The Human Microbiome Project Consortium", 2012). Previous researchers have omitted

studying the stability of the urinary tract, likely due to the clinical dogma that urine is sterile.

Conversely, emerging evidence shows that urine is not sterile (Wolfe *et al.*, 2012 Fouts *et al.*,

2012; Brubaker *et al.*, 2014; Hilt *et al.*, 2014; Pearce *et al.*, 2014). Given that urine is not sterile,

it is imperative to better understand the stability of the LUT microbiota and the associations of

these microbiota with disease. To help fill this knowledge gap, I surveyed a single asymptomatic

"healthy" female, collecting daily periurethral swabs and first-void-midstream urines

longitudinally over a 7-month time period. Microbes were detected and identified using MEQUC

and MALDI-TOF mass spectrometry, respectively.

Longitudinal Study

Overview and Rationale.

Other researchers have investigated the stability of the vaginal microbiota (Gajer, *et al.,* 2012). In

contrast, no studies have queried the stability of the GUM. Thus, I performed a pilot study to

characterize the stability of the GUM in one female, hypothesizing that these microbial

communities (i.e., those of the periurethra and the LUT) would mimic the genital microbiota and remain generally stable. This study consisted of the collection of periurethral swabs followed by first void midstream urines from one female over a longitudinal period of 7-months. These periurethral swabs and midstream urines were assessed for stability and resilience.

Results.

Comparing the Microbiota of Periurethral Swabs to Those of Voided Midstream Urine. All samples were collected from a single young healthy female over a 7-month period. The periurethra was sampled by periurethral swabs and the LUT was sampled by voided midstream urine. Comparisons were made using microbiota profiles, which present the data as the relative abundance of each bacterial genus present in each niche. Principal Component Analysis (PCA) plots were used to depict variations between the two sites. Principal Component 1 (PC1) reveals the largest amount of variation, Principal Component 2 (PC2) captures the second most variation, and Principal Component 3 (PC3) shows the third most variation. Statistically, biodiversity was calculated using Shannon's Diversity Index, Simpson's Index, ENS, and species accumulation curves.

Over the course of this 7-month period, this female's individual periurethral microbiota differed from her LUT microbiota in terms of total CFU, individual species abundance, diversity and especially composition (**Figure 6, Table 2, Table 3**). 42 unique bacterial species were detected in the periurethral swabs; species of the genus *Corynebacterium* (orange) dominated (>50%) 53.1% of the swabs, followed by *Staphylococcus* (14.0%, light blue), *Lactobacillus* (14%), *Propionibacterium* (10.9%), *Enterococcus* (6.3%), and Other (1.6%). In contrast, only 23 bacterial species were detected in the voided urines; 87.1% of the urines were dominated by the genus

Lactobacillus (dark blue) followed by *Streptococcus* (7.1%, green), *Staphylococcus* (2.9%), and *Corynebacterium* (1.4%). This pattern varied little throughout the 7-month period and shows evidence of microbial stability over time. There was little overlap between the two sites, consistent with the hypothesis that the microbiota of the periurethral swabs and midstream voided urines are distinct.
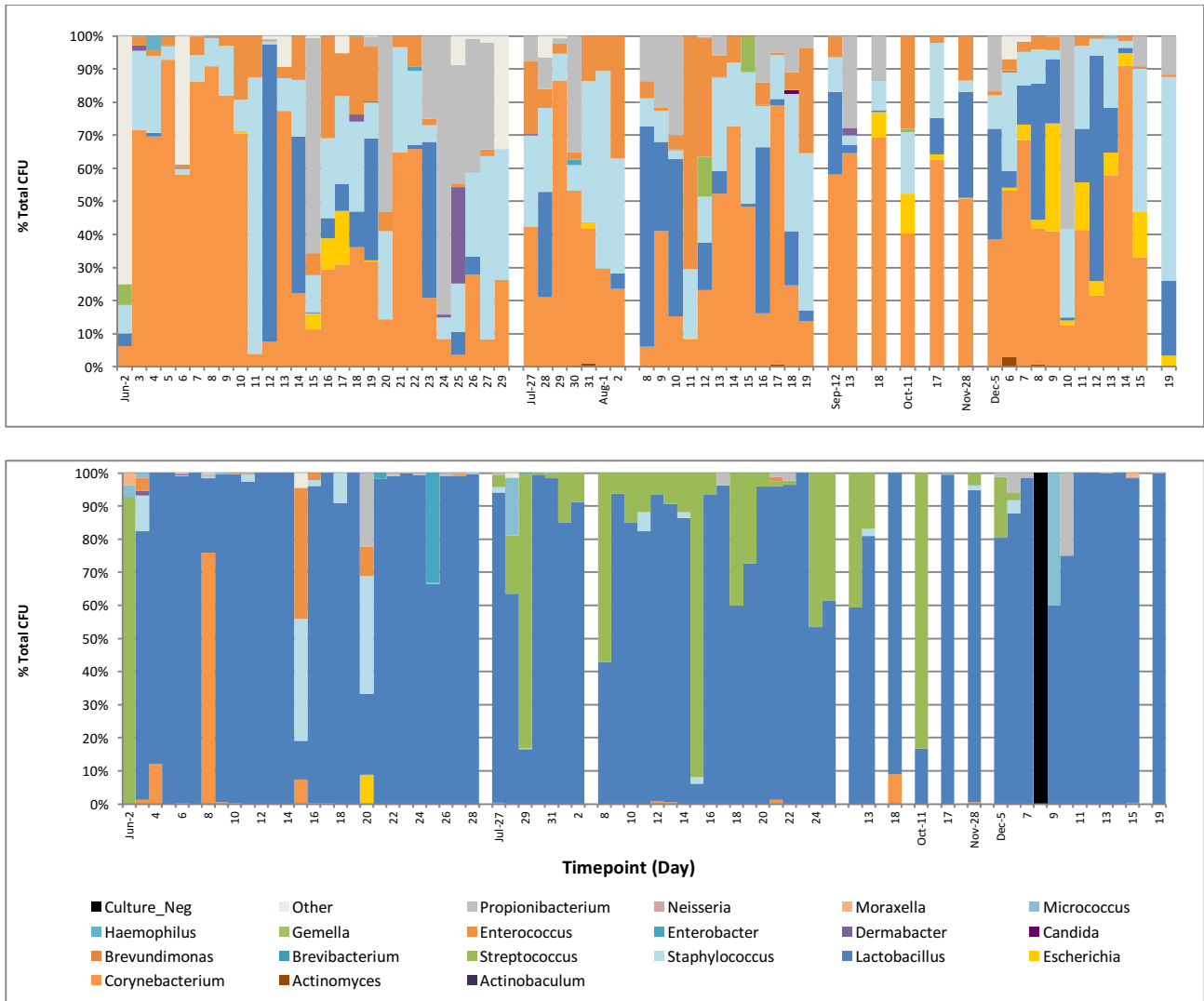
Figure 6. Genus-level Microbiota Profile of One Female's Periurethral and Voided Midstream Urine Over a 7-month Time Period
Genus-level microbiota composition based on percent CFU/mL (y-axis) for the given collection time point (x-axis). The periurethral swabs (above) contain 64 samples. The urines (bottom) contain 70 first void of the day midstream urine samples. Both the periurethral swabs and midstream voided urines were collected from the same individual. No antiseptic wipes were used. White spaces indicate gaps in collection dates.

Study ID	DUMPS001	DUMPS004	DUMPS007	DUMPS012	DUMPS015	DUMPS018	DUMPS021	DUMPS024	DUMPS029	DUMPS036	DUMPS043	DUMPS050	DUMPS057	DUMPS060	DUMPS063	DUMPS065
Date	6/2/16	6/3/16	6/4/16	6/5/16	6/6/16	6/7/16	6/8/16	6/9/16	6/10/16	6/11/16	6/12/16	6/13/16	6/14/16	6/15/16	6/16/16	6/17/16
Total CFU	8000	30900	46400	109700	257900	58000	136100	60300	93400	10300	55600	15000	105500	46000	64500	61600
Actinobaculum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomyces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corynebacterium	500	22100	32300	101900	150000	50000	123600	49500	65800	400	4200	11600	23500	5200	19000	19000
Escherichia	0	0	0	0	0	0	0	0	700	0	0	0	0	2200	6000	10000
Lactobacillus	300	0	500	0	0	0	0	0	0	0	50000	0	50000	200	4000	5000
Staphylococcus	700	7400	10800	4400	4700	4700	11800	9000	8900	8600	400	1500	18000	5100	15500	16400
Streptococcus	500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevibacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dermabacter	0	500	0	300	100	0	100	0	0	0	0	0	0	0	0	0
Enterobacter	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0
Enterococcus	0	800	800	3000	3000	3200	300	1800	18000	1300	100	500	14000	3100	20000	8000
Gemella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemophilus	0	0	2000	100	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus	0	100	0	0	0	0	300	0	0	0	200	0	0	0	0	0
Moraxella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neisseria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Propionibacterium	0	0	0	0	0	0	0	0	0	0	200	0	0	30000	0	0
Other	6000	0	0	0	100000	100	0	0	0	0	500	1400	0	200	0	3200
Culture_Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Study ID	DUMPS068	DUMPS071	DUMPS074	DUMPS077	DUMPS080	DUMPS083	DUMPS086	DUMPS088	DUMPS091	DUMPS094	DUMPS100	DUMPS107	DUMPS109	DUMPS111	DUMPS113	DUMPS115
Date	6/18/16	6/19/16	6/20/16	6/21/16	6/22/16	6/23/16	6/24/16	6/25/16	6/26/16	6/27/16	6/28/16	7/27/16	7/28/16	7/29/16	7/30/16	7/31/16
Total CFU	27900	54100	18800	9100	16100	36000	10800	28200	11100	15700	11400	31700	15700	94700	85400	10300
Actinobaculum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomyces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	300	100
Corynebacterium	10100	17200	2700	5900	10600	7500	900	1000	3100	1300	3000	13400	3300	82000	45200	4200
Escherichia	0	200	0	0	0	0	0	0	0	0	0	0	0	0	0	200
Lactobacillus	3000	20000	5000	2900	200	17000	700	2000	600	0	0	5000	5000	0	0	0
Staphylococcus	7600	5800	5000	2900	3600	1800	700	4100	2800	8700	4500	8800	4000	7600	6600	4400
Streptococcus	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevibacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dermabacter	600	100	0	0	0	0	0	100	0	0	0	100	0	0	0	0
Enterobacter	0	0	0	0	0	0	0	8200	0	0	0	0	0	0	1300	0
Enterococcus	6500	9000	1100	300	1500	700	0	300	0	300	0	7000	900	3000	2000	1400
Gemella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemophilus	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moraxella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neisseria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Propionibacterium	0	1500	10000	0	0	9000	9100	10100	4500	5100	0	2400	1500	1500	30000	0
Other	0	200	0	0	0	0	0	2500	100	300	3900	0	1000	600	0	0
Culture_Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Genus-Level Total CFU of One Female's Periurethra Over 7-Month Period

Study ID	DUMPS117	DUMPS119	DUMPS121	DUMPS123	DUMPS125	DUMPS127	DUMPS129	DUMPS131	DUMPS133	DUMPS135	DUMPS137	DUMPS139	DUMPS141	DUMPS143	DUMPS151	DUMPS153
Date	8/1/16	8/2/16	8/8/16	8/9/16	8/10/16	8/11/16	8/12/16	8/13/16	8/14/16	8/15/16	8/16/16	8/17/16	8/18/16	8/19/16	9/12/16	9/13/16
Total CFU	37500	43200	75100	27800	84200	141700	69700	84000	250300	37600	35900	40300	18300	31700	79700	158000
Actinobaculum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomyces	0	0	0	0	0	0	0	100	0	0	0	300	0	0	0	0
Corynebacterium	11100	10200	4500	11400	12900	11800	16100	43800	182100	18200	5800	31500	4500	4400	46300	102000
Escherichia	0	0	0	0	0	400	0	5800	0	300	0	0	0	0	0	0
Lactobacillus	0	2000	50100	7500	40000	0	10100	0	0	0	18000	800	3000	1000	20000	4000
Staphylococcus	22400	15000	6400	2600	2300	29500	9600	23800	48200	15000	4500	5400	7600	15100	8300	4500
Streptococcus	0	0	100	0	0	0	8500	0	0	4000	100	0	0	0	0	0
Brevibacterium	0	0	0	0	100	0	0	100	0	0	0	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida	0	0	0	0	0	0	0	0	0	0	0	0	200	0	0	0
Dermabacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3400
Enterobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus	4000	16000	3800	300	3500	100000	25000	5400	20000	100	2500	300	1000	10000	5000	300
Gemella	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0
Haemophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	200
Micrococcus	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0
Moraxella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neisseria	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0
Propionibacterium	0	0	10200	6000	25300	0	0	5000	0	0	5000	2000	2000	1200	100	43600
Other	0	0	0	0	0	0	200	0	0	0	0	0	0	0	0	0
Culture_Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Study ID	DUMPS155	DUMPS159	DUMPS163	DUMPS167	DUMPS170	DUMPS173	DUMPS176	DUMPS185	DUMPS191	DUMPS193	DUMPS195	DUMPS197	DUMPS199	DUMPS201	DUMPS203	DUMPS205
Date	9/18/16	10/11/16	10/17/16	11/28/16	12/5/16	12/6/16	12/7/16	12/8/16	12/9/16	12/10/16	12/11/16	12/12/16	12/13/16	12/14/16	12/15/16	12/19/16
Total CFU	39700	49800	183700	186700	59900	20100	168200	73000	153400	34200	49000	117300	73900	124000	26300	87800
Actinobaculum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomyces	0	100	0	0	0	600	0	500	500	0	0	0	0	0	0	0
Corynebacterium	27500	20000	115000	95000	23100	10100	115200	30000	62300	4300	20300	25300	42800	113000	8700	300
Escherichia	3100	6000	3000	200	0	200	8000	1900	50000	500	7000	5000	5000	4500	3600	2600
Lactobacillus	200	0	20000	60000	20000	1000	20000	30100	30000	300	8000	80000	10000	2000	0	20000
Staphylococcus	3500	9200	41700	6500	6000	6000	17000	7500	4000	9100	12200	6000	15400	2500	11400	54000
Streptococcus	0	500	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevibacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0
Dermabacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus	0	14000	4000	25000	800	700	5000	3000	6000	0	1500	1000	100	2000	200	700
Gemella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus	0	0	0	0	0	0	0	0	0	0	0	0	500	0	0	0
Moraxella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neisseria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Propionibacterium	5400	0	0	0	10000	0	0	0	600	20000	0	0	100	0	2400	10200
Other	0	0	0	0	0	1400	3000	0	0	0	0	0	0	0	0	0
Culture_Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Genus-Level Total CFU of One Female's Periurethra Over 7-Month Period

Study ID	DUMPS003	DUMPS006	DUMPS009	DUMPS011	DUMPS014	DUMPS017	DUMPS020	DUMPS023	DUMPS031	DUMPS038	DUMPS045	DUMPS052	DUMPS059	DUMPS062	DUMPS064	DUMPS067	DUMPS070	DUMPS073
Date	6/2/16	6/3/16	6/4/16	6/5/16	6/6/16	6/7/16	6/8/16	6/9/16	6/10/16	6/11/16	6/12/16	6/13/16	6/14/16	6/15/16	6/16/16	6/17/16	6/18/16	6/19/16
Total CFU	3000	7400	2500	2400	63400	2300	134400	57000	100400	102700	100000	100000	100000	6800	52100	100100	1100	100000
Actinobaculum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomyces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corynebacterium	0	0	300	0	100	0	102100	300	0	0	0	0	0	500	100	100	0	0
Escherichia	0	100	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0
Lactobacillus	0	6000	2200	2400	62700	2300	30100	56500	100000	100000	100000	100000	100000	800	50000	100000	1000	100000
Staphylococcus	0	800	0	0	200	0	600	100	100	2200	0	0	0	2500	900	0	100	0
Streptococcus	2500	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevibacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dermabacter	0	100	0	0	200	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus	0	300	0	0	0	0	0	0	200	100	0	0	0	2700	1100	0	0	0
Gemella	300	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus	100	100	0	0	100	0	200	100	0	0	0	0	0	0	0	0	0	0
Moraxella	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neisseria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Propionibacterium	0	0	0	0	0	0	900	0	0	400	0	0	0	0	0	0	0	0
Other	0	0	0	0	100	0	500	0	0	0	0	0	0	300	0	0	0	0
Culture_Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Study ID	DUMPS076	DUMPS079	DUMPS082	DUMPS085	DUMPS087	DUMPS090	DUMPS093	DUMPS096	DUMPS101	DUMPS108	DUMPS110	DUMPS112	DUMPS114	DUMPS116	DUMPS118	DUMPS120	DUMPS122	DUMPS124
Date	6/20/16	6/21/16	6/22/16	6/23/16	6/24/16	6/25/16	6/26/16	6/27/16	6/28/16	7/27/16	7/28/16	7/29/16	7/30/16	7/31/16	8/1/16	8/2/16	8/8/16	8/9/16
Total CFU	4500	50900	50400	100100	100600	300400	100900	100900	100400	53300	7400	180200	100500	51400	4700	6800	700	3200
Actinobaculum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomyces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corynebacterium	0	0	0	0	0	0	0	0	0	200	0	0	0	0	0	0	0	0
Escherichia	400	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus	1100	50000	50000	100000	100000	200200	100000	100000	100000	50000	0	0	0	0	0	0	0	0
Staphylococcus	1600	0	0	0	0	200	0	100	400	800	4700	30000	100000	50600	4000	6200	300	3000
Streptococcus	0	0	0	0	0	0	0	0	0	2000	1300	100	500	700	700	600	400	200
Brevibacterium	0	0	0	0	0	0	0	0	0	0	0	150000	0	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dermabacter	0	900	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0
Enterobacter	0	0	0	0	0	100000	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus	400	0	0	0	0	0	0	400	0	0	0	0	0	0	0	0	0	0
Gemella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus	0	0	0	0	0	0	0	0	0	0	1300	0	0	0	0	0	0	0
Moraxella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neisseria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Propionibacterium	1000	0	300	100	600	0	900	400	0	300	100	0	0	100	0	0	0	0
Other	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Culture_Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4. Genus-Level Total CFU of One Female's Midstream Urines Over 7-Month Period

Study ID	DUMPS126	DUMPS128	DUMPS130	DUMPS132	DUMPS134	DUMPS136	DUMPS138	DUMPS140	DUMPS142	DUMPS144	DUMPS145	DUMPS146	DUMPS147	DUMPS148	DUMPS149	DUMPS150	DUMPS152	DUMPS154
Date	8/10/16	8/11/16	8/12/16	8/13/16	8/14/16	8/15/16	8/16/16	8/17/16	8/18/16	8/19/16	8/20/16	8/21/16	8/22/16	8/23/16	8/24/16	8/25/16	9/12/16	9/13/16
Total CFU	2700	1700	22500	18200	5900	4900	7500	5400	12500	25600	2400	7800	8600	3800	10100	3100	5900	19500
Actinobaculum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomyces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corynebacterium	0	0	200	100	0	0	0	0	0	0	0	100	0	0	0	0	0	0
Escherichia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus	2300	1400	20800	16400	5100	300	7000	5200	7500	18600	2300	7400	8300	3800	5400	1900	3500	15800
Staphylococcus	0	100	0	0	100	100	0	0	0	0	0	0	0	0	0	0	0	400
Streptococcus	400	200	1500	1700	700	4500	500	0	5000	7000	100	100	100	0	4700	1200	2400	3300
Brevibacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dermabacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0
Gemella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Moraxella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neisseria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Propionibacterium	0	0	0	0	0	0	0	200	0	0	0	100	200	0	0	0	0	0
Other	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Culture_Neg	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Study ID	DUMPS156	DUMPS160	DUMPS164	DUMPS169	DUMPS172	DUMPS175	DUMPS178	DUMPS186	DUMPS192	DUMPS94	DUMPS196	DUMPS198	DUMPS200	DUMPS202	DUMPS204	DUMPS207
Date	9/18/16	10/11/16	10/17/16	11/28/16	12/5/17	12/6/17	12/7/17	12/8/17	12/9/17	12/10/17	12/11/17	12/12/17	12/13/17	12/14/17	12/15/17	12/19/16
Total CFU	1100	12010	100500	21200	8200	4900	7200	0	5000	4000	15000	3600	50700	81700	31100	51100
Actinobaculum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinomyces	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Corynebacterium	100	0	0	100	0	0	0	0	0	0	0	0	0	0	100	0
Escherichia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactobacillus	1000	2000	100000	20000	6600	4300	7100	0	3000	3000	15000	3600	50600	81700	30500	51000
Staphylococcus	0	0	0	300	0	200	0	0	0	0	0	0	0	0	0	0
Streptococcus	0	10010	400	800	1500	100	0	0	0	0	0	0	0	0	0	0
Brevibacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brevundimonas	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Candida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dermabacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterococcus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gemella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Haemophilus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Micrococcus	0	0	100	0	0	0	0	0	0	0	0	0	0	0	100	0
Moraxella	0	0	0	0	0	0	0	0	2000	0	0	0	0	0	400	0
Neisseria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Propionibacterium	0	0	0	0	0	300	100	0	0	1000	0	0	100	0	0	100
Other	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Culture_Neg	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0

Table 4. Genus-Level Total CFU of One Female's Midstream Urines Over 7-Month Period

PCA analysis confirmed the distinction between the microbiota of the voided urine samples (yellow) and the periurethral swabs (blue), as two distinct clusters were observed (**Figure 7**). The periurethral swab microbiota tended to be more diverse, as seen by a wider spread compared to the microbiota of the midstream voided urines, which displayed tighter clustering and less diversity.
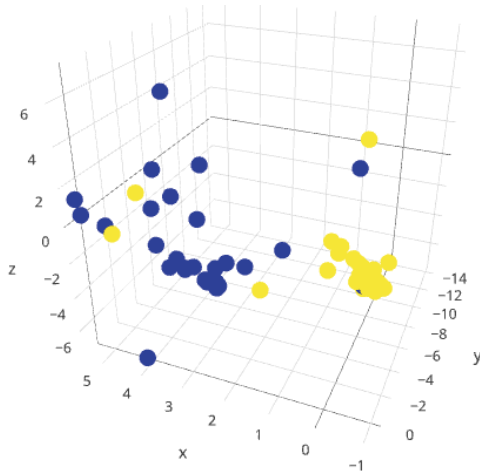

Figure 7. Principal Component Analysis Depicting Associations of Midstream Voided Urine and Periurethral Microbiota Over a 7-month Period.
The factor coefficients are based on genera of bacteria cultured, including *Corynebacterium, Dermabacter, Enterobacter, Enterococcus, Escherichia, Gemella, Haemophilus, Lactobacillus, Micrococcus, Moraxella, Propionibacterium, Staphylococcus, and Streptococcus*. This PCA plot was calculated by comparing the genus level microbiota profiles, based on relative abundance, of midstream voided urines (yellow, n = 70) and periurethral swabs (blue, n = 64) over a 7-month period.

At the species level, the diversity differed greatly between the periurethral swabs and midstream voided urines. The voided midstream urine appeared to be less diverse than the periurethral swabs, as depicted in the rarefaction curve (**Figure 8**). This curve graphically displays species richness for each group of samples and is plotted temporally. As each curve begins to plateau, it indicates total species saturation where it is unlikely any new species will be cultured.

Rarefaction curves with separate plateaus indicates a difference in species diversity. As mentioned previously, the periurethral swabs contained a total of 42 bacterial species and the midstream urines contained a total of 24 species. The midstream urine plateaued at a lower value, which suggests a lower number of unique species. Statistical analysis supported this claim as follows. When comparing the Shannon Diversity Indices (**Figure 9A**) and Simpson's Indices (**Figure 9B**) of the periurethral swabs and midstream voided urines, the midstream urine had lower richness and higher evenness compared to the periurethral swabs. This indicates that the midstream urine is dominated by a single species (*Lactobacillus crispatus*) with disproportionate abundance. In contrast, the periurethral swabs showed higher richness and more evenness when compared to midstream urine, which suggests the periurethral swabs are populated by multiple species with little dominance. Using ENS, the periurethral swabs were found to be nearly 3 times as diverse compared to the voided urines (**Figure 9A**).
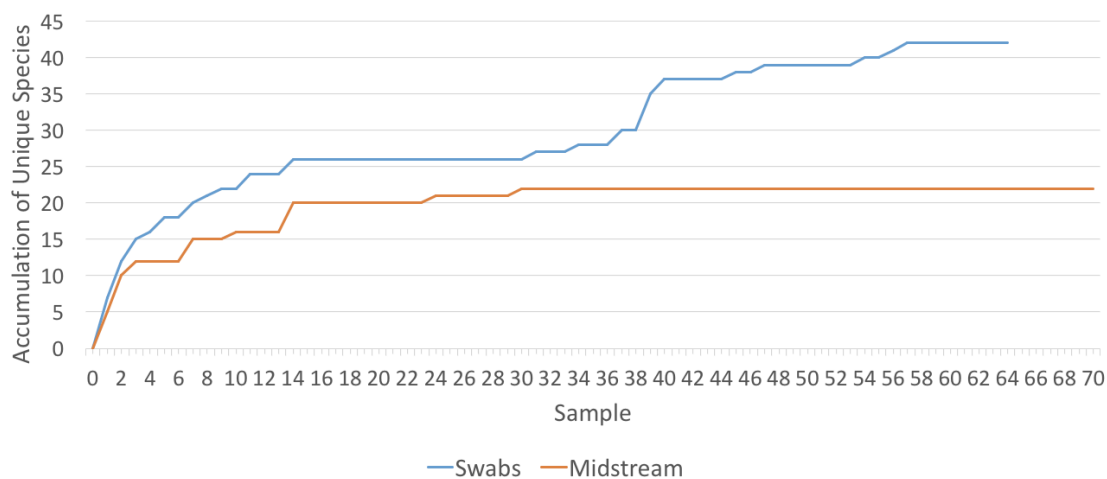

Figure 8. Rarefaction Analysis of Periurethral Swabs and Midstream Voided Urines
The periurethral swabs (n=64) contain 42 unique species and are depicted by a blue line. The midstream voided urines (n=70) contain 24 unique species and are depicted by the orange line.
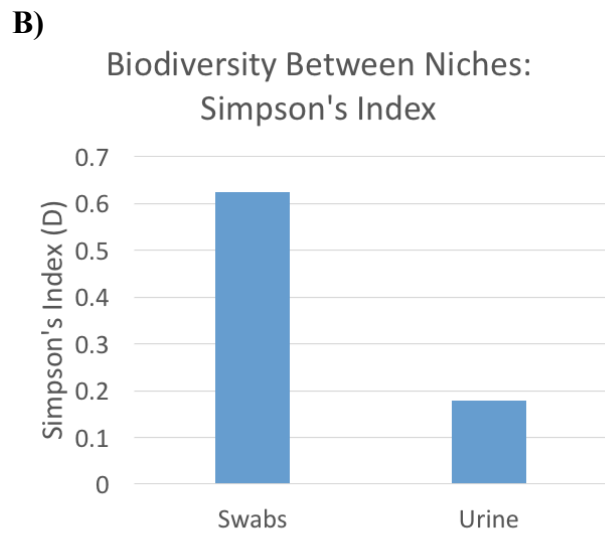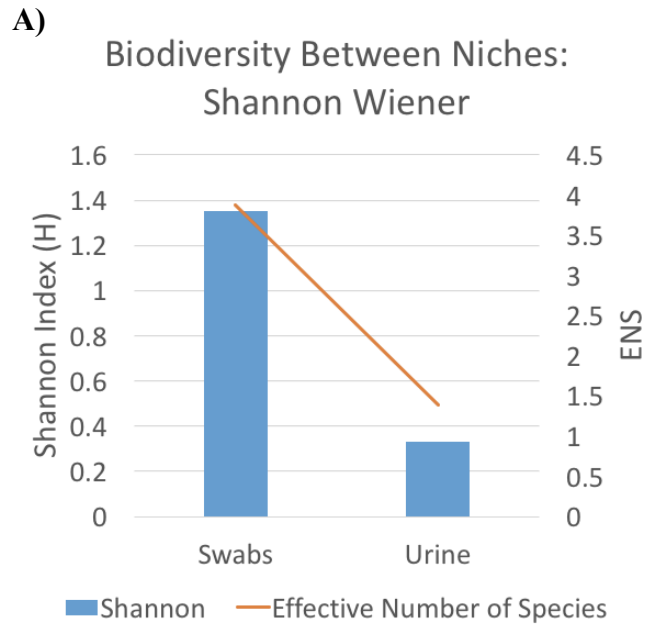
A)

B)

Figure 9. Biodiversity of the Periurethral Swabs and Midstream Voided Urine
(A) Bars depict the Shannon-Wiener Index (H) for the Microbiota of each niche. The orange line depicts the Effective Number of Species (ENS) of the microbiota for each cohort. High Shannon's Index suggests multiple species are present with no dominance. Low Shannon's Index suggests that there is likely one dominant species. ENS is the number of equally abundant species that are required to acquire the same mean proportional genera abundance. (B) Bars depict the Simpson's Index (D) of the microbiota for each niche. Higher Simpson's Index suggests that there are multiple species present at equal levels. Lower Simpson's Index suggests that the species present are very unequal.

Comparison of Clean Catch Voided Urine Over Time. Figure 6 depicts a PCA analysis of the

98 voided urines from the one female participant. The PCA analysis shows that the microbiota of

the collected samples became more closely clustered with time. Outliers or samples not closely

clustered tended to be samples that were collected near the beginning of the study (light teal),

whereas closely clustered samples tended to be collected near the end of the study (dark teal). This

evidence supports the hypothesis that improvement in clean catch occurs with practice. This may

explain why, in **Figure 7**, the microbiota of some voided samples cluster with those of the swabs.
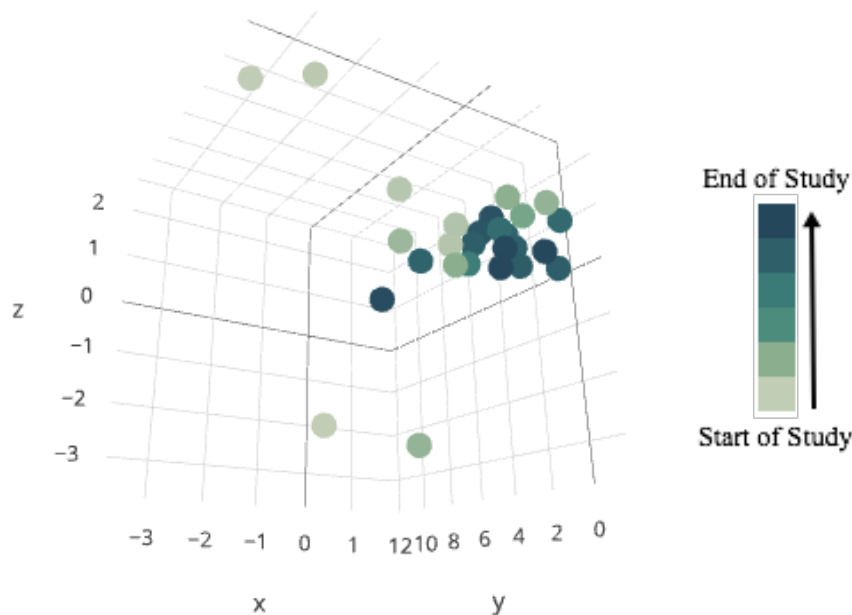
Figure 10. **Principal Component Analysis Depicting the Degree of Association Between Midstream Voided Urines Based on Longitudinal Collection Points.**
This PCA plot uses the same factor coefficients as Figure 3 and was calculated by comparing the genus level microbiota profiles of 70 midstream voided urines collected longitudinally over a 7-month period. The color ranges from light to dark teal where lightest indicates novice collection techniques and darkest indicates expert collection techniques that were acquired through practice.

CHAPTER IV

REASSESSING CLEAN CATCH

Introduction

There is emerging evidence that documents the presence of urinary microbiota in many adult women and men (Brubaker *et al.*, 2014; Hilt *et al.*, 2014; Lipsky *et al.*, 1987; Nelson *et al.*, 2010; Pearce *et al.*, 2014) and associations between these bacterial populations and various LUT disorders have been discovered (Brubaker *et al.*, 2014; Hilt *et al.*, 2014; Lipsky *et al.*, 1987; Pearce *et al.*, 2014; Pearce *et al.*, 2015, Thomas-White *et al.*, 2015). Clean-catch, a non-invasive urine collection method, is routinely used by physicians for urine cultivation and urinalysis. It is thought to provide a urine sample with the least amount of genital contamination, while allowing the physicians to treat based on the bacterial findings. Since its development (Smythe *et al.*, 1960), more sensitive assays have been advanced for culturing urinary bacteria, whereas the clean catch method itself has remained unchanged. In light of these more sensitive assays, I was convinced to reassess the protocol, establishing an optimized version of the method.

Optimizing the clean-catch method is very important. It would prevent physicians from erroneously treating urinary disorders based on genital contamination. Also, it would provide researchers with an alternative method to invasive procedures, such as catheterized urine collection, in exchange for a non-invasive urine collection method that maintains an accurate representation of the LUT microbiota. This is particularly important for community-based studies or for longitudinal studies that require daily sampling.

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To assess and optimize the clean-catch method, female volunteers were surveyed by collecting periurethral swabs followed by varying types of voided urines. Conditions that could alter microbial composition of urine (type of void, time of void and use of antiseptic wipes prior to void) were assessed. Microbes were detected using MEQUC.

Initial vs. Midstream Urine Study

Overview and Rationale.

It is important to identify the voided urine specimen, initial or midstream, that provides the least amount of genital contamination**.** The sheer force of urination through the urethra has the ability to dislodge urethral bacteria, as well as external genital bacteria, depositing them into the urine specimen. Initial stream urine, the first urine to leave the bladder, is hypothesized to contain a larger amount of urethral and genital bacteria because it is the first urine to flow through the urethra and past the periurethra and thus collects any dislodged bacteria. It is also hypothesized that, by collecting midstream urine (i.e., urine collected 3-5 seconds after the onset of urination), most of the dislodged bacteria would already be expelled and the urine would contain less urethral and genital contamination. It is important to collect a urine sample that represents the LUT opposed to the genital tract to avoid false positives due to contamination and to allow accurate interpretation of the collected data. The majority of this study consisted of the collection of periurethral swabs followed by initial and midstream voided urines from one female. Later, additional participants were recruited. To begin, urine specimens were compared to periurethral swabs to determine whether initial or midstream urine sampling results in the least amount of genital contamination.

Results.

Initial and Midstream Voided Urines Exhibit Some Resemblance. A total of 79 voided urines (9 initial, 70 midstream) were collected from a single healthy female over the course of a 7-month time period. All urine specimens were processed using the MEQUC protocol and identified using MALDI-TOF mass spectrometry. **Figure 11** depicts a PCA comparing initial stream voided urine to midstream voided urine samples. All voided urines clustered together, showing that they are similar in terms of bacteria detected. However, initial stream urines seemed to form a subcluster within the cluster of midstream urines.
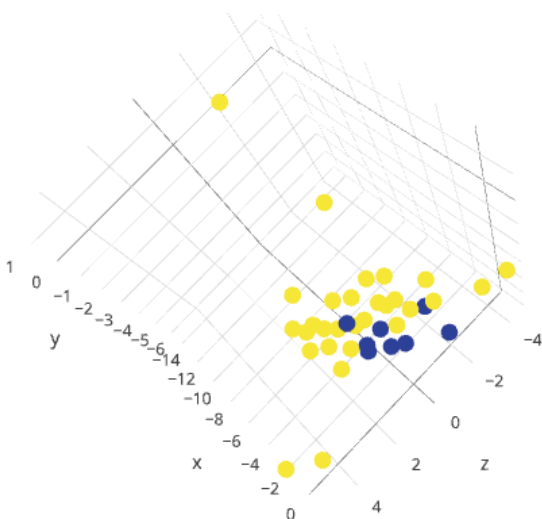

Figure 11. Principal Component Analysis Depicting the Degree of Association Between the Microbiota in Initial Stream Voided Urines and Midstream Voided Urines.
The factor coefficients are based on genera of bacteria collected including *Corynebacterium*, *Dermabacter*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Gemella*, *Haemophilus*, *Lactobacillus*, *Micrococcus*, *Moraxella*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus*. This PCA plot was calculated by comparing the genus level microbiota profiles of initial stream voided urines (blue) and midstream voided urines (yellow).

Initial Stream Urines More Closely Resemble Periurethral Swabs. To determine if the subclustering of initial stream urines was microbiologically relevant, I focused only on paired urine samples and periurethral swabs. A total of 18 voided urines (9 initial, 9 midstream) and 9

periurethral swabs were collected from a single healthy female over the course of 9 days. As above, all urine and periurethral specimens were processed using the MEQUC protocol and identified using MALDI-TOF mass spectrometry. **Figure 12** compares the microbiota of the initial stream urine, midstream urine, and periurethral swabs using radial graphs. Radial graphs depict the number of species present using spokes; the length of each spoke represents abundance of that bacterial species. The microbiota of the initial stream urine appeared more diverse compared to those of the midstream urine, whereas greater overlap of the initial stream urines with the periurethral swabs suggests that these two samples are similar.

Sample 1

Sample 2

Sample 3

Sample 4

Sample 5

Sample 6

Sample 7

Sample 8
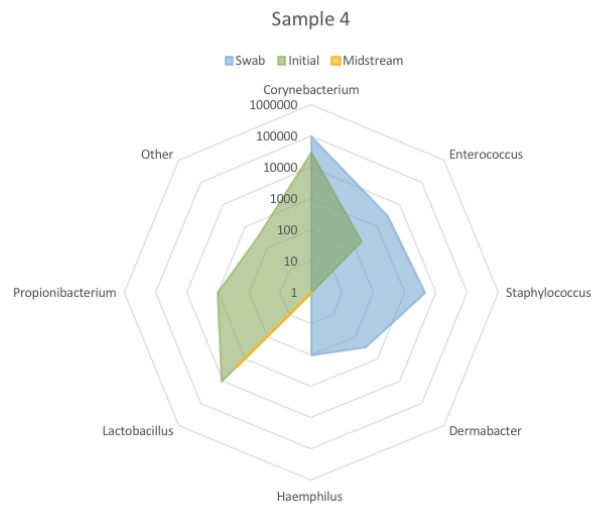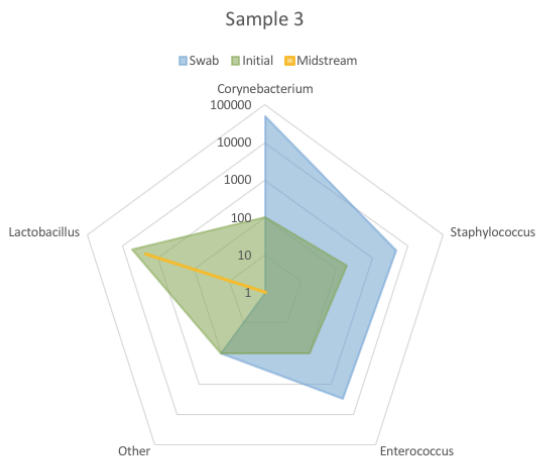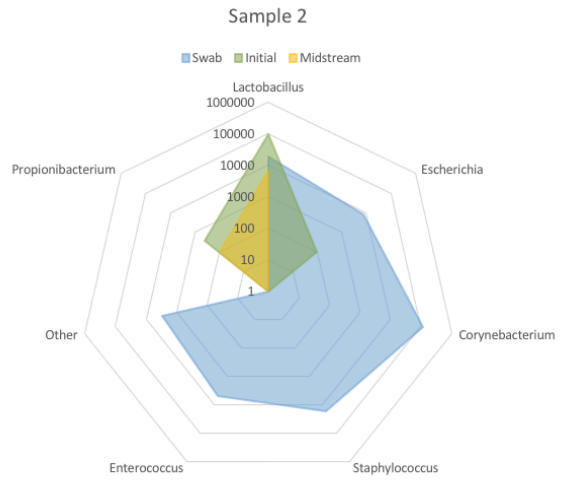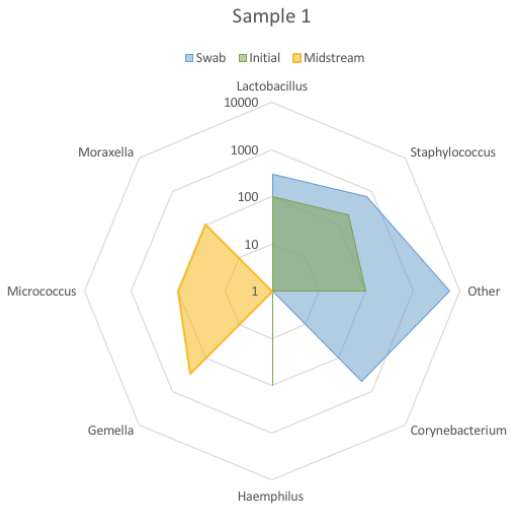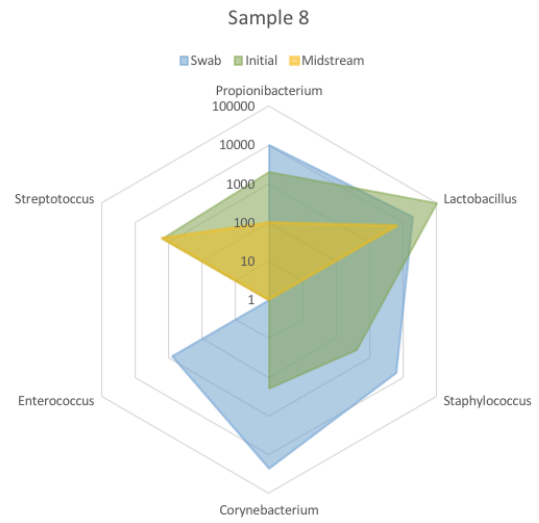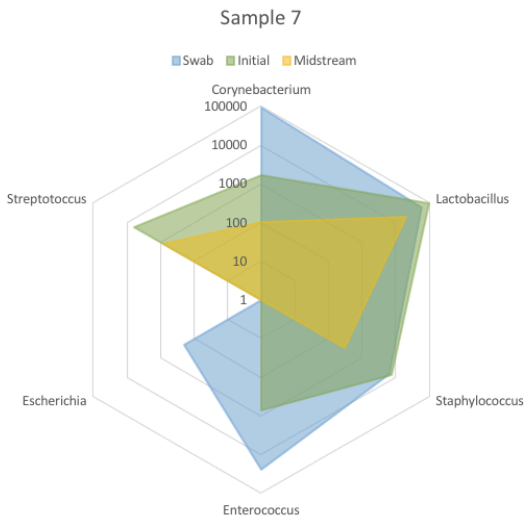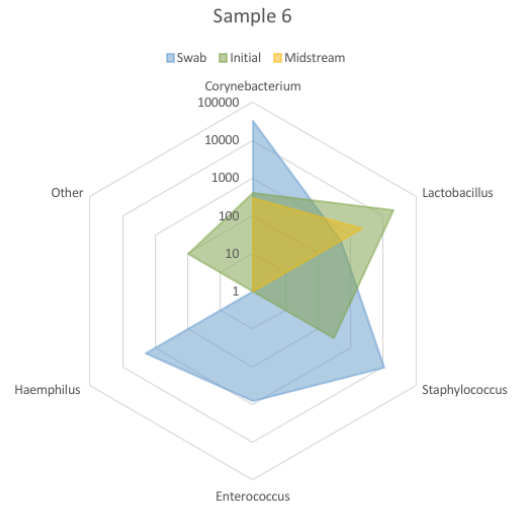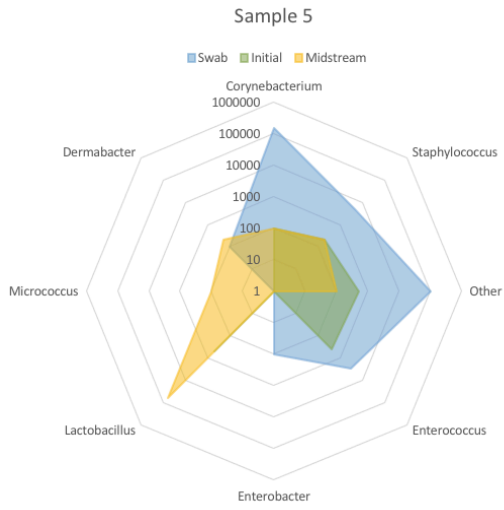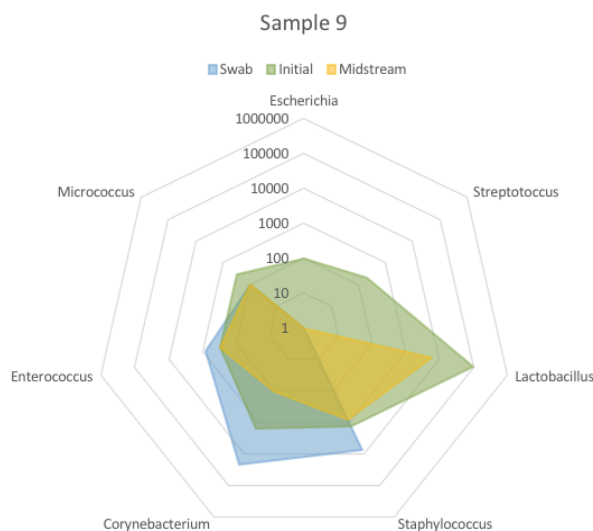
Figure 12. Radar graphs Displaying Bacterial Species Cultured from Periurethral Swabs and Voided Urines
The radar graph consists of radii, called spokes, which represent each bacterial species cultured. The CFU for each bacterial species is represented by the length of each spoke. Each spoke is connected with a line to create a star-like appearance which allows bacteria from each site to cluster. This clustering allows for easy visualization of any overlap between the sites. Samples 1-9 were collected from one female participant and each represents a single sample collection point. Over a 9-day period Periurethral swabs (blue) were collected prior to voided urine collection. Only first voids of the day, including initial stream voided urine (green), and midstream voided urine (yellow), were collected.

Initial Stream Urines Are More Diverse Than Midstream Urines. The radar graphs suggest

differences in the diversity of the two different types of voided urines. The Shannon-Wiener Index

and Simpson's Index were used to determine differences in biodiversity because they are a measure

of species evenness and richness, respectively. ENS is the number of equally abundant species that

are required to acquire the same mean proportional genera abundance. Using these indices, I found

that the midstream urine had lower richness and lower evenness compared to the initial stream

urine (**Figure 13**). This indicates that the midstream urine is dominated by a single species with

disproportionate abundance. In contrast, the periurethral swabs showed higher richness and higher

evenness when compared to midstream urine, which suggests the initial stream urines are populated by multiple species with few dominant species. Using ENS, the initial stream urines were found to be almost twice as diverse compared to the voided urines (**Figure 13a**). Taken together, the data support the use of midstream urine as the appropriate specimen for collection, as microbiologically it least resembles periurethral swabs.

A)
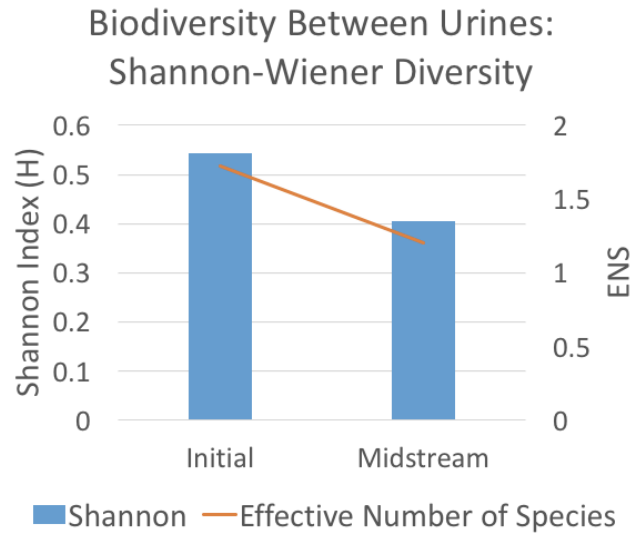
B)
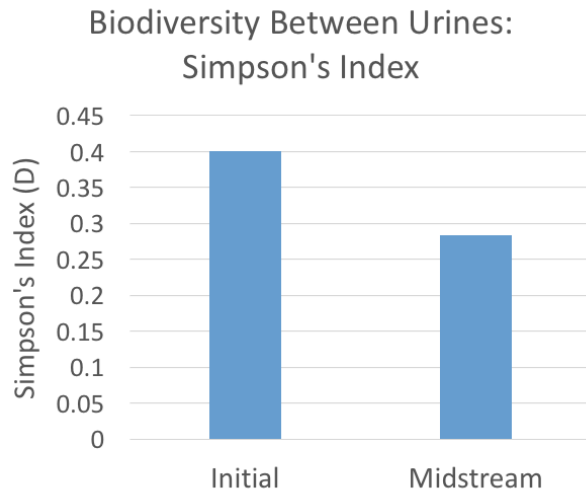
Figure 13. Biodiversity of Initial Stream and Midstream Voided Urines
A) Bars depict the Shannon-Wiener Index (H) for the Microbiota of each urine sample. The red line depicts the Effective Number of Species (ENS) of the microbiota for each cohort. (B) Bars depict the Simpson's Index (D) of the microbiota for each urine sample.

Antiseptic Wipes Study

Overview and Rationale.

Historically, clean catch involves the use of an antiseptic wipe prior to urine collection. Antiseptic wipes are saturated in Castile soap and hydrogen peroxide and are thought to eliminate any external genital bacteria prior to collection, thereby reducing genital contamination of voided urine specimens. However, castile soap is only composed of natural plant based oils and has limited bactericidal capabilities. Castile soap, along with other types of soap, can create charged molecules that can capture and attach to non-water soluble particles including some bacteria. With the addition of water, charged molecules that have already attached to bacteria become free-floating and can be rinsed away. Ironically, no water is used during the clean catch wiping process, so it is possible that these wipes merely spread and relocate bacteria to other areas of the genitalia. Furthermore, it is possible that during urine collection, the urine would act like water and sweep these free-floating charged molecules into the urine stream more easily, thereby causing an increase in genital contamination. This study consisted of the collection of female periurethral swabs followed by the collection of midstream urine with or without the prior use of an antiseptic genital wipes in a small cohort of females. Urine specimens were compared to periurethral swabs to determine whether wipes or no wipes resulted in the least amount of genital contamination.

Results.

Female Participants Have Either Distinct or Non-Distinct Periurethral and Midstream Voided Urines. A total of 8 female participants partook in a 17-day study by collecting periurethral swabs and first voided midstream urines daily. Urines were cultured using $100\mu L$ urine samples, whereas periurethral swab specimens were cultured using 10 or $100\mu L$ periurethral swab

specimens. Thus, the detection limit of urine specimens was 100 CFU/mL and periurethral swabs was 1,000 CFU/mL. To accurately identify contamination, females that took part in this study were required to have distinct periurethral and urinary microbiota profiles. If their periurethral and urinary microbiota profiles were too similar, we were not able to distinguish whether the urinary sample represents the LUT or contains vaginal contamination. Alternatively, if the two sites were very distinct, we could speculate that the urine sample did not contain vaginal contamination. This was achieved by comparing previously collected microbiota profiles (17 periurethral swabs and 17 voided midstream urines). Of the 8 females that were screened, three had distinctly different niches: non-*Lactobacillus*-dominant periurethral swabs and *Lactobacillus*-dominant urines (**Figure 14, A-C**). The remaining 5 participants were non-distinct, having *Lactobacillus*-dominant periurethral swabs and midstream voided urines (**Fig. 14, D-H**). Only individuals with distinct microbiota were analyzed.

A)

Periurethral Swab

Midstream Urine

B)

C)

Time Point

Time Point

D)

E)

F)

G)

H)
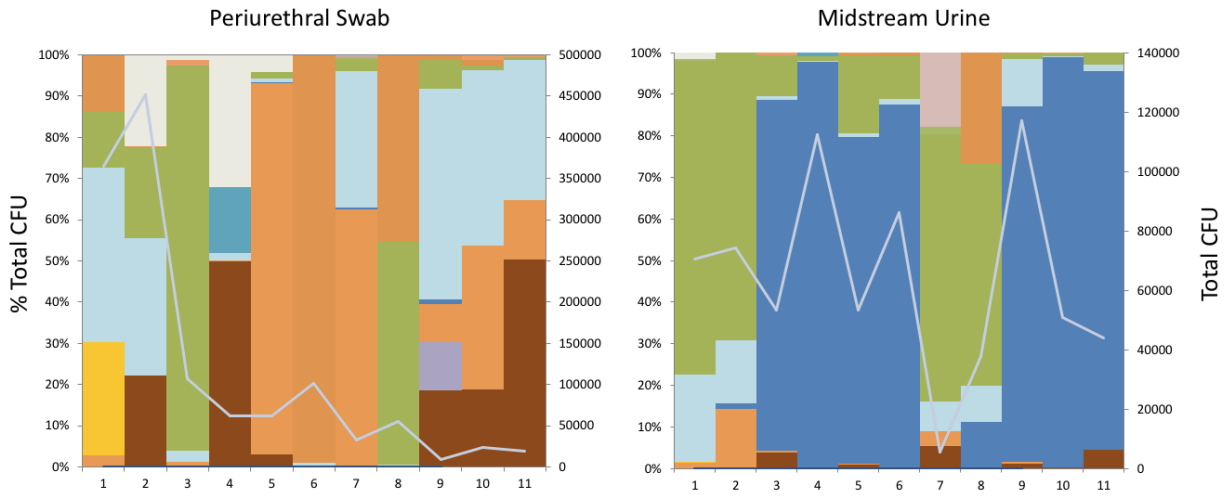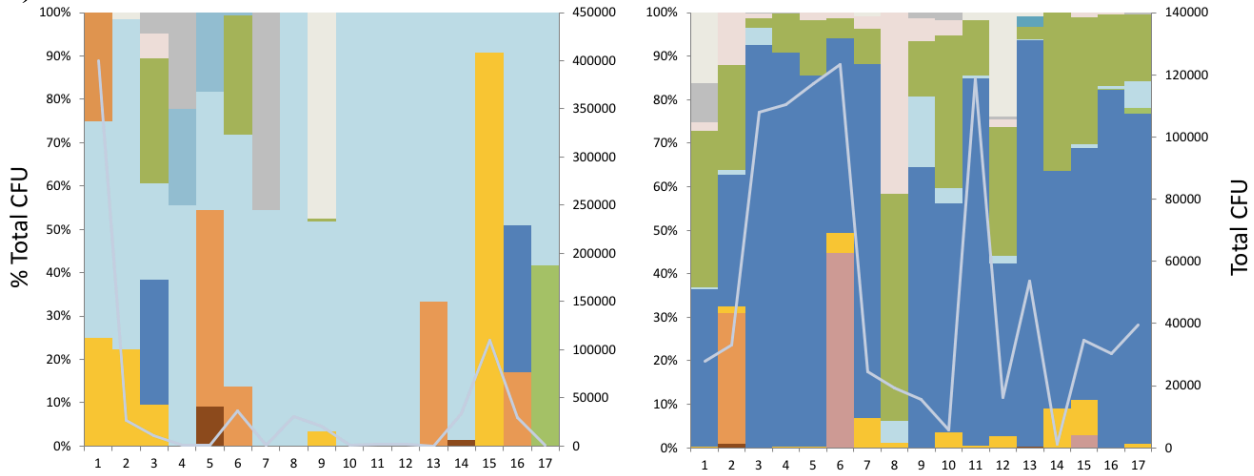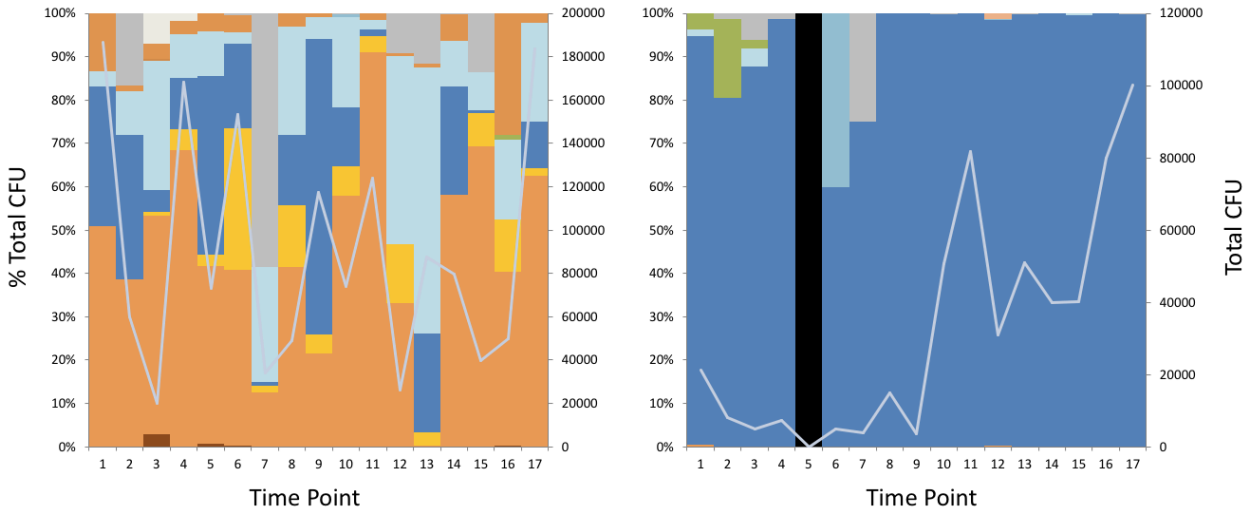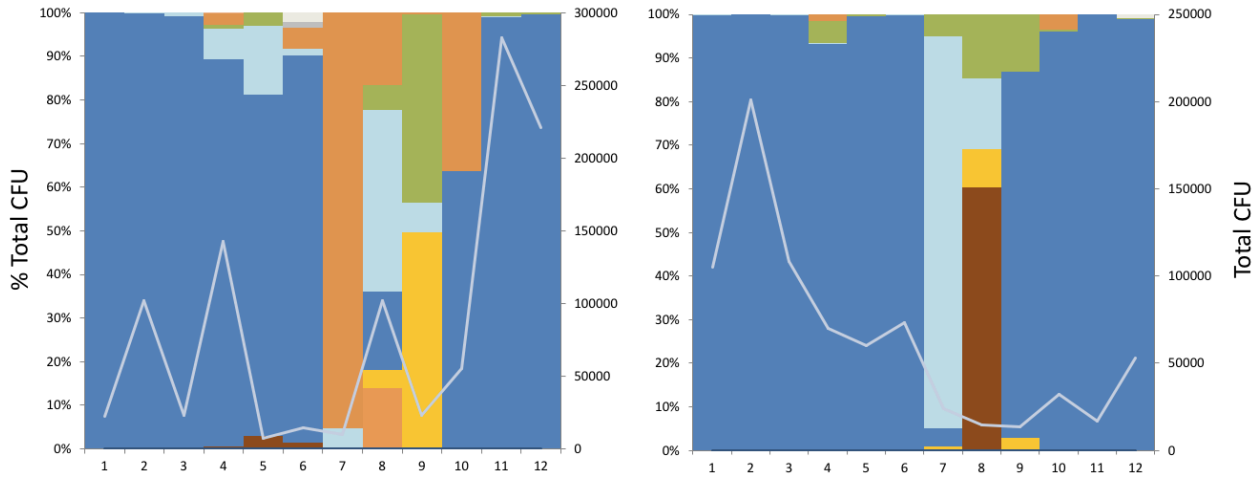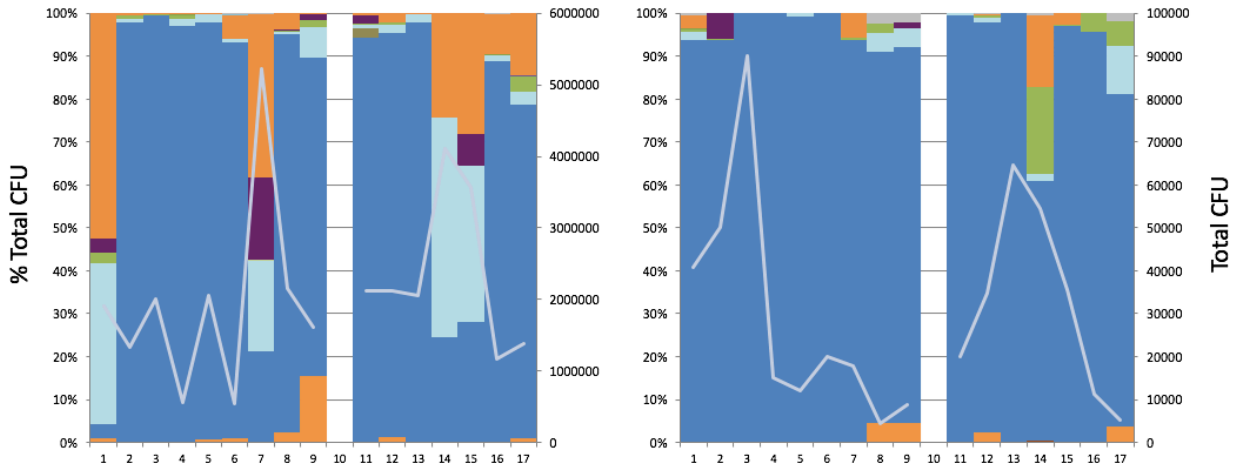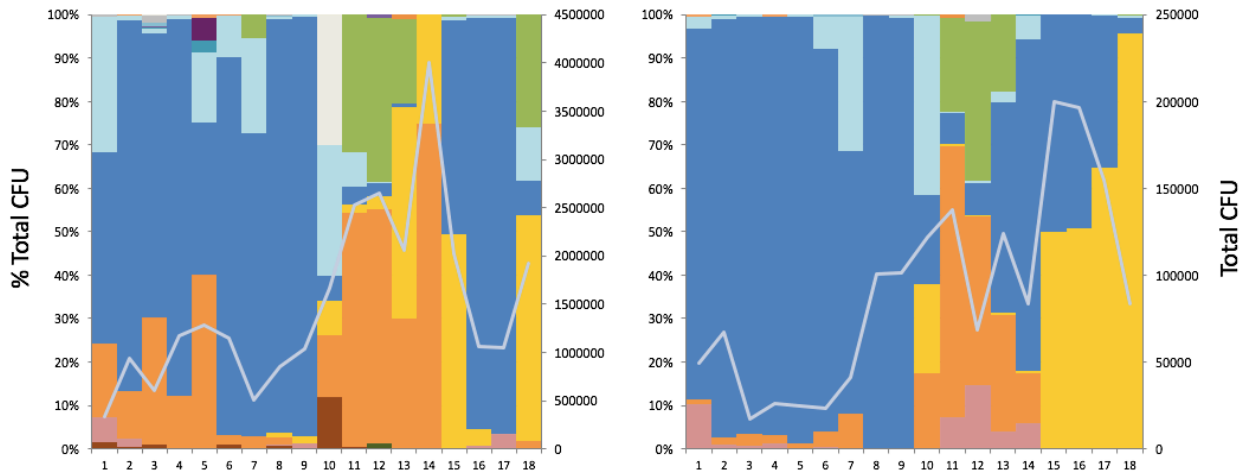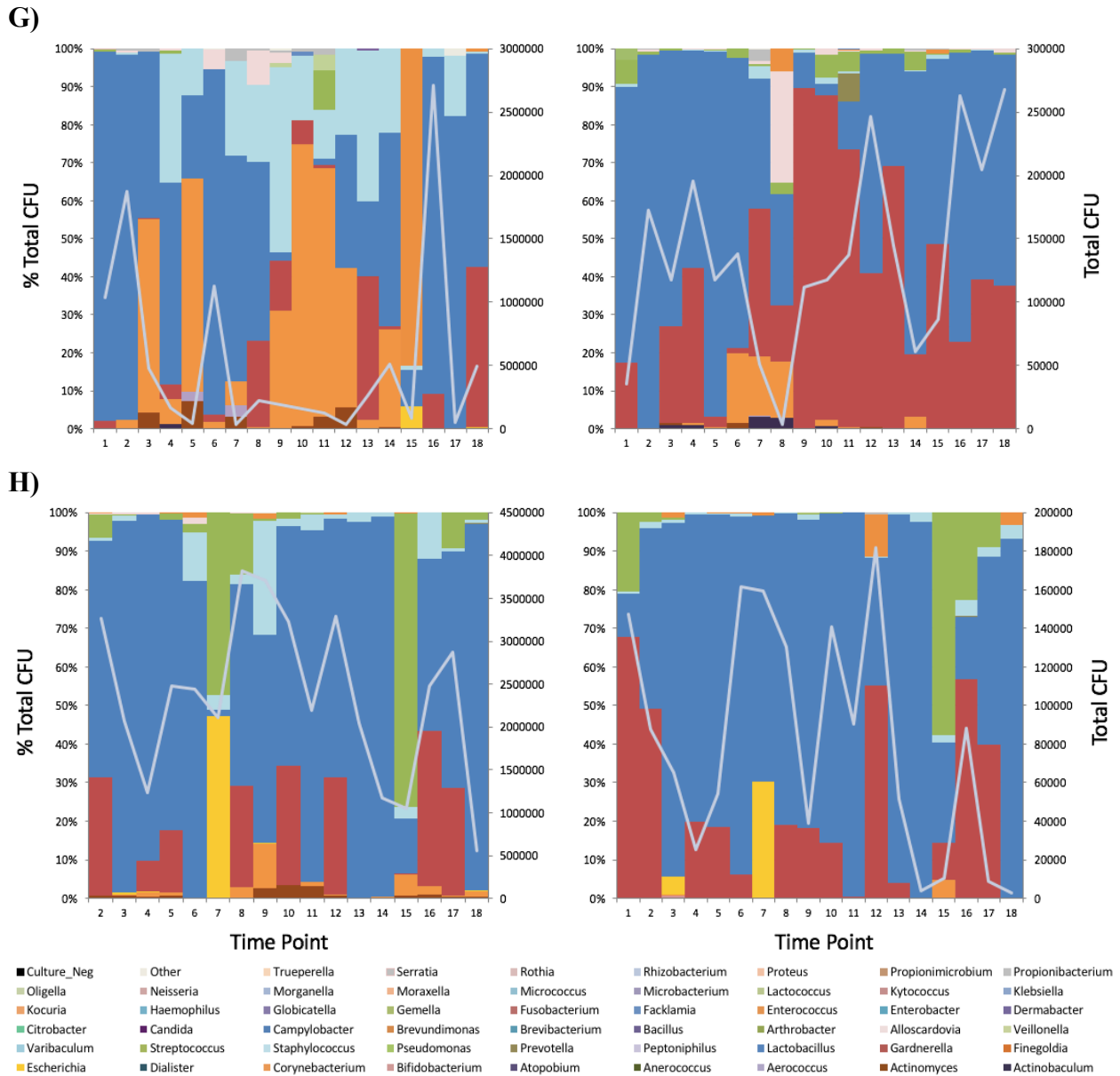
Figure 14. Genus-level Microbiota Profiles of 8 Female's Periurethral Swab and Midstream Voided Urine

Genus-level microbiota composition based on percent CFU/mL (1° y-axis) and total CFU (2° y-axis) for the given collection time point (x-axis). The periurethral swabs (left) and midstream voided urines (right) compares the periurethral swabs and midstream voided urines collected at a single time point from the same individual. A-C show distinct microbiota profiles and D-H show non-distinct microbiota profiles.

Antiseptic Wipes Cause a Change in Microbiota Cultured from Midstream Voided Urines and Periurethral Swabs. All samples were collected from young healthy females over the course of 2 days. Radar graphs (**Figure 15**) were used to show the bacterial species present in each sample, periurethral swabs or voided midstream urine, before and after the use of antiseptic wipes.

Preceding the use of antiseptic wipes, the microbiota of the periurethral swabs and midstream voided urines were clearly distinct. Following the use of antiseptic wipes, changes occurred in bacterial abundance and diversity. In most cases, a shift occurred in organisms cultured from both the periurethral swabs and urines (**Table 6**). In each instance, we cultured organisms from the urine that were not cultured from the urines prior to use of the antiseptic wipes, but were cultured from the swabs (**Figure 15, A-C**). The diversity of swabs also increased following the use of antiseptic wipes in all 4 participants with distinct urine and periurethral microbiota (**Table 6**). On average, there were 7.6 unique species cultured from the swabs before wipes and 10.33 unique species cultured following wipes.

(A)
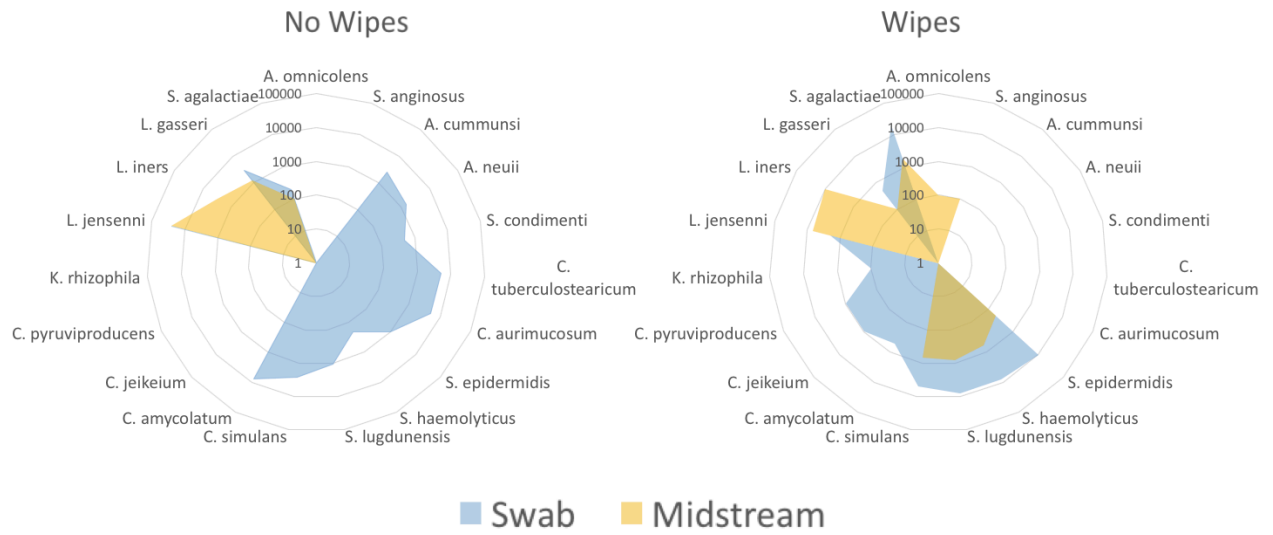
(B)
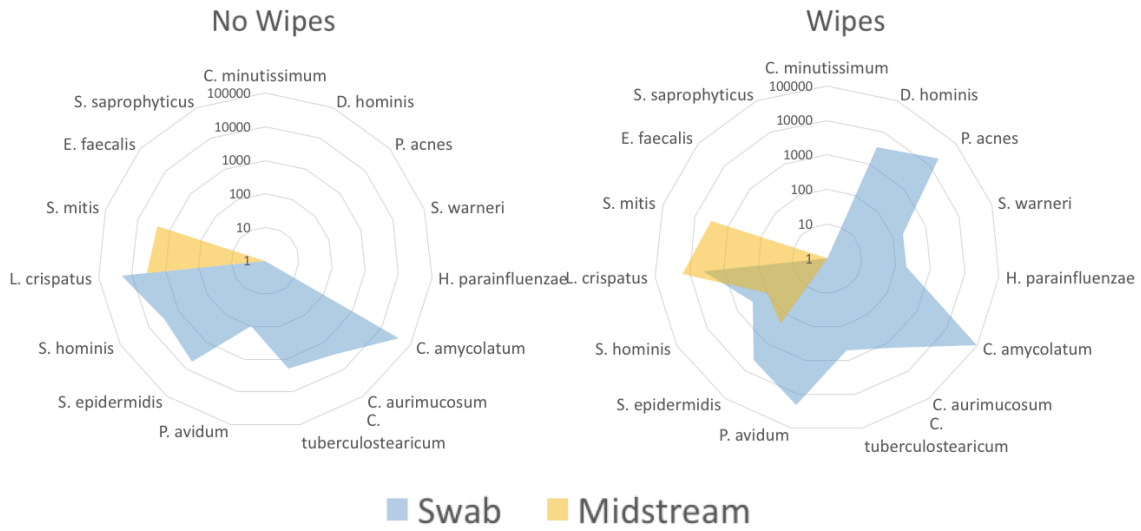
(C)
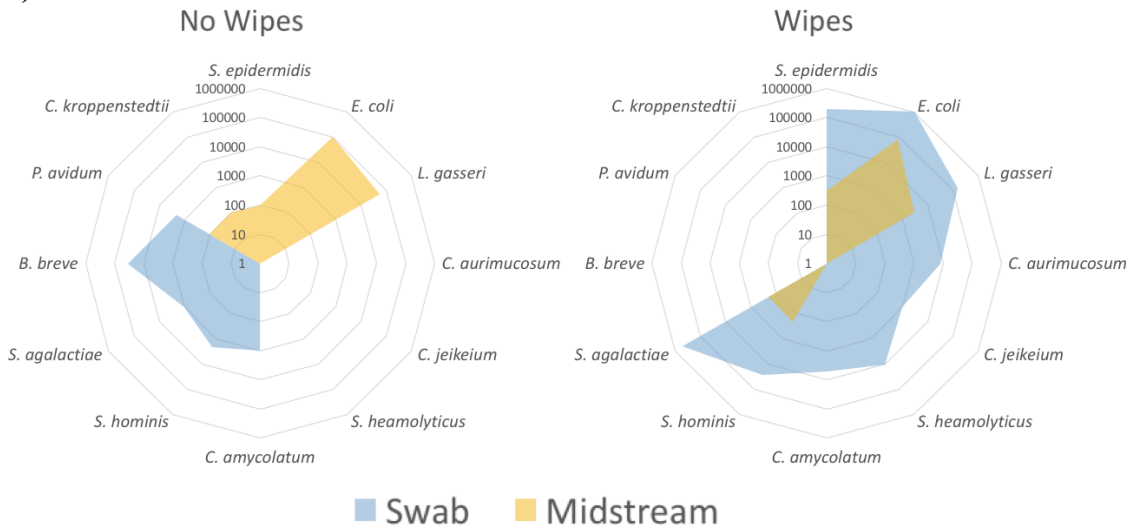
Figure 15. Radar Graphs Displaying Bacterial Species Cultured from Periurethral Swabs and Voided Midstream Urines from 3 Participants Before and After the Use of Antiseptic Wipes.

Figure 15. Radar Graphs Displaying Bacterial Species Cultured from Periurethral Swabs and Voided Midstream Urines from 3 Participants Before and After the Use of Antiseptic Wipes.

The radar graph consists of radii, called spokes, which represent each bacterial species cultured. The CFU for each bacterial species is represented by the length of each spoke. Each spoke is connected with a line to create a star-like appearance which allows bacteria from each niche to cluster. This clustering allows for easy visualization of fluctuations that may occur following the use of antiseptic wipes. (A-D) Each represents a single individual. Midstream voided urine (yellow) and periurethral swabs (blue) were collected over a 2-day time period. On day 1 (left), no wipes were used prior to sample collection. On day 2, (right) antiseptic wipes were used prior to sample collection.

Voided Midstream Urine				
Participant	Total Species Cultured		Following Wipe Use	
	No Wipes	Wipes	Gained Species	Lost Species
A	5	5	2	2
B	2	5	3	0
F	5	11	6	0
Average	4	7	3.6	0.6

Periurethral Swabs				
Participant	Total Species Cultured		Following Wipe Use	
	No Wipes	Wipes	Gained Species	Lost Species
A	5	9	6	2
B	7	11	4	0
F	12	11	4	5
Average	8	10.3	4.6	2.3

Table 6. Effects of Antiseptic Wipes on Total Species Cultured from Periurethral Swabs and Midstream Voided Urines

Time of Void Study

Overview and Rationale.

Due to the variability in an individual's daily routines, urine specimens may be collected (i.e. doctor's visit) at different times throughout the day. It is important to determine if time of day has an impact on the bacteria found in voided midstream urine. Bacteria can replicate exponentially; some species double in as little as 20 minutes. Therefore, urines with longer incubation times are expected to have a higher CFU/mL than urines with shorter incubation times. This study will consist of the collection of midstream voided urine from one female at three different time points throughout the day: first void, afternoon void, and last void. The composition of the urine at varying time points were compared for changes in bacterial abundance and/or diversity.

Results.

Time of Day Does Not Impact the Bacterial Abundance or Diversity of Midstream Voided Urines in One Female. A single healthy female was surveyed over a 7-day period. A total of 21 midstream voided urines were collected (First void, 7; Mid-day void, 7; Last void, 7). All urine specimens were processed using the MEQUC protocol and identified using MALDI-TOF mass spectrometry. **Figure 16A** depicts the total CFU from each time point; I did not observe any significant variations in total CFU. **Figure 16B** depicts the total unique species cultured from each time point; I did not see any significant variations in the number of unique species cultured.

(A)

Five Number Summary (Total CFU)			
	First Void	Midday Void	Last Void
Upper Whisker	102,700	100,800	101,900
3rd Quartile	100,200	100,150	100,200
Median	100,000	80,000	83,000
1st Quartile	54,050	30,550	50,000
Lower Whisker	40,200	1,000	17,200
Nr. of Data Points	7	7	7

(B)

Five Number Summary (Distinct Species)			
	First Void	Midday Void	Last Void
Upper Whisker	5	4	4
3rd Quartile	4.5	2.5	3
Median	2	2	3
1st Quartile	1.5	1	1
Lower Whisker	1	1	1
Nr. of Data Points	7	7	7

Figure 16. Boxplots Displaying Microbial Characteristics of Midstream Voided Urine Based on Time of Day from a Single Female.
Boxplots display the data as a distribution of the five number summaries (minimum, first quartile, median, third quartile, and maximum). The lower cap of the box depicts the first quartile value and the upper cap depicts the third quartile value. The single line that dissects the box depicts the median value. The whiskers (vertical line ending in a horizontal line) depicts the spread of the data. (A) These boxplots show the changes in total CFU cultured from midstream voided urine based on time of collection (first void, mid-day void, last void). (B) These boxplots show the changes in distinct species cultured from midstream voided urine based on time of collection (first void, mid-day void, and last void).

CHAPTER V

DETERMINING THE EFFECTS OF ALCOHOL CONSUMPTION

ON THE GENITOURINARY TRACT

Introduction

In several recent studies, it has been shown that the consumption of alcohol has an affect on the

microbial abundance and diversity of the gastrointestinal tract (GIT) (Engen *et al.*, 2015; Mutlu *et*

al., 2012; Purohit *et al.,* 2008; Queipo-Ortuno *et al.*, 2012). However, few studies have focused on

the affects of alcohol within other human sites that contain microbial communities. There is

emerging evidence that documents the presence of urinary microbiota in many adult women and

men. These bacterial populations are found in healthy individuals as well as in individuals with

various lower urinary tract disorders and diseases (Brubaker *et al.,* 2014; Hilt *et al.,* 2014; Pearce

et al., 2014; Pearce *et al.,* 2015; Thomas-White *et al.*, 2015). If alcohol is able to alter the

homeostasis of the GIT microbiome, it may also influence the genitourinary microbiota and could

potentially be an additional factor in genitourinary disease.

Defining the Physiological Conditions and Bacterial Composition of Urine

Pre- and Post-Alcohol Consumption

Overview and Rationale.

During our previous studies, we noticed what appeared to be an effect of alcohol consumption on

the LUT microbiota of one female. The mechanism that caused these changes remained unclear;

however, it was possible that alcohol by-products were involved. If alcohol plays a role in

altering the GUM, we should be able to measure a change either in the microbial composition and/or on the physiological conditions of the urine pre- and post-alcohol consumption. Further knowledge about the effects of alcohol on human physiology would be beneficial to human health. In particular, there may be significant clinical effect for the many people with urinary tract disorders. Understanding normal fluctuations of the LUT microbiota, and the impact of external/lifestyle factors on the microbial composition, may have significant implications on urinary, genital, and sexual health.

Periurethral swabs and voided midstream urines were collected longitudinally from 8 female participants who consumed alcohol on a regular basis (≥1/month). Participants kept a daily alcohol consumption diary including amount of alcohol consumed, types of alcohol consumed, and length of alcohol consumption. To analyze the physiological conditions of urine, a routine dipstick was used to measure glucose, bilirubin, ketones, specific gravity, blood, pH, protein, urobilinogen, nitrite, and leukocytes. Periurethral swabs and urine were analyzed for microbial content by MEQUC. Urinary characteristics, such as microbial contents and physiological composition, were compared to alcohol consumption.

Results.

There is a Decrease in the Genus *Lactobacillus* Cultured from Voided Midstream Urines Post-Alcohol Consumption. A total of 8 female participants who regularly consumed alcohol partook in a 17-day study by collecting periurethral swabs and first voided midstream urines daily. Participants kept a daily log, including amount of alcohol consumed, types of alcohol consumed, and times of alcohol consumption. Participants were separated based on their dominant urotype (i.e. *Lactobacillus*) (**Table 7**). Participants were eligible for analysis if they had no alcohol

consumption within 3 days prior to an episode of binge of drinking. Additionally, participants were required to have distinct periurethral and urinary microbiota profiles. Of the 8 individuals who participated, 3 individuals fit these criteria and their data were analyzed.

Over the course of the study, the periurethral swabs remained relatively stable in terms of CFU/mL and distribution of microbes. In contrast, total CFU/mL in midstream voided urine decreased drastically following the first day of binge drinking ($\geq$4 alcohol drinks/day). Individuals with urotypes *L. crispatus* (participant's B and C) and *L. jensenii* (participant A) were acutely influenced by alcohol consumption (**Figure 17**), as *Lactobacillus* CFU/mL dropped to zero following binge drinking and took 1.33 days to return to baseline. *Streptococcus* CFU/mL remained relatively constant even in the face of binge drinking. These data are consistent with an effect of binge drinking on some LUT microbiota of some females, specifically the genus *Lactobacillus*.

Individuals with non-distinct periurethral and LUT microbiota saw a complete loss of *Lactobacillus*, a partial decrease in *Lactobacillus*, or no change in *Lactobacillus* **(Figure S3)**. Complete loss of *Lactobacillus,* as seen in Participant F, suggests that this participant may have obtained a clean catch and that the two sites are merely similar. The partial decrease in *Lactobacillus*, such as seen in participant D, may suggest clean catch was not accomplished. This less dramatic decrease in "LUT" *Lactobacillus* could be due to cross contamination with periurethral microbiota. There might have been a substantial decrease in urinary *Lactobacillus*, but no effect on the periurethral *Lactobacillus*; when combined, one would observe a partial decrease. No loss in *Lactobacillus*, such as seen in participant G, may suggest that alcohol does not affect

this individual's urinary *Lactobacillus*. Alternatively, it may be possible that this individual has

little *Lactobacillus* in their LUT and I only captured cross-contamination from the periurethra.

Participant	L. crispatus	L. jensenii	L. gasseri	L. iners	L. vaginalis	L. mucosae
B	X					
C	X					
E	X	X				
F	X		X	X	X	
G	X		X		X	
H			X			X
A		X	X	X		
D	X	X	X		X	

Table 7. *Lactobacillus* Species Cultured from Midstream Voided Urine of Healthy Females. All species of *Lactobacillus* present for each participant are represented by an "x". Dominant urotypes are enclosed by a blue box. *Lactobacillus crispatus* dominant (B-C, E-F); *Lactobacillus jensenii* dominant (A, D); *Lactobacillus gasseri* dominant, (H); Co-dominant *L. crispatus/L. gasseri*, (G).
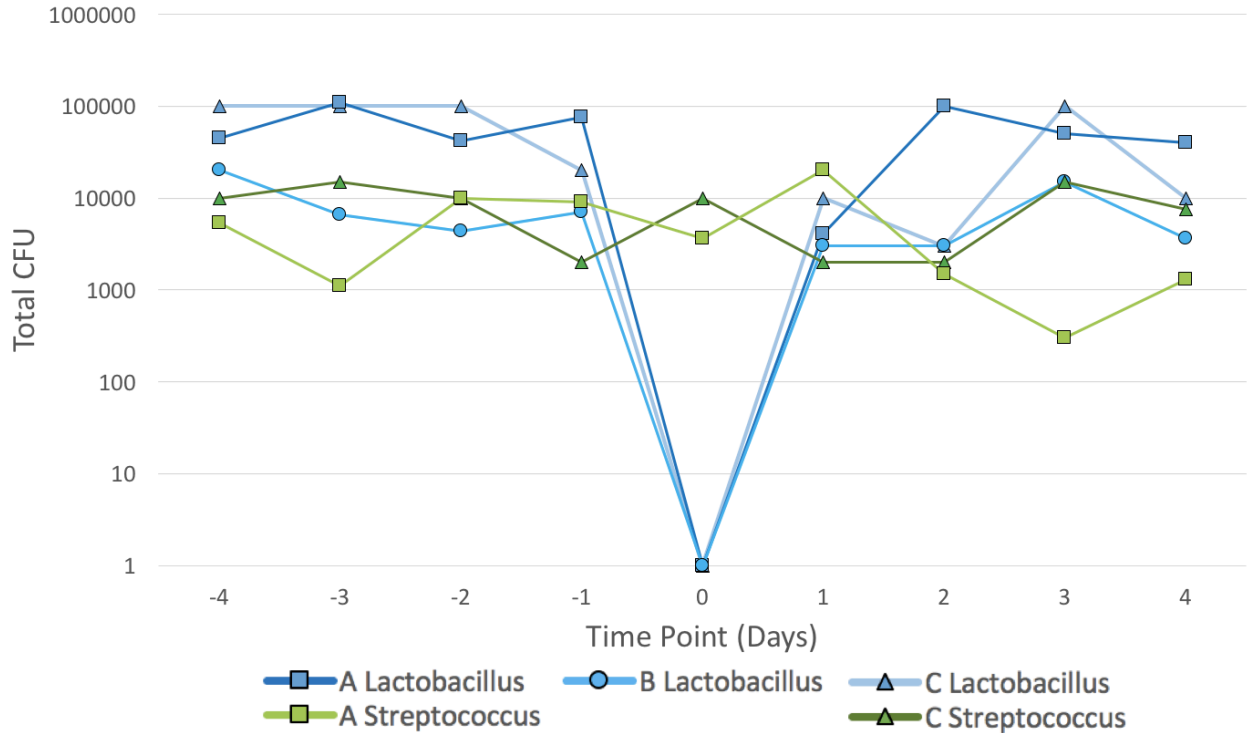
Figure 17. Line Graph Depicting Urinary *Lactobacillus* CFU in Relationship to Binge Drinking

Longitudinal data depicting the relationship between *Lactobacillus* (△) and *Streptococcus* (□) from voided midstream urine before and after binge drinking. Participants (A-C) had distinct periurethral and voided midstream urines. Data is based on total CFU (y-axis) for a given time point (x-axis). Time point 0 illustrates the first morning void following an episode of binge drinking. Participant B did not culture *Streptococcus* from the lower urinary tract. A decrease in *Lactobacilli* CFU is seen following episodes of binge drinking while *Streptococcus* CFU remains stable.

Measuring the By-Products of Alcohol Metabolism in Urine

Pre- and Post-Alcohol Consumption

Overview and Rationale.

Alcohol metabolites are found in urine post-alcohol consumption (Kitasawa *et al*., 1994; Tsukamoto *et al.*, 1993). These metabolites may have an effect on the resident microbiota of the lower urinary tract.

To analyze alcohol and its metabolites in the urine, we used colorimetric assays that detected ethanol, acetaldehyde, and acetate. Urinary characteristics, such as concentrations of alcohol and its metabolites, was compared to alcohol consumption.

Results.

Acetaldehyde and Acetate Were Detected in Female Midstream Voided Urine Following the Consumption of Alcohol but Ethanol Was Not. The urine of 8 female participants who regularly consumed alcohol was tested for alcohol metabolites at various time points over a 17-day period. Levels of metabolites were then compared to alcohol consumption using participant's daily logs. The colorimetric kits were able to detect both acetaldehyde and acetate in urine following alcohol consumption, but were not able to detect ethanol at any point (**Table 8**). Acetaldehyde was detected in 4 out of 6 individuals who consumed alcohol. Acetate was detected one time and in a participant who did not consume alcohol.

			A	E	F	D	G
Week 1	Friday (Day 6)	Ethanol	-	-	-	-	-
		Acetaldehyde	-	-	-	-	-
		Acetate	-	-	-	-	-
	Saturday (Day 7)	Ethanol	-	-	-	-	0
		Acetaldehyde	-	-	-	-	6.83
		Acetate	-	-	-	-	-
	Sunday (Day 8)	Ethanol	-	-	-	-	0
		Acetaldehyde	4.80	8.41	-	-	6.28
		Acetate	-	-	-	-	-
Week 2	Friday (Day 13)	Ethanol	-	-	-	-	-
		Acetaldehyde	-	-	-	-	-
		Acetate	-	-	0	-	-
	Saturday (Day 14)	Ethanol	-	-	-	-	-
		Acetaldehyde	-	-	44.37	-	-
		Acetate	-	0	0	0	0
	Sunday (Day 15)	Ethanol	-	-	-	-	-
		Acetaldehyde	-	0	1048.03	0	-
		Acetate	-	0	11.64	0	0
	Monday (Day 16)	Ethanol	-	-	-	-	-
		Acetaldehyde	-	-	-	-	-
		Acetate	-	0	-	0	0

Table 8. Levels of Alcohol Metabolites Detected in Midstream Voided Urine
Alcohol metabolites were detected throughout the 17-day study using colorimetric assays. Type and concentration of metabolites are as follows: Ethanol (mM), Acetaldehyde (μM), and Acetate (mM). Number values indicate assay was performed while dashes indicate assay was not completed on that day. The shading of the values is related to alcohol consumption (Red, no alcohol consumed; Green, non-binge drinking; Yellow, binge-drinking).

Determining the Affects of Alcohol Metabolites on Bacterial Isolates Cultured

Pre- and Post-Alcohol Alcohol Consumption

Overview and Rationale.

The mechanism that causes a change in bacterial abundance is still unclear; however, it is possible that alcohol and its metabolites are involved. This study consisted of exposing urinary isolates collected pre- and post- alcohol consumption to varying concentrations of ethanol, acetaldehyde, and acetate. To determine sensitivity profiles to various metabolites, the bacteria were quantified by using serial dilutions at various metabolite concentrations for varying time points (up to 24 hours). I predicted urine collected post-alcohol consumption would contain higher concentrations of alcohol metabolites as well as microbial isolates that are less sensitive to alcohol metabolites compared to urine collected pre-alcohol consumption.

Results.

Biological Levels of Ethanol, Acetaldehyde, and Acetic Acid Did Not Affect Viable CFU of
L. crispatus **or** *L. jensenii* **Isolates Collected Pre- and Post-Alcohol Consumption.** Ethanol and acetaldehyde MICs were determined for select isolates. All measured values are listed in **Table 9**. At 24 hours, *L. crispatus* isolates collected post-alcohol consumption had a greater ethanol minimal inhibitory concentration (MIC) compared to isolates collected pre-alcohol consumption (**Fig. 18, top**). *L. jensenii* isolates collected post-alcohol consumption had a greater acetaldehyde MIC at 6 hours compared to isolates collected post-alcohol consumption (**Fig. 18b, bottom**). However, at 24 hours, *L. jensenii* post-alcohol isolates had lower acetaldehyde MIC compared to pre-alcohol isolates (**Fig. 18b, bottom**). There were no other changes in ethanol, acetaldehyde or acetic acid MICs between pre- and post-alcohol isolates. All MICs were significantly above the

measured values and literature values of ethanol and acetaldehyde detected in urine. The concentrations of acetic acid tested against isolates far exceeded the measured and literature values of acetate in the bladder. At the levels we tested, acetic acid MIC was not determined for any of the isolates. I conclude that *Lactobacillus crispatus* and *Lactobacillus jensenii* isolates were not affected by biological levels of ethanol, acetaldehyde, or acetic acid levels found in urine.

(A)

Effects of Ethanol on an isolate of *L. crispatus* Collected Pre-Alcohol Consumption

Effects of Ethanol on an isolate of *L. crispatus* Collected Post-Alcohol Consumption

Effects of Ethanol on an isolate of *L. jensenii* Collected Pre-Alcohol Consumption

Effects of Ethanol on an isolate of *L. jensenii* Collected Post-Alcohol Consumption

(B)

Effects of Acetaldehyde on an isolate of *L. crispatus* Collected Pre-Alcohol Consumption

Effects of Acetaldehyde on an isolate of *L. crispatus* Collected Post-Alcohol Consumption

Effects of Acetaldehyde on an isolate of *L. jensenii* Collected Pre-Alcohol Consumption

Effects of Acetaldehyde on an isolate of *L. jensenii* Collected Post-Alcohol Consumption

(C)

Effects of Acetate on an Isolate of *L. crispatus* Collected Pre-Alcohol Consumption

Effects of Acetate on an Isolate of *L. crispatus* Collected Post-Alcohol Consumption

Effects of Acetate on an isolate of *L. jensenii* Collected Pre-Alcohol Consumption

Effects of Acetate on an isolate of *L. jensenii* Collected Post-Alcohol Consumption

Figure 18. Minimal Inhibitory Concentrations Curves of Alcohol and its Metabolites for Isolates of *L. crispatus* and *L. jensenii* Collected Pre- or Post-Alcohol Consumption
Isolates were subjected to varying concentrations of alcohol metabolites (x-axis). Total CFU for each isolate (y-axis) were calculated at separate time points. (A) Minimal inhibitory assays using ethanol against *L. crispatus* (top) and *L. jensenii* (bottom) comparing isolates collected pre-alcohol consumption (left) to post-alcohol consumption (right). (B) Minimal inhibitory assays using acetaldehyde against *L. crispatus* (top) and *L. jensenii* (bottom) comparing isolates collected pre-alcohol consumption (left) to post-alcohol consumption (right). (C) Minimal inhibitory assays using acetic acid against *L. crispatus* (top) and *L. jensenii* (bottom) comparing isolates collected pre-alcohol consumption (left) to post-alcohol consumption (right).

(A)

Isolate	Time point (hours)	Ethanol (mM)		Acetaldehyde (mM)		Acetic Acid (mM)	
		Pre-Alcohol	Post-Alcohol	Pre-Alcohol	Post-Alcohol	Pre-Alcohol	Post-Alcohol
L. crispatus	0	n/a	n/a	n/a	n/a	n/a	n/a
	3	-	-	553	553	-	-
	6	4,282	4,282	277	277	n/a	n/a
	24	2,141	4,282	138	138	n/a	n/a
L. jensenii	0	n/a	n/a	n/a	n/a	n/a	n/a
	3	-	-	138	138	-	-
	6	2,141	2,141	69	138	n/a	n/a
	24	2,141	2,141	69	35	n/a	n/a

(B)

	Ethanol (mM)	Acetaldehyde (mM)	Acetate (mM)
Measured Levels	0	0 - 0.00841	11.64
Literature Values	7.4 - 55.1	0.000016 - 0.000055	0.25 - 1.79

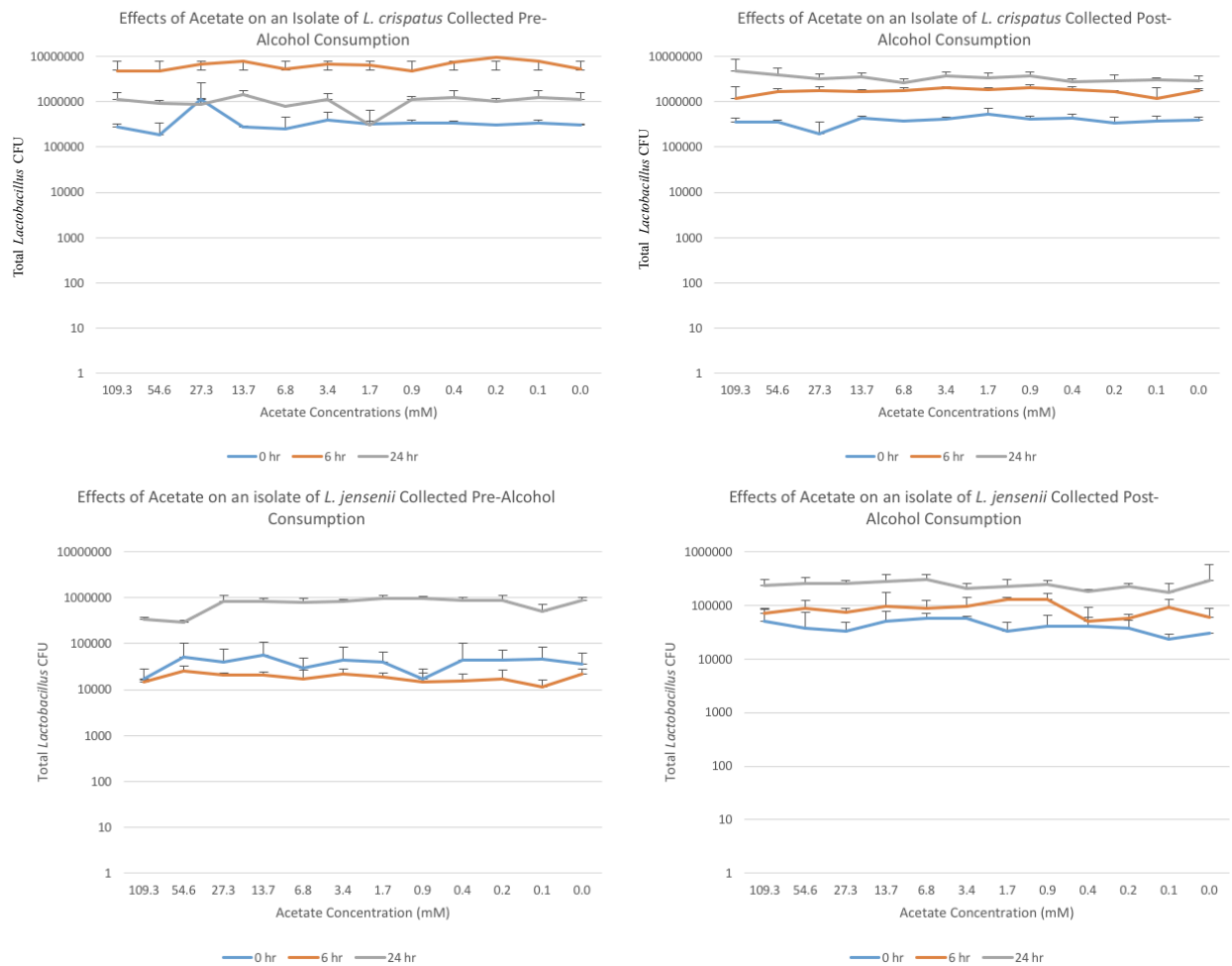**Table 9. Minimal Inhibitory Concentrations of Alcohol and its Metabolites for isolates of *L. crispatus* and *L. jensenii* Collected Pre- or Post-Alcohol Consumption**
(A) This table depicts the concentration of alcohol metabolite needed to cause complete inhibition of bacterial growth relative to time. Blue boxes indicate changes in MIC between isolates collected pre- and post-alcohol consumption. (B) This table indicates the actual concentration of alcohol metabolites measured in urine using colorimetric assays and compares those values to recorded literature values.

CHAPTER VI

CAPTURING A URINARY TRACT INFECTION

Introduction

It is estimated that about 50% of women will develop at least one UTI during their lifetime (Wien

and Kavossi 2007). Typically, UTIs are diagnosed based on either the presented symptoms of the

patient or through routine laboratory work ups. UTI symptoms reported by patients may include

urinary frequency, urgency, dysuria, pelvic pain, incontinence, and hematuria (Wien and Kavossi

2007). Laboratory work ups, such as standard urine culture (SUC) and routine urine dipsticks, can

also be used to detect UTIs.

Detecting an Acute Urinary Tract Infection in a Young Women

Overview and Rationale.

The SUC has a limited sensitivity. It is designed to detect relatively large numbers of known

uropathogens (typically 10^5 CFU/ml) that grow quickly with limited nutrient requirements in

ambient atmospheric conditions, especially *E. coli* (Price *et al*., 2016). SUC requires 24 hours to

culture and interpret the results, which delays patient care and ultimately allows for the progression

of disease. A faster alternative, a routine urine dipstick, may be used to diagnose UTIs, as results

are available in just a few seconds. Still, the rate of false negatives is substantial, as the dipstick

misses up to 77% of UTIs from urine culture-positive UTI patients (Mambatta et el 2015). With

today's advancing assays, such as MEQUC and colorimetric assays, it may be possible to detect a

UTI before routine diagnostics.

The following information was observed retrospectively from the previously described alcohol study (**Chapter V**). Briefly, the periurethral swabs and voided midstream urines collected over an 18-day period from a single female participant and were compared. This participant kept a daily alcohol consumption diary including amount of alcohol consumed, types of alcohol consumed, and length of alcohol consumption. The participant also voluntarily recorded key factors such as first day of menses and onset of UTI. To analyze the physiological conditions of urine, a routine dipstick was used to measure glucose, bilirubin, ketones, specific gravity, blood, pH, protein, urobilinogen, nitrite, and leukocytes. Colorimetric assays were used to analyze ethanol, acetaldehyde, and acetate in urine. Periurethral swabs and urine were analyzed for microbial content by MEQUC. Urinary characteristics, such as microbial content and metabolite composition, were compared to recorded lifestyle factors, such as start of menses and UTI.

Results.

The Start of Menses Disrupts the Periurethral and Urinary Microbiota Profiles.

Microbiota profiles (**Figure 19**) from both periurethra and urine remained relatively stable for Days 1-9. On Day 10, the patient self-reported the onset of menses, which coincided with a change in periurethral and urinary microbiota (**Figure 19**), as well as large amounts of blood detected by routine dipstick (**Table 10**).

MEQUC Detected *E. coli* Before Self-Reported UTI.

MEQUC did not detect *E. coli* in the urine between days 1-8 (**Figure 19**). *E. coli* was first detected in the urine on Day 9 (i.e, 9 days before self-reported UTI) and every subsequent day (**Table 11**). MEQUC detected *E. coli* below the 10^5 CFU/mL threshold of SUC (Days 9-14). Only on day 15 did *E. coli* levels reach that threshold.

Routine Dipstick Detected Known UTI Metabolites on the Same Day as Self-Reported UTI.

Considering known UTI markers (nitrite, leukocytes, blood), the routine dipstick did not detect any relevant changes until Day 18 (**Table 10**). On Day 18, the routine dipstick detected a small amount of blood present in the urine, which coincided with the patients self-reported onset of UTI.

Acetaldehyde and Acetate are Detected in Urine Prior to Self-Reported UTI and Coincide with the Presence of *E. coli*. Acetaldehyde and Acetate were detected using colorimetric assays (**Table 10**) and were only measured on Days 13-15. We saw a rapid increase in acetaldehyde starting (Day 14) and the presence of acetate (Day 15), which coincided with an increase in *E. coli* from 700 CFU/mL (Day 13) to 100,000/mL CFU (Day 15).

Detected Levels of Acetaldehyde During UTI are Capable of Killing *Lactobacillus spp*. The measured acetaldehyde levels recorded during UTI progression were initially measured at 44.37μM on day 14 and within 24 hours increased to 1,048.03μM by day 15 (**Table 10**). When comparing acetaldehyde levels to the MIC assays (**Fig. 18B**), after just 3 hours, *L. crispatus* had a MIC of 553μM and *L. jensenii* had a MIC of 138μM. However, by 24 hours, *L. crispatus* had a MIC of 138μM and *L. jensenii* has a MIC of 69μM, which are far below the measured acetaldehyde levels.

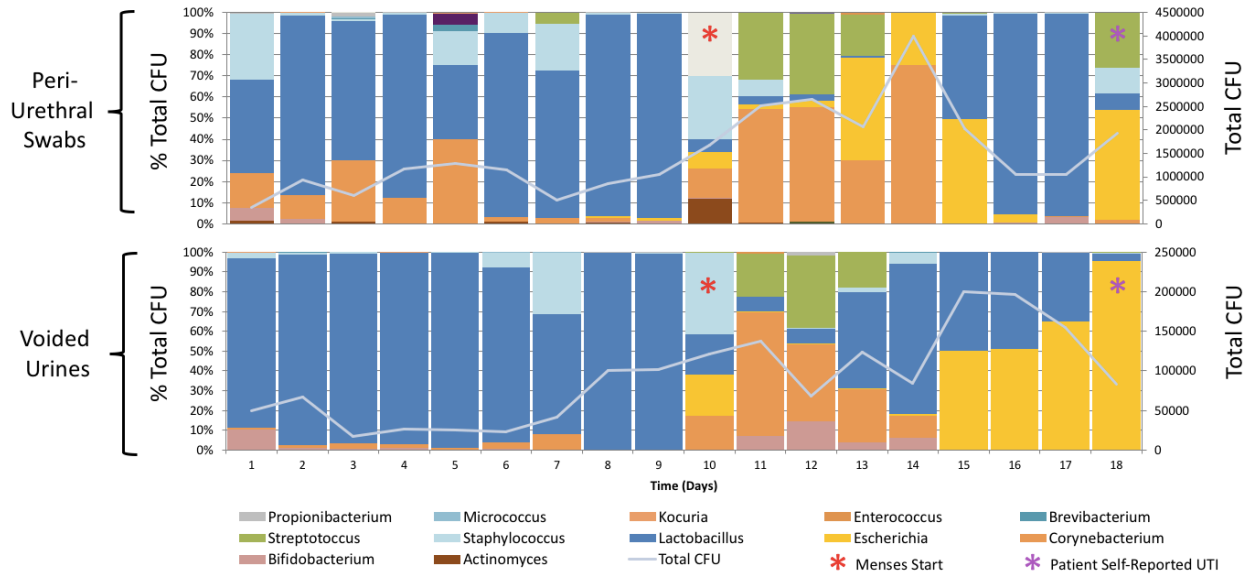**Figure 19. Genus-level Microbiota Profile of One Female's Periurethral Swab and Midstream Voided Urine**

Genus-level microbiota composition based on percent CFU/mL (primary y-axis) for the given collection time point (x-axis). The periurethral swabs (above) midstream voided urines (bottom) were collected from one female individual. Red asterisk indicates the start of menses and purple asterisk indicates self-reported UTI.

Metabolite	Day																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
GLU	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BIL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KET	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SG	1.03	1.02	1.01	1.02	1.015	1.03	1.025	1.03	1.03	1.03	1.03	1.03	1.03	1.03	1.03	1.03	1.03	1.005
BLO	0	0	0	0	0	0	0	0	0	+++ High	+++ High	0	0	0	0	0	0	+ Low
pH	6	6	6	6	6	5.5	6.5	6	6	6	6	5	5	6	6	6	6	6
PRO	0	0	0	0	0	0	0	0	0	30+	0	0	0	0	0	0	0	0
URO	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
NIT	0	0	0	0	0	0	0	8 High	8 High	8 High	0	0	0	0	0	0	0	0
LEU	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ALD	-	-	-	-	-	-	-	-	-	-	-	-	-	44.37 μM	1,048.03 μM	-	-	-
ACE	-	-	-	-	-	-	-	-	-	-	-	-	0 mM	0 mM	11.64 mM	-	-	-

Table 10. Metabolites Measured in Urine During the Development of a UTI
This table depicts metabolites measured in the urine from a single individual over the course of 18-days. Glucose (GLU), Bilirubin (BIL), Ketones (KET), Specific Gravity (SG), Blood (BLO), pH, Protein (PRO), Urobilinogen (URO), Nitrite (NIT), and Leukocytes (LEU) were measured using a routine urinary dipstick. Acetaldehyde (ALD) and Acetate (ACE) were measured using colorimetric assays. Patient self-reported the start of menses (Day 10) and UTI (Day 18). A dash (-) indicates specific metabolite a was not tested for that time point.

E. coli	Day																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CFU	0	0	0	0	0	0	0	0	100	25,000	800	300	700	600	100,000	100,000	100,000	80,000

Table 11. Changes in *E. coli* CFU During the Development of a UTI
This table depicts changes in lower urinary tract *E. coli* (CFU/mL) from a single individual over the course of 18-days. Patient self-reported the start of menses (Day 10) and UTI (Day 18).

CHAPTER VII

DISCUSSION

In **Chapter III**, the GUM (the periurethral and LUT microbiota) of one healthy female participant appear to be generally stable throughout a 7-month period, sustaining a dominant genus with minor fluctuations that generally return to baseline within 1-2 days. But what causes those fluctuations? Regarding the periurethral microbiota, these fluctuations may be associated with external factors, such as sexual activity as seen by **Gajer** *et al.* (2012) or the use of antiseptic wipes (**Chapter IV**). The LUT microbiota also experienced extreme fluctuations and these correlated with the use of antiseptic wipes use and alcohol consumption, both key factors that were explored in **Chapters IV and V**. **Figures 6-9** illustrate that the microbiota of midstream voided urine can be distinctly different from those of the periurethral swab, which suggests that clean catch may be acquired through voided urine. This suggests that future studies may be able to use midstream voided urine, avoiding invasive catheterized urine samples, to accurately sample the female LUT. Additionally, clean catch appears to be a learned process (**Figure 10**). Therefore, simply allowing individuals practice may allow for sample collection that avoids the invasive catheter urine sample. This not only improves patient care, but is economically favorable.

Once I had determined that the GUM can be stable and that a clean catch is possible, I sought an optimized method of clean catch urine collection by focusing on additional factors

(**Chapter IV**). I decided to relate the traditional methods of clinical clean catch with a few caveats. One important factor of clean catch is the time of collection. It was hypothesized that urine collected as the first void of the day would have an increase in bacterial abundance due to the 6-8 hours of urinary retention as the participant slept. Surprisingly, I found that the time of collection had no impact on the bacterial abundance (**Figure 16**). This could have important implications on the health care field as many physicians see patients throughout the day and this would allow for accurate sampling independent of the time of collection. However, the sample size is very small and these non-fluctuating conditions may only be observed in this single individual. This individual predominately had monoculture samples and it is possible that there could be changes in individuals who culture multiple organisms. Other aspects to consider are an individual's hydration habits, diet, and urinary frequency that could change the outcome. For example, if a participant consumes heavy amounts of caffeine (diuretics), would we see a change throughout the day? Or, if a participant voids every hour, would we see a change between first void of the day and afternoon voids? These situations need additional analysis and increasing the number of individuals for this study is required to make further conclusions.

Clinically, midstream urine has been defined as the "cleanest" type of void. An alternative type of void is initial stream urine. By comparing these two voids, it was determined that midstream urine less resembled the periurethral swabs (**Figure 12**). It is important to note that not every midstream sample collection resulted in a "cleaner" catch that was distinctly different from initial stream. However, midstream urine more often gave a cleaner sample than initial stream voids. These findings indicate that midstream urine should remain the preferred sample type.

Collectively, I found that antiseptic wipes tend to increase the diversity of the urine sample, specifically increasing the amount of previously cultured periurethral bacteria (**Figure 15**). The periurethral swabs also displayed changes in bacterial diversity. Some bacterial species were completely eliminated, whereas others were newly cultured. It is hypothesized that these newly cultured organisms may have come from a nearby distinct niche, such as the perineum or deep vaginal site, and that the wipes mechanically relocated the bacteria to the area of the periurethra. Alternatively, it is possible that some bacteria are merely undetectable prior to wipe use due to low CFU and that the total CFU barely passes this threshold the following day. It is important to note that during the course of the study, all participants cultured *E. coli* from their periurethral swabs and that wipes tended to relocate genital bacteria to the area of the urethra. Furthermore, if one relocates bacteria with motile capabilities (e.g., *E. coli* or *Proteus*) to the area of the urethra, they are now increasing the chance that these bacteria will travel up the urethra and cause a UTI. This finding will have a major impact on the world of clinical practices and research. Removing wipes from the clean catch protocol may be advisable to ensure the cleanest urine sample possible and to prevent possible self-inoculation and infection.

It is important to note the discovery that some females have unique periurethral and urinary microbiota profiles, while others do not (**Figure 14**). While this was not an aspect I initially focused on, it quickly became apparent that this must be considered when analyzing current data and for future studies. Regarding clean catch, one can determine if clean catch has been acquired if one can compare bacteria from the periurethra and urine. Only individuals with distinct microbiota profiles can be compared because if the sites are too similar one would not be able to determine which bacteria came from which site.

In **Chapter V**, I assessed the effects of alcohol on the GUM. In a small cohort of females, it was found that *Lactobacillus* was acutely affected by excessive alcohol consumption, while other bacteria found in the bladder, such as *Streptococcus*, remained unaffected. I originally hypothesized that alcohol by-products were to blame; however, further analysis revealed this is not the case. It is possible that other by-products of alcohol metabolism play a role either directly or by triggering other bodily reactions. Lastly, it is already known that alcohol consumption triggers immunological responses and it is possible that these immunological factors damage bacteria, either directly by targeting specific bacterial species or indirectly by damaging the urothelial cells lining the bladder to which bacteria adhere for survival.

One participant from the alcohol study developed a UTI (**Chapter VI**). I was able to capture the participant's baseline GUM and visualize changes that coincided with both menstruation and UTI (**Figure 19**). Interestingly, the changes involved with menses coincided with patient's self-reported menses, but UTI development appeared before the patient's self report. This novel dataset reveals that UTI development can be a gradual process and that the causative agent can be detected 10 days prior to patient's self report and clinical diagnosis. Acetaldehyde concentrations in the urine were high enough to inhibit *Lactobacillus* growth, suggesting this as possible mechanism of virulence for *E. coli* in UTI, because it inhibits protective bacteria, such as *Lactobacillus*, and increases the LUT's susceptibility to *E. coli* colonization. It may be of interest to develop a rapid acetaldehyde detection kit, such as a urine dipstick, that measures acetaldehyde levels in the urine for early UTI detection. In conclusion, this novel case study provides valuable insight into UTI development and it appears that there may be room for improvement in UTI

diagnosis, both in early detection and diagnostics used. This also poses the question whether feminine hygiene practices (e.g., tampons, pads) play a role in UTI.

Collectively, these data give us insight on improving urine collection methods while revealing aspects that should be avoided to obtain an accurate sample. Previous work completed by Loyola Urinary Education & Research Collaborative (LUEREC) included individuals with pre-existing urinary disorders who regularly visited the clinic and thus it was possible to obtain a catheter urine sample. However, due to the newly established clean catch method, we may be able to sample from a larger cohort of individuals found in the public and this would allow us to increase our study population to individuals who are not regularly seen in the clinic. Also, these data suggest that it may be time to adopt a new optimized method of clean catch as well as new methods for detecting UTI.

APPENDIX:

SUPPLEMENTAL FIGURES

		CFU	Influence	(CFU*Influence)
Sample 1 PC1 Score:	**Enterococcus (A)**	0	-14	0
	Lactobacillus (B)	4000	-8	-32000
	Escherichia (C)	6000	0.3	1800
	Staphylococcus (D)	15500	6	93000
	Corynebacterium (E)	19000	12	228000
	Enterococcus (F)	20000	9	180000
			Sum	**470800**

		CFU	Influence	(CFU*Influence)
Sample 1 PC2 Score:	**Enterococcus (A)**	0	-0.2	0
	Lactobacillus (B)	4000	-2	-8000
	Escherichia (C)	6000	12	72000
	Staphylococcus (D)	15500	10	155000
	Corynebacterium (E)	19000	0.3	5700
	Enterococcus (F)	20000	-0.1	-2000
			Sum	**222700**

Sample 1 Coordinates:
(470500, 222700)

		CFU	Influence	(CFU*Influence)
Sample 2 PC1 Score:	**Enterococcus (A)**	3200	-14	-44800
	Lactobacillus (B)	5000	-8	-40000
	Escherichia (C)	10000	0.3	3000
	Staphylococcus (D)	16400	6	98400
	Corynebacterium (E)	19000	12	228000
	Enterococcus (F)	8000	9	72000
			Sum	**316600**

		CFU	Influence	(CFU*Influence)
Sample 2 PC2 Score:	**Enterococcus (A)**	3200	-0.2	-640
	Lactobacillus (B)	5000	-2	-10000
	Escherichia (C)	10000	12	120000
	Staphylococcus (D)	16400	10	164000
	Corynebacterium (E)	19000	0.3	5700
	Enterococcus (F)	8000	-0.1	-800
			Sum	**278260**

Sample 2 Coordinates:
(316600, 278260)

Figure S1. Calculating PCA Scores

PCA scores are calculated using the total CFU for each unique bacterial genus or species present multiplied by the statistically calculated influence score. The sum of these products is calculated to give an individual coordinate. PCA-1 scores will give the x-coordinate, PCA-2 scores will give the y-coordinate, and PCA-3 scores (not pictured) will give the z-coordinate. When coordinates are combined, a single point can be plotted on a scatter plot.
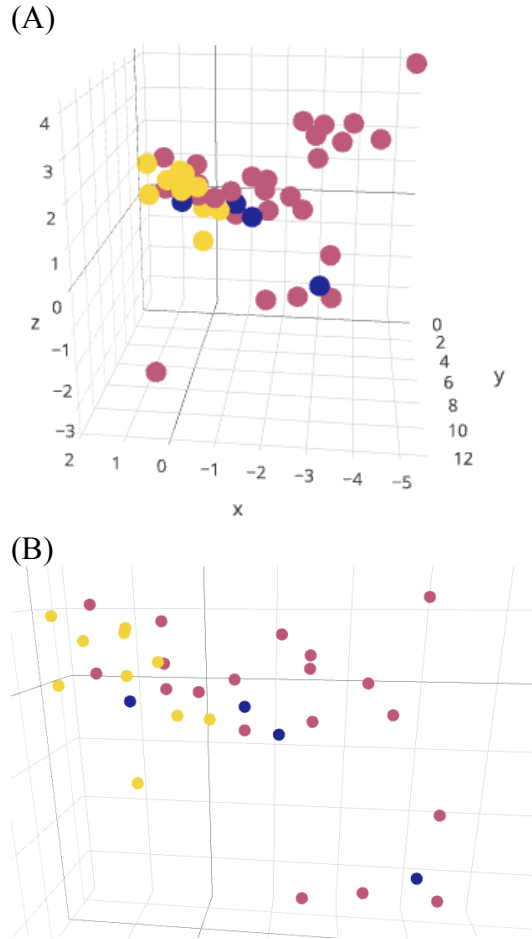
(A)

(B)

Figure S2. Principal Component Analysis Depicting the Degree of Association Between Initial Stream Voided Urines, Midstream Voided Urines, and Periurethral Swabs.
A total of 79 voided urines (9 initial, 70 midstream) and 64 periurethral swabs were collected from a single healthy female over the course of a 7-month time period. The factor coefficients are based on genera of bacteria collected including *Corynebacterium, Dermabacter, Enterobacter, Enterococcus, Escherichia, Gemella, Haemophilus, Lactobacillus, Micrococcus, Moraxella, Propionibacterium, Staphylococcus*, and *Streptococcus*. (A) This PCA plot was calculated by comparing the genus level microbiota profiles of initial stream voided urine (purple), midstream voided urine (yellow), and periurethral swabs (pink). (B) This is a magnified version of PCA plot in panel A that eliminates one periurethral outlier.
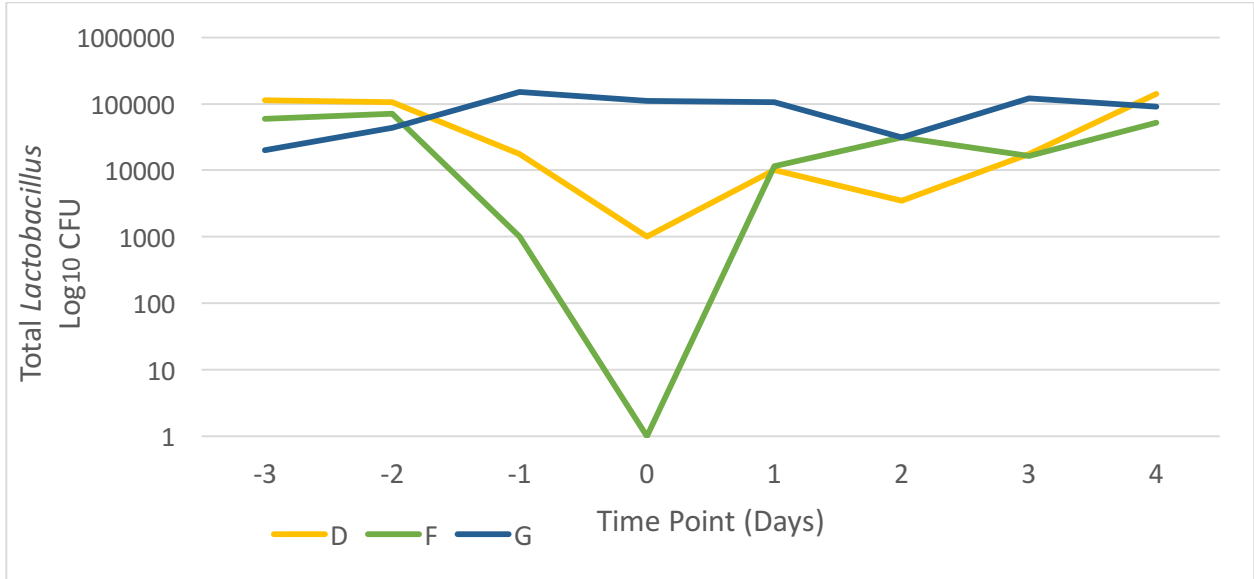
Figure S3. Line Graph Depicting Urinary *Lactobacillus* CFU in Relationship to Binge Drinking
Longitudinal data depicting the relationship between *Lactobacillus* from voided midstream urine before and after binge drinking. Participants (D, F, G) had non-distinct periurethral and voided midstream urines. Data is based on total CFU (y-axis) for a given time point (x-axis). Time point 0 illustrates the first morning void following an episode of binge drinking.

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VITA

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