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Longitudinal Urinary Microbiome Studies: A Need to Transition to Voided Urine

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LONGITUDINAL URINARY MICROBIOME STUDIES:

A NEED TO TRANSITION TO VOIDED URINE

A THESIS SUBMITTED TO

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LIST OF ABBREVIATIONS

CFU	Colony Forming Units
EQUC	Expanded Quantitative Urine Culture
IRB	Institutional Review Board
MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectroscopy
RUTI	Recurrent Urinary Tract Infections
rRNA	Ribosomal RNA
SCC	Standard Clean Catch
SPA	Supra-Pubic Aspirate
SUC	Standard Urine Culture
TUC	Transurethral Catheterization
UTI	Urinary Tract Infection

ABSTRACT

It is now established that the bladder is not sterile; it contains communities of microbes (microbiota). While the healthy bladder microbiota have been defined using expanded quantitative urine culture (EQUC) and urines obtained by transurethral catheterization (TUC), longitudinal and population studies have not previously been possible. These studies cannot be done using TUC urines, as it would be impractical to catheterize participants daily. Instead, we must transition to using voided urines. In order to make this switch, we have addressed three main issues raised by using voided urine. First, we showed that EQUC out performs standard urine culture (SUC) on voided urines in a clinically relevant population. Paired voided and TUC urines were provided by women diagnosed with recurrent urinary tract infections. Both urines were cultured with EQUC and SUC for analysis, and we found that EQUC detects more diverse bacteria at higher rates, including potential uropathogens. Next, we defined the constituents of the urethral and peri-urethral microbiota by collecting TUC urine, a urethral brush, a peri-urethral swab, and voided urine samples from each participant. Although this approach allowed us to characterize the microbiota of the entire lower urinary tract, we could not deconstruct voided urine. Finally, we identified a "cleaner" catch method. The current standard clean catch method has been shown to contain significant post-bladder contribution, making it unsuitable for urinary microbiome (urobiome) research. Therefore, we tested a novel urine collection device (Peezy, by Forte Medical) and found that it is capable of reducing post-bladder contribution to voided urines. Combined, these studies provided crucial insight for interpreting voided urine for urobiome research.

CHAPTER ONE:

INTRODUCTION

In 2012, Wolfe and coworkers reported the use of 16S ribosomal RNA (rRNA) gene sequencing to provide evidence of bacterial DNA (microbiome) in urine taken by suprapubic aspiration from the bladders of adult women undergoing urogynecological surgery (Wolfe et al 2012). However, these organisms detected by sequencing were not able to be cultured by standard urine culture (SUC) method; therefore, in 2014, they developed the expanded quantitative urine culture (EQUC) to show that these microbes were alive (Hilt et al 2014). Thus, the prevailing dogma, that the adult female bladder is sterile, was not true. This paradigm shift requires a re-evaluation of a vast range of urinary disorders. For example, urinary tract infection (UTI) may not be strictly due to a pathogenic invasion of a sterile environment, as commonly thought. Although suprapubic aspiration samples the bladder directly, this method of urine collection is impractical for microbiome research due to its highly invasive nature. For this reason, Wolfe and co-workers also examined urines collected by transurethral catheterization (TUC), and determined that this less invasive urine collection method yielded results similar to suprapubic aspiration (Wolfe et al 2012). Analysis of TUC urines by 16S rRNA gene sequencing and EQUC allowed several studies to define the bladder microbiota and associations with some lower urinary tract symptoms (Pearce et al 2014, Pearce et al 2015, Thomas-White et al 2016, Karstens et al 2016).

Although the use of TUC urine has allowed the research community to establish that the bladder is not sterile, it limits urinary microbiome (urobiome) research to patient populations; therefore, we must shift to using non-invasive sampling techniques. A common practice in

clinics is to collect midstream voided urine using the standard clean catch (SCC) method; however, this SCC method has been shown to contain high amounts of vulvovaginal contribution when cultured (Immergut et al 1981, Lifshitz et al 2000, Baerheim et al 1992). Since there is much overlap between vaginal and urinary microbiota (Thomas-White et al 2018), the current SCC method is unacceptable for urobiome research, as it does not yield urine that resembles the bladder microbiota.

To expand urobiome research to general populations and longitudinal studies, one must find a method to better interpret voided urine by determining the lower urinary tract origin of microbiota or be able to obtain voided urine that is representative of the bladder by means of a better collection method.

Literary Review

Function, Anatomy, and Histology of the Female Urinary Tract

The female urinary tract is divided into upper and lower portions. The function of the upper urinary tract, which includes the kidneys and ureters, is to remove waste products (e.g., urea) from the blood (Hickling et al 2016). Blood enters the kidneys through the renal artery, is filtered by glomeruli in nephrons, and exits back into the circulatory system through the renal vein. Waste and excess water removed from the blood is transferred from the nephrons by tubules to the renal pelvis (Hickling et al 2016). Urine collected in the renal pelvis drains into long, muscular tubes called ureters. Ureters, which are lined with transitional epithelium, connect the renal pelvis to the bladder, allowing urine to drain from the kidneys into the bladder (Hickling et al 2016).

The lower urinary tract, which includes the bladder and urethra, functions to store and excrete waste from the body (Hickling et al 2016). The bladder is made of three layers (Yoshitaka et al 2017). The innermost lining of the bladder is the mucosa, made up of

transitional epithelium that stretch as the bladder fills with urine. The outermost layer includes the adventitia/serosa. This layer is comprised of connective tissue and simple squamous epithelium that cover the bladder muscles. Between these layers lies the muscularis, or the muscle layer of the bladder that includes the detrusor muscle (Yoshitaka et al 2017). When the urination signal is produced, these muscles contract to expel urine from the body (Abelson et al 2018).

As the detrusor muscle contracts, urine is forced to exit through the sphincter located at neck of the bladder (Yoshitaka et al 2017). The urethra connects the neck of the bladder to the outside of the body (Hickling et al 2016). The female urethra is 3-5 centimeters long (Abelson et al 2018). The proximal portion (closest to bladder) of the female urethra is lined with transitional epithelium (Carlile et al 1987). As the urethra gets more distal to the bladder, the epithelium changes: in the bladder-proximal region, it is transitional epithelium (similar to the bladder); in the bladder-distal portion, it is stratified squamous epithelium (similar to vaginal skin); in between, it is pseudostratified columnar epithelium (Carlile et al 1987). The external opening of the urethral is in close proximity to the vaginal tract, the labia minora and the labia majora (Hickling et al 2016).

History of Female Urobiome Research

In the 19th century, early microbiologists observed that a tightly closed vial of urine incubated in ambient conditions would not become cloudy, but if the vial was left open, microorganisms would grow rapidly, causing the urine to become turbid (Duclaux 1920). Whilst this observation was paramount to disproving spontaneous generation, the scientists wrongfully concluded that "healthy" urine was sterile (Roberts 1881, Bloom et al 1994). This "urine is sterile" dogma has persisted into the current century.

On the basis of this dogma, Dr. Edward Kass developed a non-invasive urine culture technique to diagnose pyelonephritis (Kass 1956, Kass 1957). His method involved the use of mid-stream voided urine plated onto blood agar and MacConkey media incubated under aerobic conditions for one day, which is conducive to the growth of *Escherichia coli*, the most common cause of pyelonephritis. To distinguish contamination from infection, he set a threshold of 10^5 colony forming units (CFU) of any uropathogen in 1 milliliter (mL) of urine (Price et al 2016). This SUC method worked well for diagnoses of pylonephritis; however, it was expanded to cystitis without substantiation (Brubaker and Wolfe 2017). Because SUC was designed to reproducibly detect *E. coli* at high CFUs, many clinicians debate its relevance for diagnosing UTI. For example, some clinicians argue that the 10^5 CFU threshold is erroneous, as it has not been shown if certain uropathogens cause symptoms at lower thresholds (Stark and Maki 1984, Hooton et al 2013). Moreover, SUC is not designed to detect fastidious or anaerobic microbes (Price et al 2016), biasing the culture methods towards *E. coli* despite the knowledge that other uropathogens exist. Since the use of SUC to detect bladder infection was instituted without validation, the current perception of cystitis is greatly biased.

This dogma was not rigorously tested until 1979 when Dr. Rosalind Maskell observed that patients with lower urinary tract symptoms repeatedly had culture-negative urine samples but became symptom-free upon antibiotic treatment (Maskell et al 1979). By incubating urine cultures in conditions with increased carbon-dioxide for longer lengths of time, Maskell was able to isolate slow-growing and fastidious microbes from urine deemed "no growth" by SUC in women diagnosed with dysuria and interstitial cystitis. Maskell concluded that microbes could be associated with bladder disorders other an acute UTI and that SUC was incapable of culturing many of these microbes (Maskell 2010). Unfortunately, Maskell's findings disregarded as contamination or rejected (Maskell 1988).

In 2012, Wolfe and colleagues reported the use of 16S ribosomal RNA (rRNA) gene sequencing to obtain DNA evidence of bacteria in SUC-negative urine samples collected by transurethral catheter (TUC) from women with and without lower urinary tract (LUT) symptoms (Wolfe et al 2012). An enhanced urine culture method called Expanded Quantitative Urine Culture (EQUC) showed these bacteria were alive (Hilt et al 2014), supporting Maskell's conclusions. The authors concluded that the adult female bladder possesses a resident community of microbes that they called the bladder microbiota. Using these two complementary approaches, several studies have described the bladder microbiota and identified microbial associations with some lower urinary tract disorders. This paradigm shift requires re-evaluation of UTI, as it may not be strictly due to a pathogenic invasion of a sterile environment, as commonly thought (Brubaker and Wolfe 2016, Price et al 2018).

Current Knowledge of the Female Urogenital Microbiota

In 2014, Hilt and coworkers used 16S rRNA sequencing and EQUC to examine the bladder microbiota of asymptomatic women. This study found that asymptomatic controls had a high prevalence of *Lactobacillus* and *Streptococcus* species (Hilt et al 2014). The dominance of these genera in the bladder has been confirmed by multiple studies (Brubaker and Wolfe 2016). Similarly, the vaginal microbiome of asymptomatic individuals is often dominated by *Lactobacillus* species (Lloyd-Price et al 2016). In 2018, Thomas-White and coworkers used metagenomic analysis to describe the relatedness of microbiota isolated from the bladder and vaginal tract within an individual. This study demonstrated that similar species isolated from the bladder and vaginal tract within an individual were highly related (Thomas-White et al 2018).

Furthermore, Hilt and coworkers showed in 2014 that the microbiota of control patients differed from that of patients diagnosed with overactive bladder syndrome. Women with this condition were less likely to have *Lactobacillus* species present in bladder urine and more likely

to have microbiota belonging to more diverse genera, such as *Corynebacterium* and *Aerococcus* (Hilt et al 2014). Thus, this paramount study suggested that unique microbiota profiles can be associated with disease. Bladder microbiota have since been implicated in a variety of disease states. For example, women with urge urinary incontinence have a higher prevalence of *Aerococcus urinae*, an emerging uropathogen, accompanied by a decrease in *Lactobacillus* species (Pearce et al 2014, Pearce et al 2015). Abundance of microbiota seems to play a role in this condition as well. Women with lower alpha diversity scores had significantly increased symptom severity (Karstens et al 2016), demonstrating that bladder disorders are not only influenced by the presence of bacteria, but the community structure as well. Conversely, some bladder conditions may not directly result from a bacterial component. For example, microbiota profiles from asymptomatic controls and interstitial cystitis/painful bladder syndrome patients do not differ (Bresler et al 2019).

Similarly to microbiota of other body sites, the bladder microbiota have been shown to be affected by external factors. For example, a subset of patients undergoing urogynecologic surgery are more likely to develop post-operative UTI (Thomas-White et al 2018). Patients with baseline samples depleted of *Lactobacillus iners* and positive for Gram-negative uropathogens are more susceptible to developing UTI after surgery (Thomas-White et al 2018). Moreover, sexual activity has been shown to alter the bladder microbiota. In 2019, Price sampled eight women daily for three months (Price 2019). This study demonstrated that although the bladder microbiota fluctuate daily, it remains relatively stable over time. More dramatic fluctuations overlapped with reports of sexual activity. Sexual activity was accompanied by a sharp increase in *Streptococcus* species, which dissipated quickly in the following days (Price 2019). Together, these findings demonstrate that disruption of the urinary microbiota community structure can lead to development of infection.

Urine Collection Techniques and Sample Description

Since the mid 20th century, physicians have been collecting urine by suprapubic aspiration for UTI diagnosis to avoid contamination of bladder urine (Guze and Beeson 1956, RR Bailey 1969). Whilst this method of urine collection bypasses contamination, it is highly invasive, as it requires the insertion of a needle through the abdomen and directly into the bladder. For this reason, many groups sought to establish methods of urine collection that were more efficient and equally efficacious, such as transurethral catheterization (Savige et al 1983, Pollack et al 1994). Furthermore, groups throughout the late 20th century began to characterize differences between TUC and voided urine, using patient populations to demonstrate that TUC and voided urines produce similar results when infection is present (Immergut et al 1981, Walter 1989). Although these studies attempted to identify differences between collection methods, the detection methods were biased by the "bladder is sterile" dogma.

In 2012, Wolfe and coworkers established that, similar to SPA, TUC sampled the bladder directly and showed DNA evidence of microbiota in asymptomatic women (Wolfe 2012). Typically, urine obtained directly from the bladder by either SPA or TUC contains microbes in low abundance and low diversity (Brubaker 2017). In contrast, midstream voided urine has been shown to contain considerably more microbes including microbes that originate outside the lower urinary tract (*e.g.*, those that originate in the vulva or vagina (Wolfe et al 2012). Because SPA and TUC urine collection methods limit study participants to patient populations, researchers are forced to establish methods that better permit accurate interpretation of data obtained from voided urines. For example, bioinformaticians have developed software (SourceTracker, Decontam) that attempts to identify contaminants in high biomass biological samples. However, it was recently reported that substantial overlap exists between the bladder and vaginal microbiota (Thomas-White et al 2018). This finding reduces the likelihood that

subtractive methods (*e.g.*, SourceTracker) will successfully remove vulvo-vaginal contaminants from voided urines unless the baseline bladder microbiota have been previously defined (*e.g.*, Decontam) (Kartstens et al 2018). Instead, researchers must improve/develop non-invasive urine collection techniques.

The current standard clean catch (SCC) method involves using a cleansing peri-urethral wipe, discarding the initial urine stream into the toilet, and collecting midstream voided urine into a sterile cup. Researchers have modified various aspects of this procedure in the hope of obtaining lower contamination rates. For example, it has been hypothesized that instead of cleansing the peri-urethral skin, the sterilizing wipe introduces skin and vaginal contaminants to the peri-urethral area; however, studies testing this hypothesis have shown that the use of a cleansing wipe has no effect on contamination rates of voided urine (Baerheim 1992, Lifshitz 2000). Another proposed modification has been to replace the hard-plastic collection container with a sterile soft-plastic bag in an attempt to prevent splash-back. Again, these studies have shown no decrease in contamination rates (Verliat-Guinaud 2015), even when holding labia apart (Southworth et al 2019, Price 2018). Finally, since there is no standardized instruction for discarding the initial urine stream, some have hypothesized that providing detailed instructions for this procedure would decrease contamination rates. Yet, Teo and coworkers found no improvement of contamination rates when providing written instruction (Teo et al 2016). With no success modifying the standard "clean catch" procedure, some individuals/companies have turned to development of novel urine collection devices.

One company, Forte Medical, has attempted to resolve this issue by developing a device called the Peezy. The Peezy is a small plastic urinal-type device designed to assist women with voided urine collection by discarding a standardized amount of the initial urine stream without interrupting urine flow (Edwards). The procedure for Peezy usage is very straightforward: a

woman uses a peri-urethral wipe, holds the device by the designated area, and voids. The device allows the initial stream (about 10 milliliters of urine) to pass through the device. This initial stream causes the expansion of a cellulose sponge that, when engaged, forces the midstream urine into a sterile urine collection tube. Excess urine flows out through a secondary opening. While Peezy has been shown to be a much more user-friendly collection method than the SCC method, previous studies of Peezy's efficacy have produced ambiguous results (Jackson 2005, Collier 2014).

Recurrent Urinary Tract Infections

Clinical indications of UTI include dysuria or painful urination, frequency and urgency of urination, and hematuria or blood in the urine (Nicolle 2005). UTIs disproportionately affect women and most women will be diagnosed with at least one UTI in their lifetime. Elevated UTI prevalence in women is most likely due to female lower urinary tract anatomy. For example, the male urethra is nearly five times longer than the female urethra, which is typically about three centimeters in length (Abelson 2018). The short length of the female urethra is thought to facilitate ascension of uropathogens into the bladder (Haddock 2015).

Diagnostic methods for UTI include urinalysis and urine culture. Urinalysis examines voided urine for leukocyte esterase or nitrites, indicative of activated white blood cells and Gram-negative bacteria, respectively (Madeo et al 2015). If urine is positive for white blood cells or nitrites, patients are prescribed antibiotic treatment and urine may be sent for culture. Clinical microbiology labs use SUC to diagnose for UTI, and urine is considered positive if a pure culture of a suspected uropathogen (most often *Escherichia* coli) is detected at 100,000 CFU/ml (Sfeir et al 2018). Once cultured, these microorganisms are tested for antibiotic sensitivity. Although UTI symptoms have considerable overlap with other LUT or genital tract conditions, such as sexually transmitted infections, physicians often treat these symptoms empirically (Tomas et al 2015,

NICE 2015). This means that antibiotic treatment is often prescribed based on symptoms without diagnostic testing. Empiric treatment of UTI may be contributing to antibiotic resistance, as UTI is one of the most common reasons for antibiotic prescription (Gupta et al 2001).

About 2-5% of women with UTI will develop recurrent UTI (RUTI), defined as three or more UTI episodes per year or two episodes within six months (Epp et al 2010). It is unknown why these women are predisposed to this condition, but several genetic and lifestyle factors have been identified as possible risks for RUTI development, such as frequent sexual activity and having the first UTI before age fifteen (Scholes et al 2000). The mechanism of RUTI development is unidentified, but a common hypothesis is that uropathogens persist in reservoirs between infections (Anderson et al 2003, Rosen et al 2007, Hunstad et al 2010), as patients commonly present with the same uropathogen at each episode (Kodner et al 2010). Due to the persistent nature of RUTI, these women routinely receive antibiotics, which can lead to adverse side effects and raise the possibility of antibiotic resistance. This has caused many to investigate alternative treatment methods, such as probiotics and vaginal estrogen, which have both been shown to promote colonization of commensal organisms in the bladder (Akgul et al 2018, Caretto et al 2017).

CHAPTER TWO:

METHODS AND MATERIALS

Participants

Following Loyola institutional review board (IRB) approval for all aims, participants gave verbal and written consent for the collection and analysis of their urine for research purposes. For the first aim, participants were asymptomatic female Loyola University Medical Center students and employees. For the second aim, participants were women with a clinical history of recurrent urinary tract infections seeking treatment from the Urogynecology Department at University of California San Diego. For the third aim, participants were women presenting to the Urogynecology Clinic of Loyola University Outpatient Center for initial evaluation of pelvic floor symptoms. All participants answered Pelvic Floor Disability Index (Barber et al 2005) and Urinary Tract Infection Symptom Assessment (Clayson et al 2005) questionnaires to assess the severity of any pelvic floor conditions and possibility urinary tract infection, respectively.

Collection of Urines Samples

Bladder urine samples were collected by transurethral catheterization. The urethral meatus was prepped with a routine betadine swab before a sterile Bard Clean-Cath Ultra 6" female catheter, 14Fr for intermittent catheterization was placed into the urethra and advanced until urine was returned. Urine specimens were collected in a sterile BD Vacutainer. SCC midstream voided urines were collected by standard clinic protocol (Lifshitz 2000). Participants were instructed to use wash their hands with soap and water, use a sterilizing peri-urethral wipe, discard the initial urine stream, and then collect midstream urine into a sterile Becton Dickinson (BD) Vacutainer cup. An aliquot of catheterized and SCC midstream urines was immediately transferred to a gray-

top tube containing boric acid and refrigerated for preservation. Paretcipants contributing modified clean catch (MCC) specimens were collected identically to SCC specimens, except that participants were given a sterile plastic bag and instructed to hold the labia apart during midstream urine collection. Participants contributing Peezy midstream urines were instructed to wash their hands with soap and water, unpackage the Peezy device and attach the sterile collection tube containing a boric acid preservative, use a sterilizing peri-urethral wipe (if appropriate), hold the Peezy device by the designated area, and void completely into the device. Participants then allowed the device to drain fully before unscrewing and capping the sterile midstream collection tube.

Collection of Urethral and Peri-urethral Samples

Urethral brush samples were collected by prepping the urethral meatus with a routine betadine swab and inserting a sterile brush (BD ESwab Collection and Transport System) into the urethra. This brush was advanced until no longer visible (1/2 inch), rotated 360 degrees, then withdrawn. Peri-urethral samples were collected by swabbing (BD ESwab Collection and Transport System) the peri-urethral vagina 5mm from the urethral meatus.

Sample Culture Methods

The SUC method involved inoculation of 1 μL of urine onto 5% sheep blood agar plate and MacConkey agar plate. Plates were incubated aerobically at 35°C for 24 hours. Thus, the level of detection for SUC is 10^3 CFU/mL, represented by 1 colony of growth on either plate. The EQUC method involved inoculation of 100 μL of catheterized urine, or 10 μL of voided urine, urethral, and peri-urethral samples, onto 5% BAP, chocolate agar, colistin and nalidixic acid (CNA) agar, CDC anaerobe 5% BAP plates. Plates were incubated at 35°C for 48 hours in aerobic (BAP), 5% CO_2 (BAP, chocolate agar, CNA), or anaerobic conditions (CDC anaerobe 5% BAP). Thus, the

level of detection for EQUC is 10 CFU/mL for catheterized urine, or 100 CFU/mL for voided

urine, urethral, and peri-urethral samples, represented by 1 colony of growth on any of the plates.

Identification of Bacterial Isolates

Each morphologically distinct colony type in both SUC and EQUC procedures was

counted and isolated on a different plate of the same medium to prepare a pure culture that was

used for identification with Matrix-Assisted Laser Desorption/Ionization Time-of Flight

(MALDI-TOF) mass spectroscopy. MALDI Biotyper 3.0 software Realtime Classification was

used to analyze the samples. In the Realtime Classification program, log score identification

criteria are used as follows. A score between 2.000 and 3.000 is species-level identification, a

score between 1.700 and 1.999 is genus-level identification, and a score that is below 1.700 is an

unreliable identification. A Realtime Classification log score was given for each bacterial isolate

sample for every condition from which it was isolated.

Statistical Analyses

Culture data, consisting of species detected and CFU/mL, were analyzed by various

diversity measures. Alpha diversity measures were used to compare species frequency, abundance,

and evenness within populations/sample types. Beta diversity measures (Bray-Curtis Analysis and

Principal Component Analysis) were used to compare microbial community diversity between

populations/sample types. In RStudio 1.1.423 (Boston, MA), non-parametric Wilcoxon signed-

rank, Wilcoxon rank-sum, Kruskal-Wallis, and Chi-square or Fisher's Exact tests were used to test

for significance.

CHAPTER THREE:

CHARACTERIZING THE URINARY MICROBIOTA OF WOMEN WITH RECURRENT

URINARY TRACT INFECTIONS

Rationale

Episodes of RUTI and acute UTI are typically diagnosed by SUC which was designed to

culture *E. coli* for diagnosis of pyelonephritis (Price 2017); however, EQUC performs better than

SUC on TUC urine samples (Price 2016), identifying bacteria in 90% of urine samples deemed

"no growth" by SUC and reproducibly detecting more non-*E.coli* uropathogens than SUC. It must

be determined if this EQUC finding holds true for voided urines as well. The unsuccessful

treatment of RUTI suggests the possibility of repeatedly undetected non-*E.coli* pathogens.

Therefore, if EQUC out performs SUC on voided urine, it would become possible to obtain a more

comprehensive understanding of the LUT microbiota of women with RUTI.

Experimental Design

For this study, we are recruiting adult women with RUTI ($\geq$3 UTIs in prior 12 months or

$\geq$2 UTI in prior 6 months). In addition to demographic variables and current UTI self-report

status, participants contributed voided and TUC urine specimens (**Figure 1**). These specimens

were submitted for culture via SUC as well as EQUC. Bacterial isolates were identified by

MALDI-TOF mass spectrometry. Culture results were then analyzed for species presence,

abundance, and correlation with symptoms. Comparisons were made between microbiota

composition of TUC and voided urine as well as SUC and EQUC results.

**Participants (N=37) gave self-report UTI status
and answered demographic questionnaires
prior to sample collection**

TUC Urine

Voided Urine

**Culture by SUC
and EQUC**

**Culture by SUC
and EQUC**

Bacterial isolates identified by MALDI-TOF MS

Analyzed by microbiota frequency and abundance
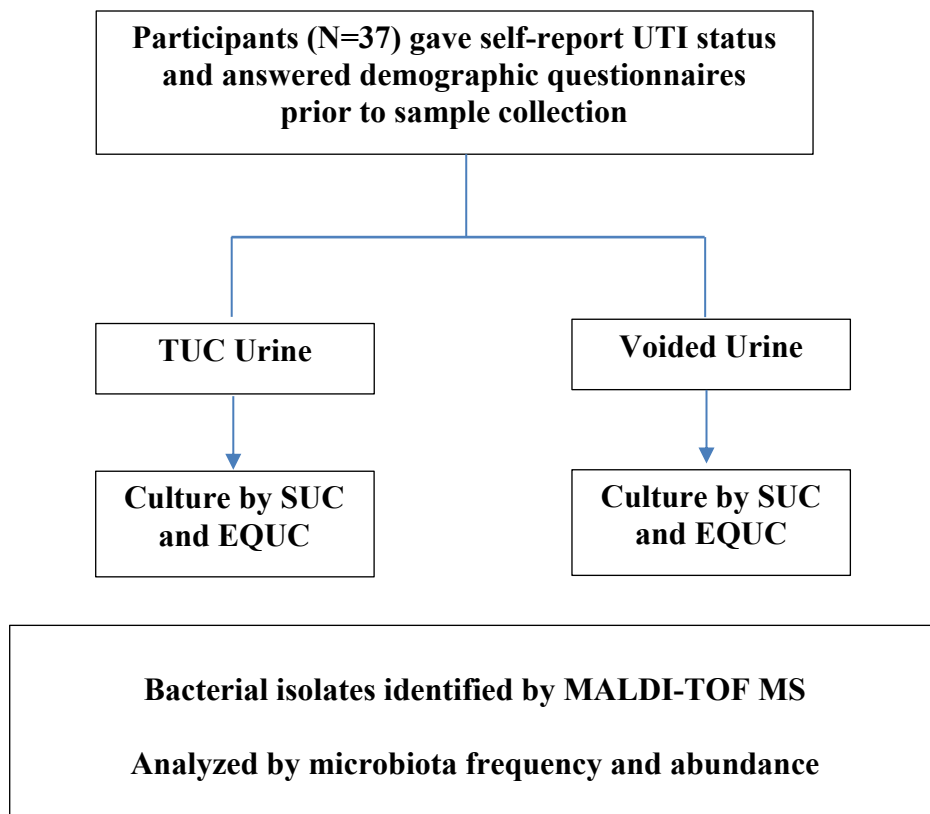
Figure 1. RUTI Experimental Design.

Results

EQUC Detects More Total Microbiota than SUC from Paired TUC and Voided Urines

Thus far, 37 participants with an average age of 70 years have contributed TUC and

voided urines. Most participants are Caucasian (**Table 1**, 81%), postmenopausal (86%), have

taken antibiotics for a UTI within the last 30 days (73%), and are currently using vaginal

estrogen (62%). These 37 participants have reported an average of 52 lifetime UTIs and an

average RUTI duration of nine years. At the time of specimen collection, 13 women self-

reported UTI, while 24 did not.

To determine the efficacy of EQUC on voided urine samples, culture results obtained via

SUC and EQUC from paired TUC and voided urines were compared. Compared to SUC, EQUC

reproducibly detected more unique bacterial species in both catheterized (21 versus 7) and

voided urine (55 versus 22) samples (**Figure 6A**). More unique bacterial species were cultured

from voided urine than TUC urine by EQUC (55 versus 21) and by SUC (22 versus 7); thus,

voided urine contained multiple species unseen in TUC urine. Similarly, EQUC detected

microbiota at higher abundances from both urine types than SUC (**Figure 3B**).

Table 1. RUTI Participant Profile

Participant Profile (N=37)	
Median Age (Range)	70 (32-91)
Ethnicity (%): Caucasian Hispanic Asian	 30 (81%) 6 (16%) 1 (3%)
Menopausal Status: Pre Post	 5 (14%) 32 (86%)
Antibiotic Use in Last 30 Days: Yes No	 27 (73%) 10 (27%)
Vaginal Estrogen use: Yes No	 23 (62%) 14 (38%)
Self-report UTI Status: Yes No	 13 (35%) 24 (65%)
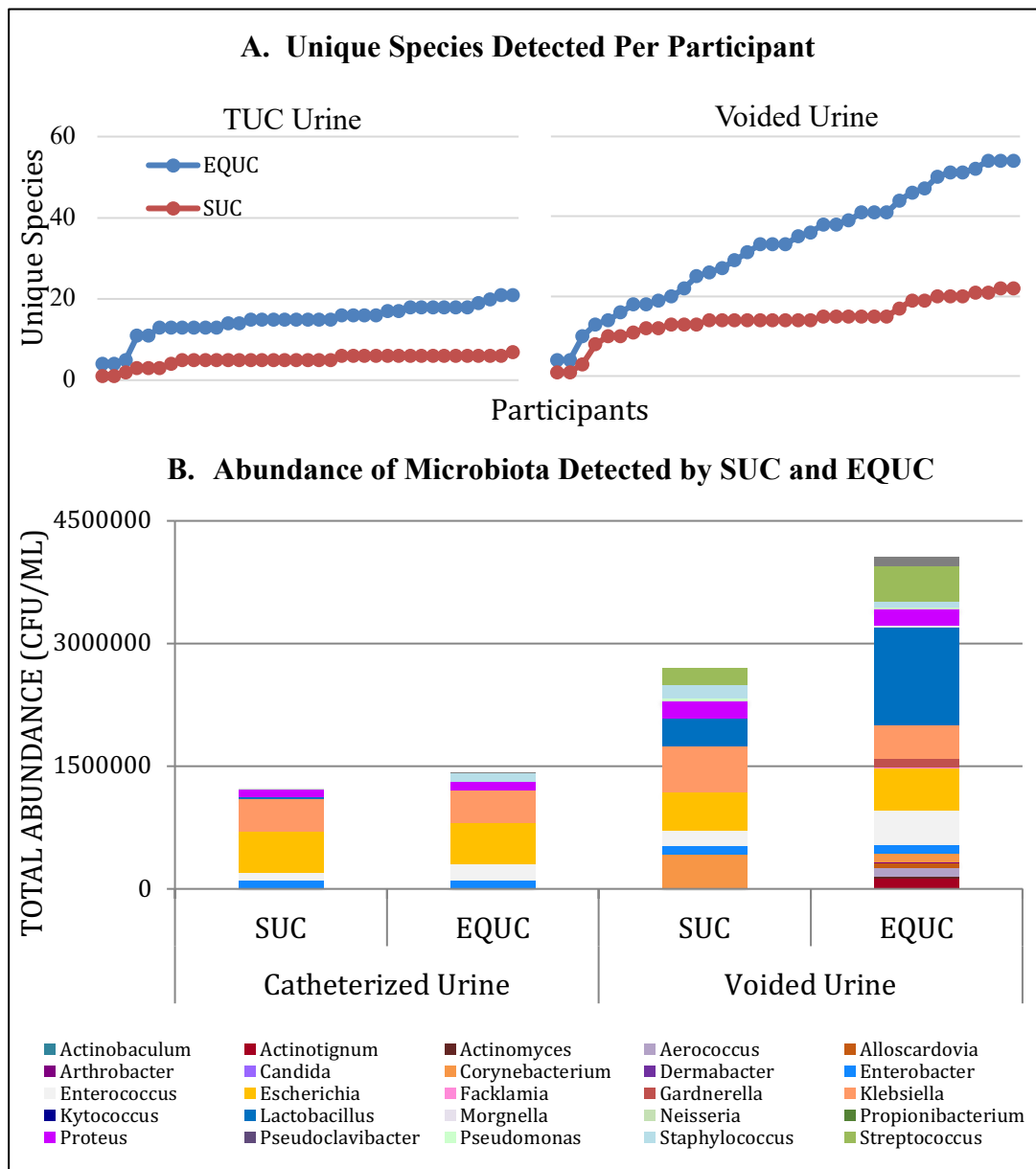
Figure 2 – Comparison of Bacterial Detection Between EQUC and SUC. (A) Rarefaction curves showing number of unique species identified with EQUC and SUC for TUC and voided urine specimens per participant (x-axis). (B) Total abundance (CFU/ml) of bacterial genera detected by EQUC and SUC for TUC urine and voided urine specimens.

EQUC Detects More Total Potential Uropathogens than SUC from Paired TUC and Voided Urines

Comparable to total microbiota, EQUC also consistently detected more potential

uropathogens from both urine types compared to SUC. EQUC detected 23 instances of potential

uropathogens from TUC urine, whereas SUC detected only 12 (**Figure 3**). Similarly, EQUC

detected 65 instances of potential uropathogens from voided urine versus 25 instances detected by SUC. Thus, more total potential uropathogens were observed in voided urine (71) than in TUC urine (25) and some species detected in voided urine were not detected in TUC urine specimens.

Furthermore, in this cohort, the most prevalent uropathogen was *Enterococcus faecalis*, detected by EQUC 6 and 13 times from TUC and voided urine, respectively. *E. coli* was the second most prevalent uropathogen, detected by EQUC 5 and 11 times from TUC urine and voided urine, respectively.
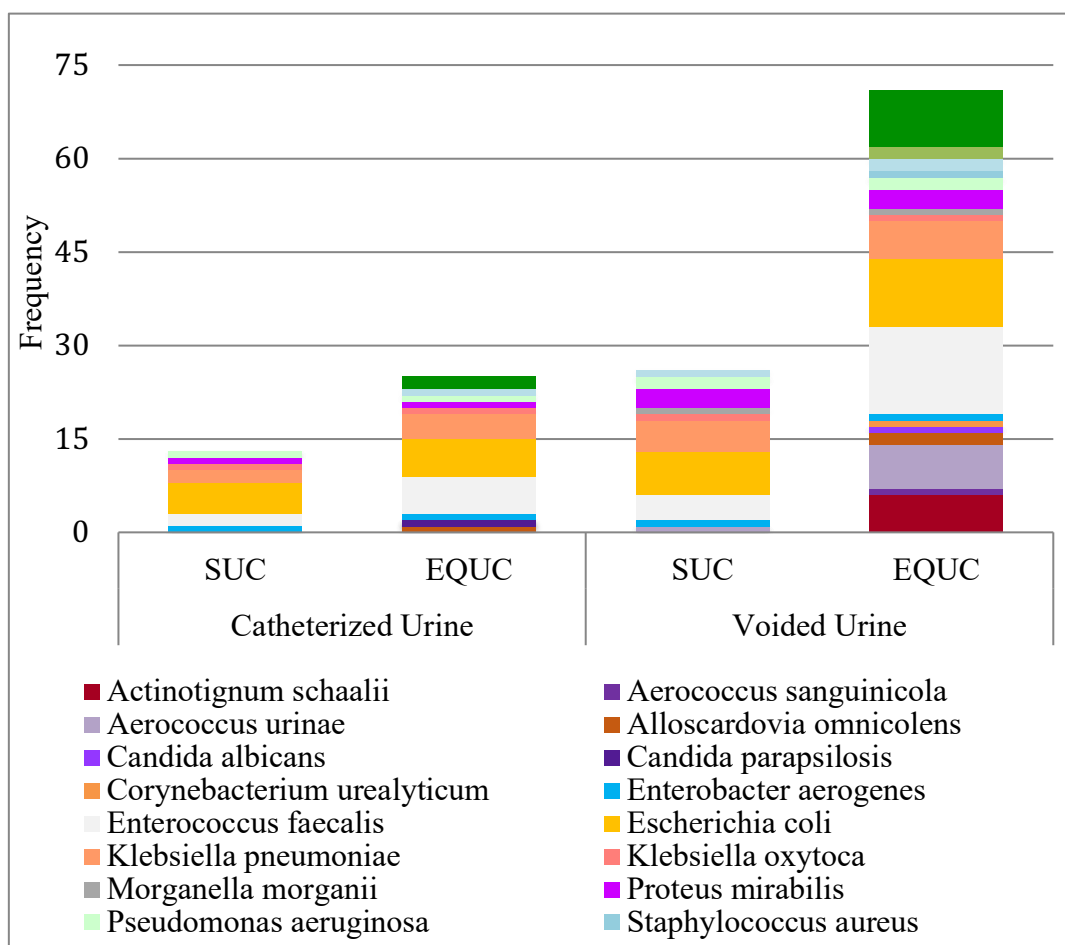
Figure 3 – Frequency of Uropathogens. Frequency of potential uropathogens detected by EQUC and SUC for TUC urine and voided urine.

Enterococcus faecalis **Is More Likely to be Associated with RUTI symptoms than** *Escherichia coli*

Because *E. faecalis* was the most prevalent potential uropathogen, it was further investigated to determine its possible role as the causative agent of UTI symptoms in this cohort. At the time of specimen collection, 13 women self-reported UTI, whereas 24 did not. The detection of *E. faecalis* was related to self-report UTI status: 7/13 in positive self-report vs. 6/24 in negative self-report (p=0.08, **Table 2**). *E. coli,* the second most prevalent uropathogen, was detected by EQUC in 11 women, 6 times from catheterized urine and 10 times from voided urine. Only 3 of these 11 women self-reported UTI (p=1.0), and 2 of these 3 women were also culture-positive for *E. faecalis*.

Table 2 – Association of *E. faecalis* and *E. coli* with Self-Reported UTI

Uropathogen Detected	UTI-Self Report Status		*P*-value
	Yes (N=13)	No (N=24)	
E. faecalis	7	6	0.08
E. coli	3	8	1.00

RUTI Microbiota Profiles Differ from UTI Microbiota Profiles

Because *E. coli*, the most common causative agent of acute UTI, was not found to be the main uropathogen involved in RUTI, microbiota profiles of acute UTI (Dune 2017) and RUTI cohorts were compared to elucidate other differences. Participants in each cohort were assigned urotypes based on the dominant organism detected.

Compared to acute UTI, TUC urine from women with RUTI was less likely to be dominated by *E. coli* (**Figure 4A**, p<0.001). Conversely, TUC urine from women with RUTI was more likely to be dominated by *E. faecalis* (p=0.10) or be culture-negative (p=1E-6) than TUC urine from women with acute UTI. Similarly, the RUTI cohort displayed a diminished frequency of *E. coli* (p=1E-5, **Figure 4B**).
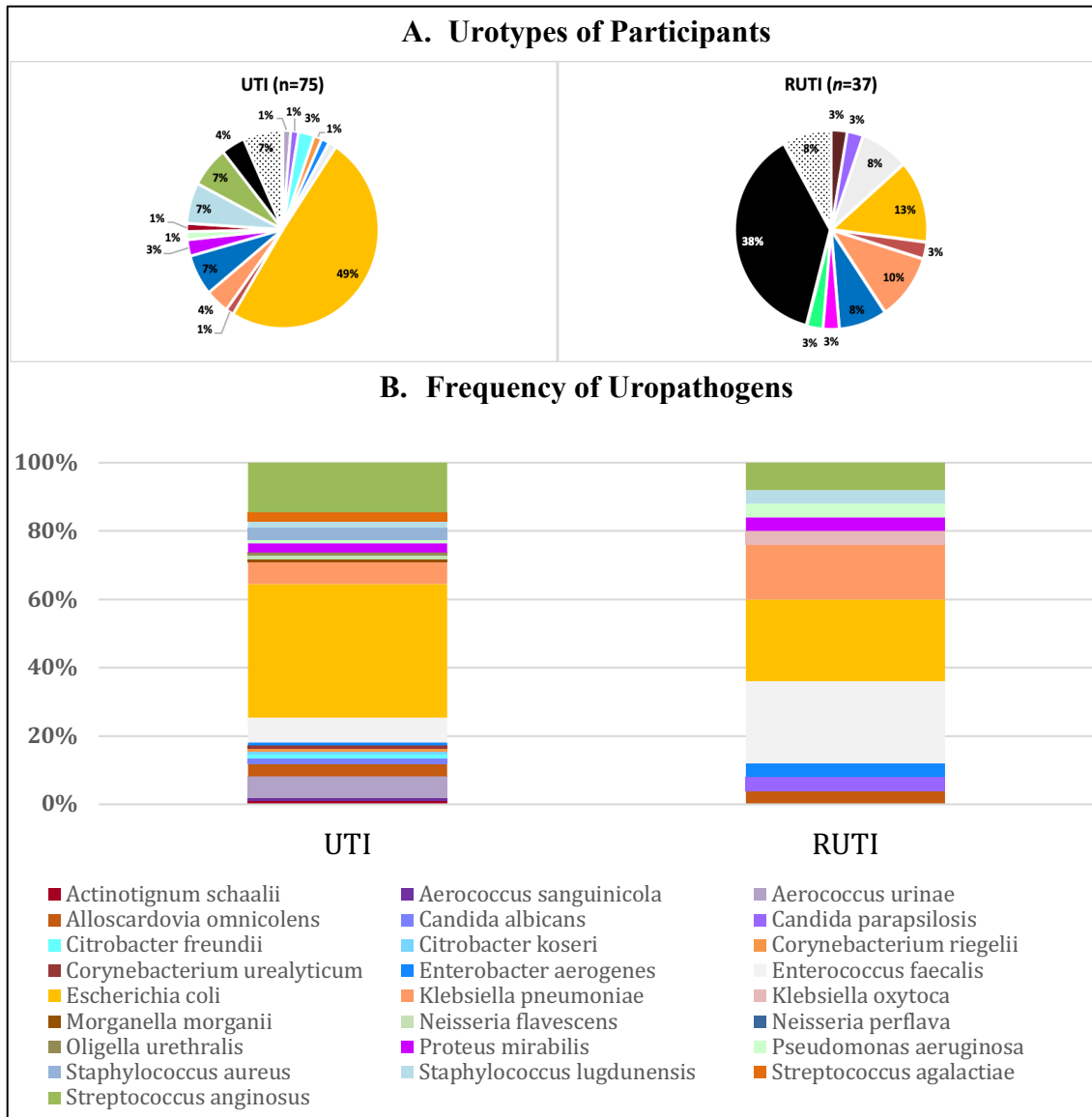
Figure 4. Comparison of Acute UTI and RUTI Microbiota Profiles. (A) Urotypes for each cohort were determined by the dominant organism detected. Chi-square and Fisher's exact tests were used to test for significance. (B) Frequency of uropathogens detected in each cohort. Chi-square and Fisher's exact tests used to test for significance.

Longitudinal Analysis Provides Insights to Possible RUTI Microbial Mechanisms

Since not much is known about the mechanism of disease, women from this RUTI cohort were asked to return to the clinic for follow-up visits. Of the 37 total participants, 5 women have returned to the clinic for follow up visits. One participant returned twice, creating a total of 6 return visits for this cohort (**Figure 5**).

Although no overall trends can be elucidated from this small longitudinal population, several interesting observations can be made. First, three participants (26, 36, and 40) had the same species of uropathogen cultured from the same specimen type at baseline and return visits. Second, the remaining two participants (4 and 6) had the same species of uropathogen isolated from TUC urine specimens that was previously seen in only voided specimens. Last, three participants (4, 26, and 36) had uropathogens present in return specimens that were undetected at the baseline visit.
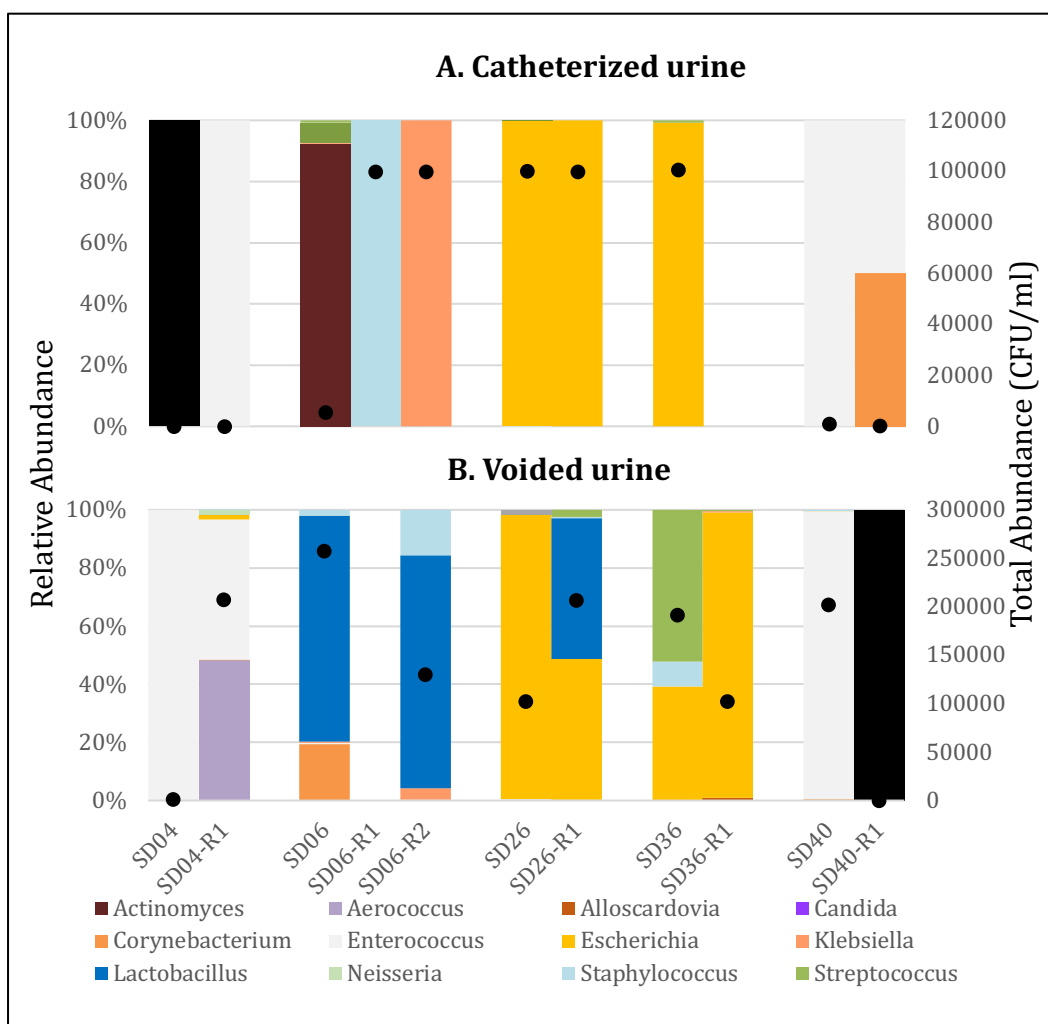
Figure 5. Microbiota Profiles for Longitudinal RUTI Samples. Paired TUC urine (A) and voided urine (B) specimens. The first bar for each participant represents the baseline sample obtained at the initial visit; subsequent bars represent subsequent visits. Black dots denote the total bacterial abundance of each sample.

Discussion

SUC consistently failed to culture numerous uropathogens detected by EQUC, including *E. faecalis*, the most prevalent uropathogen in this cohort. Although *E. coli* is commonly thought to be the leading causative agent of uncomplicated UTI, it was only the second most common uropathogen detected in this RUTI cohort and was less likely to be associated with symptomatic RUTI than *E. faecalis* (p=0.02). Moreover, *E. faecalis* was isolated more frequently from voided urine then catheterized urine specimens. These data suggest that *E. faecalis* might be the main player in RUTI and may be capable of causing urethritis in these women. This finding demonstrates the possibility of unique urethral microbiota.

To date, longitudinal analysis of returning participants in this cohort shows that uropathogens involved in RUTI may be capable of (1) persisting/recurring despite treatment; (2) advancing up the lower urinary tract between episodes; (3) persisting in a manner that allows the establishment of additional uropathogens at subsequent episodes. These observations need to be confirmed with larger sample sizes and sequence analysis is necessary to determine if these "recurring/persisting" uropathogens are of the same strain.

CHAPTER FOUR:

CHARACTERIZING THE MICROBIOTA OF THE FEMALE URETHRA

Rationale

Currently, voided urine obtained by a clean catch cannot be used to distinguish between microbiota of the bladder and more distal regions of the LUT, such as the urethra. Furthermore, we have begun to use the peri-urethra, or the skin surrounding the external urethral opening, as a control to check for contamination of LUT urine (Southworth et al 2018, Price 2019). It is currently unknown whether the microbial compositions of these three locations (bladder, urethra and peri-urethra) differ. Since urine flows down from the bladder through the more distal regions daily, it is sensible to assume that the microbiota in all three locations have considerable overlap. However, data from previous studies suggest that the microbiota of the bladder and peri-urethra can differ greatly in terms of bacterial abundance and composition (Lloyd-Price 2016, Price 2019). The LUT epithelia change from transitional urothelium in the bladder to nonkeratinizing, stratified squamous epithelium in the distal urethra (Carlile 1987). This epithelial gradient and the imbalance in abundance of microbiota along the LUT suggest the existence of different `microbial niches. Characterizing the urethral microbiota would allow us to determine if microbiota grow preferentially within the LUT. Analyzing these trends may allow us to determine the LUT location where bacteria cultured from voided urine most likely originated.

Experimental Design

For this study, we recruited 50 adult women presenting to the clinic for initial evaluation of their pelvic floor symptoms. From each participant, we collected demographic variables as well as mid-stream voided urine, peri-urethral swab, transurethral brush, and catheterized urine

(**Figure 6**). Each sample type was submitted for expanded quantitative urine culture. Bacterial isolates were identified by MALDI-TOF mass spectrometry. Microbiota of each sample type were compared by diversity measures, frequency, and abundance of microbiota.

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┌─────────────────────────────────────────────┐
│  Participants (N=50) answered demographic    │
│   questionnaires prior to sample collection  │
└─────────────────────────────────────────────┘
                      │
          ┌───────────────────────┐
          │     Voided Urine      │
          ├───────────────────────┤
          │    Peri-urethral      │
          │        Swab           │
          ├───────────────────────┤
          │    Urethral Brush     │
          ├───────────────────────┤
          │      TUC Urine        │
          └───────────────────────┘
                      │
                      ▼
┌─────────────────────────────────────────────┐
│      All sample types cultured via EQUC,     │
│   bacterial isolates identified by MALDI-TOF MS │
│                                              │
│  Analyzed by diversity measures and CFU comparison │
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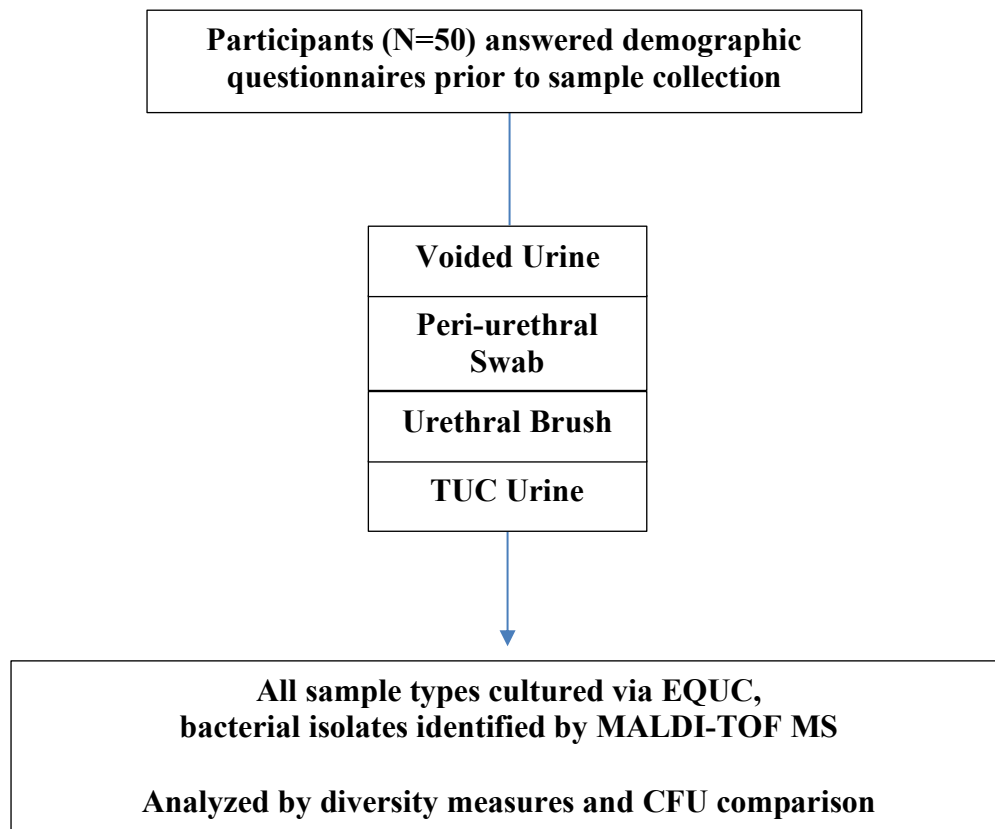
Figure 6. Lower Urinary Tract Niche Experimental Design.

Results

Urethral Microbiota are Distinct from Bladder Microbiota and More Similar to Peri-urethral Microbiota

50 eligible women were recruited for this study. One participant was unable to provide all four specimens and was therefore excluded from analysis. The cohort of 49 women was mostly white (71%) with a median age of 55 (range 21-85) (**Table 3**). They were also mostly post-menopausal (76%), but approximately equal in terms of sexual activity (53% active versus 47% inactive).

Examination of microbiota profiles for each individual show that some participants have the same genera isolated from each sample type, whilst some participant's LUT samples differ drastically (**Figure 7A**). This range of similarity was quantified with Bray-Curtis analysis, which compares species overlap and relative abundance between samples. Bray-Curtis analysis showed that the microbiota in the TUC specimens were highly dissimilar compared to the urethral microbiota (B-U, median=0.99, $p<0.0001$), the peri-urethral microbiota (B-PU, median=0.99, $p<0.0001$), and the voided urine microbiota (B-V, median=0.97, $p<0.0001$) (**Figure 7B**). In contrast, the urethral microbiota were only moderately dissimilar to peri-urethral microbiota (U-PU, median 0.56, $p=0.31$). Although these specimens were statistically similar, their relationship to the voided urine specimens differed; the urethral microbiota were dissimilar to voided urine specimens (U-V, median=0.73, $p=0.001$), whereas the peri-urethral microbiota were not (PU-V, median=0.55, $p=0.16$).

Table 3. Lower Urinary Tract Niche Participant Profile

Participant Profile (N=49)	
Median age (range)	55 (21-85)
Ethnicity (%): Caucasian Black Hispanic Other	 36 (74%) 6 (12%) 6 (12%) 1 (2%)
Menopausal status (%): Pre Post	 12 (24%) 37 (76%)
Sexually active (%): Yes No	 26 (53%) 23 (47%)

A. Microbiota Profiles

Legend:
- Actinobaculum
- Actinomyces
- Actinotignum
- Aerococcus
- Aeromicrobium
- Alloscardovia
- Aspergilus
- Arthrobacter
- Brevibacterium
- Burkholderia
- Candida
- Corynebacterium
- Dermabacter
- Enterobacter
- Enterococcus
- Escherichia
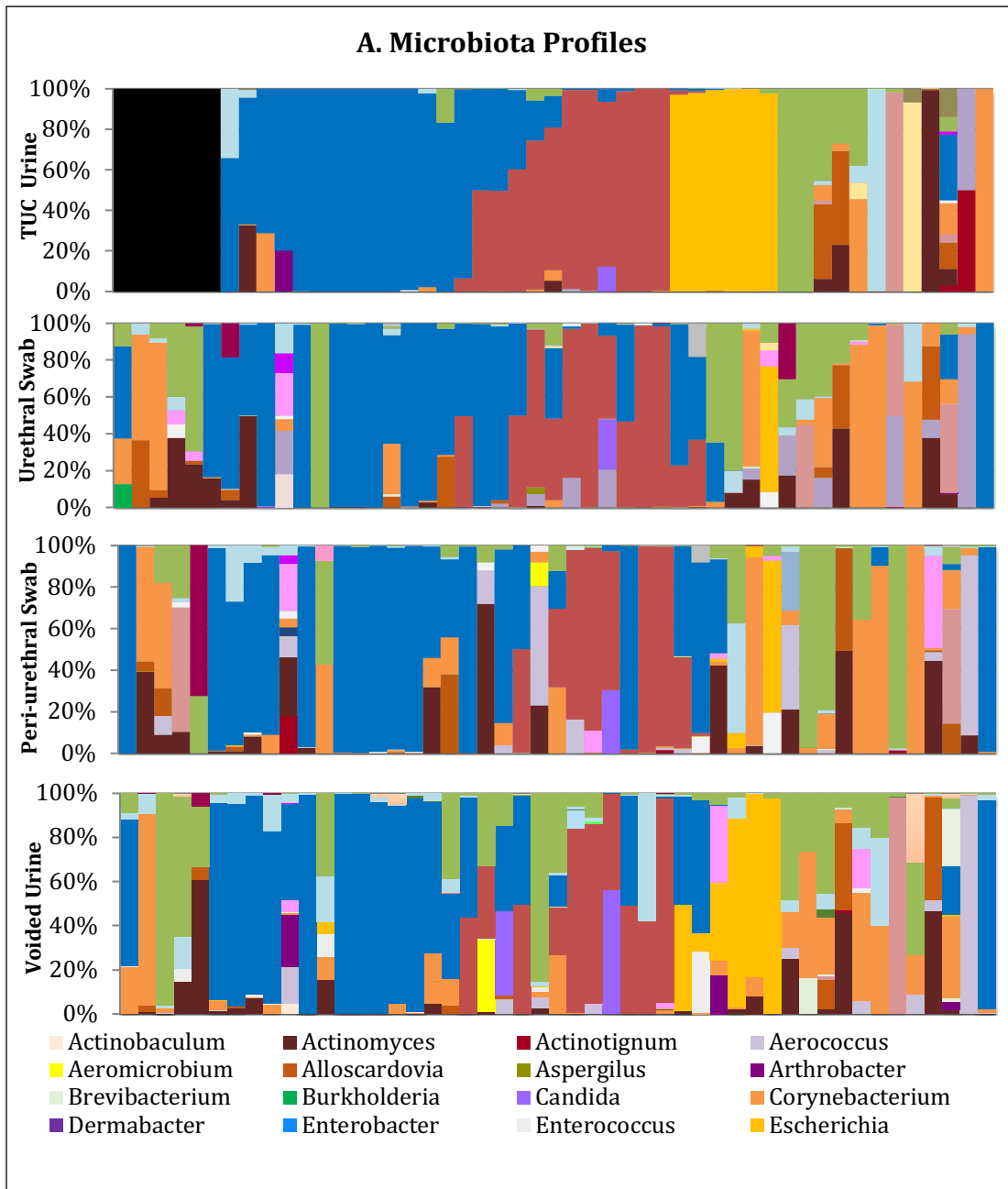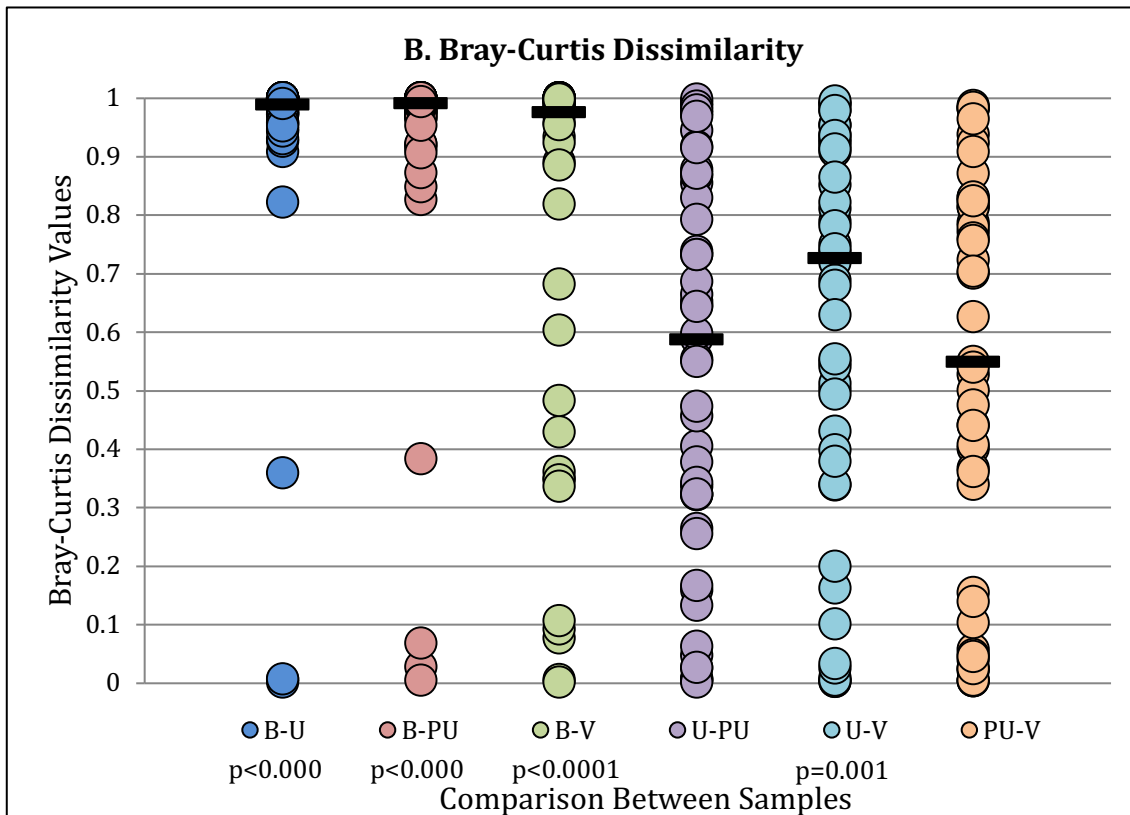
Figure 7. Diversity Comparison Between Lower Urinary Tract Samples. A) Microbiota profiles comparing LUT samples. TUC urine, urethral swab, peri-urethral swab, and voided urine specimens for each participant. B) Quantification of microbiota turnover by Bray-Curtis Dissimilarity Index. Comparisons between sample types on X-axis (B=Bladder, U=Urethra, PU=Peri-urethra, and V=Voided urine). With a dissimilarity cutoff of 0.5, chi-square and Fisher's exact tests were used to test for significance. Significant *p*-values denoted under sample comparison.

Frequency and Abundance of Microbiota Differ Throughout the LUT

To determine the cause of these differences between sample types, frequency and abundance of microbiota were compared across the samples. Whether or not specimen types were similar or different, all shared the same top ten most frequently isolated genera, but not always with the same distribution. For example, *Lactobacillus* and *Gardnerella* were isolated with similar frequency from all specimen types (p=0.98), whereas *Staphylococcus* was isolated from the urethral and peri-urethral specimen more frequently than from the TUC samples but less frequently than from voided urine specimen (p<0.001). Other patterns also were apparent. For example, *Escherichia*

was isolated more frequently from voided urine than the other specimen types, from which

Escherichia was isolated with similar frequency. Several genera (*Actinomyces, Aerococcocus,*

Alloscardovia and *Corynebacterium*) were isolated less frequently from the TUC urine, but with

similar frequencies from the other specimen types (**Figure 8A**). Differences in distribution also

were observed in terms of relative abundance. For example, *Escherichia* was more abundant and

Lactobacillus was less abundant in TUC urine, whereas *Corynebacterium* and *Streptococcus* were

more abundant in the other three specimen types (**Figure 8B**). Finally, total abundance increased

as the specimen type became more distal to the bladder.
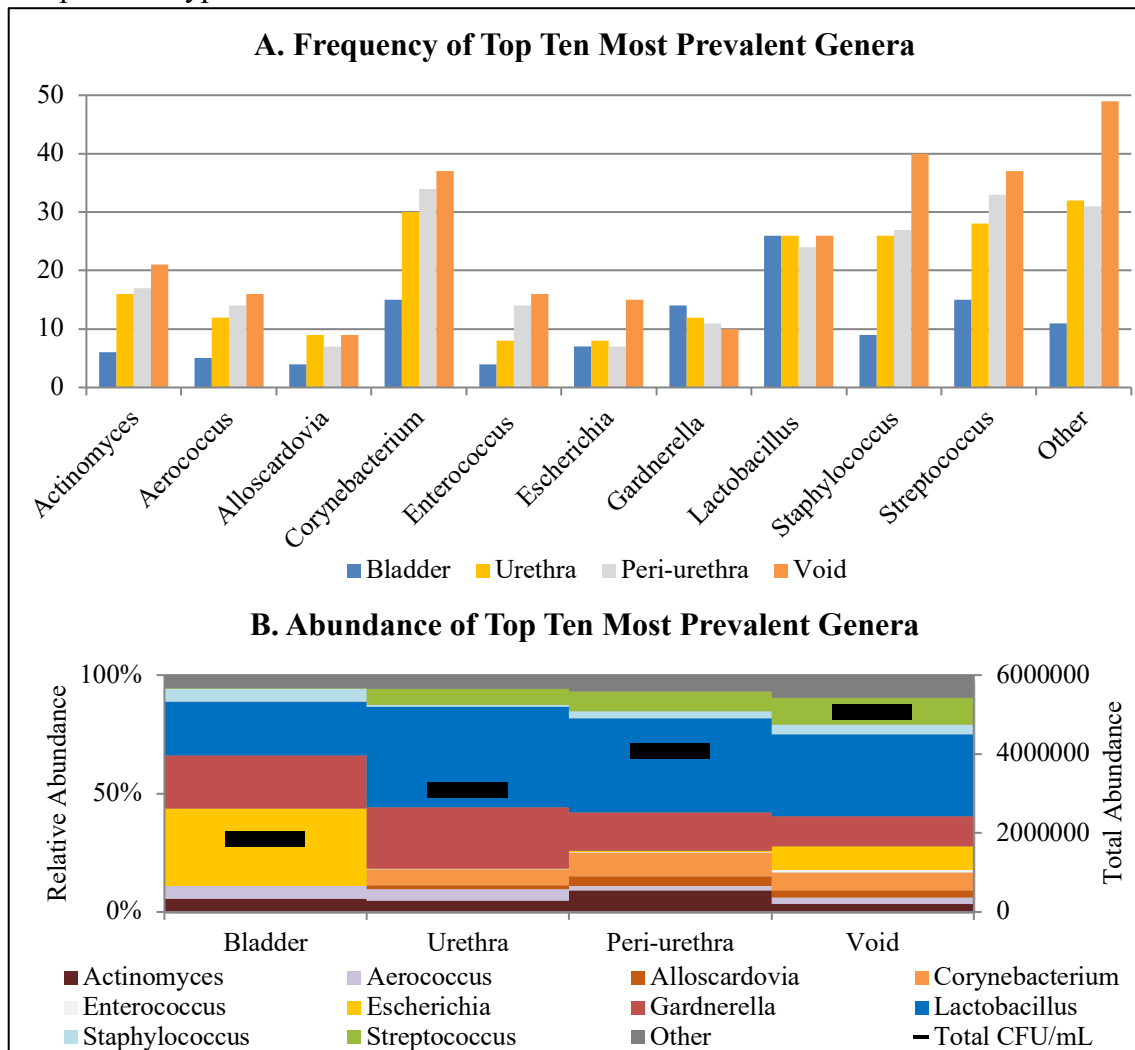
Figure 8. Comparison of Microbiota Distribution Across LUT Samples. A) Frequency of most prevalent genera across LUT samples. Chi-square and Fisher's exact tests used to test for significance. B) Relative abundance of microbiota across LUT samples. Kruskal-Wallis test used to test for significance.

LUT Microbiota Differ Based on Demographics

To analyze the effect of demographics on lower urinary tract microbiota, we divided the study participants into two groups based on age (**Table 4**). Women below the age of 55 were significantly younger (p<1E-8), more likely to be pre-menopausal (p<0.0001), and more likely to be sexually active (p=0.002) than women aged 55 or older.

Table 4. Demographic Breakdown of Lower Urinary Tract Niche Study Participants

PARTICIPANT PROFILE (N=49)				
Cohort (N)	Total (N=49)	Aged <55 (N=23)	Aged 55+ (N=26)	*P*-value
Median Age (Range)	55 (21-85)	44 (21-54)	65 (55-85)	<1E-8
Ethnicity (%):				
Caucasian	36 (74%)	13 (57%)	22 (85%)	0.06
Black	6 (12%)	3 (13%)	3 (12%)	1.00
Hispanic	6 (12%)	7 (30%)	0 (0%)	<0.01
Other	1 (2%)	0 (0%)	1 (3%)	1.00
Menopausal Status (%):				
Pre	12 (24%)	12 (52%)	0 (0%)	<1E-4
Post	37 (76%)	11 (48%)	26 (100%)	
Sexually Active (%):				
Yes	26 (53%)	18 (78%)	8 (31%)	<0.01
No	23 (47%)	5 (22%0	18 (69%)	

All specimen types of women below the age of 55 had higher frequencies of *Gardnerella* and *Lactobacillus* species (p<0.01 for all comparisons) than women aged 55 and older (**Figure 9A**). Similarly, urethral and peri-urethral specimens of women below the age of 55 contained higher abundances of *Gardnerella* (p<0.01 for all comparisons) and *Lactobacillus* (p<0.001 for all comparisons) than women aged 55 and over (**Figure 9B**). Increased frequency and abundance of these genera also was seen in TUC urine (p<0.01 for all comparisons) and voided urine (p<0.001 for all comparisons) specimens from women under the age of 55.

Conversely, urethral, peri-urethral, and voided urine specimens of women aged 55 and over displayed increased abundance of more diverse organisms than women under 55. These more abundant genera included *Actinomyces* (p<0.01 for all comparisons), *Alloscardovia*

(p=0.05, p=0.03, and p=0.02 respectively), *Corynebacterium* (p=0.01 for all comparisons) and, *Streptococcus* (p=0.02 p=0.09, and p<0.01 respectively). Additionally, TUC urines for women 55 and over had an increased abundance of *Alloscardovia* (p=0.05), *Staphylococcus* (p=0.04), and *Streptococcus* (p=0.04), but not *Actinomyces* (p=0.63) or *Corynebacterium* (p=0.64). These data demonstrate that demographic factors influence LUT microbiota in women.

A. Comparison of Frequency of Microbiota
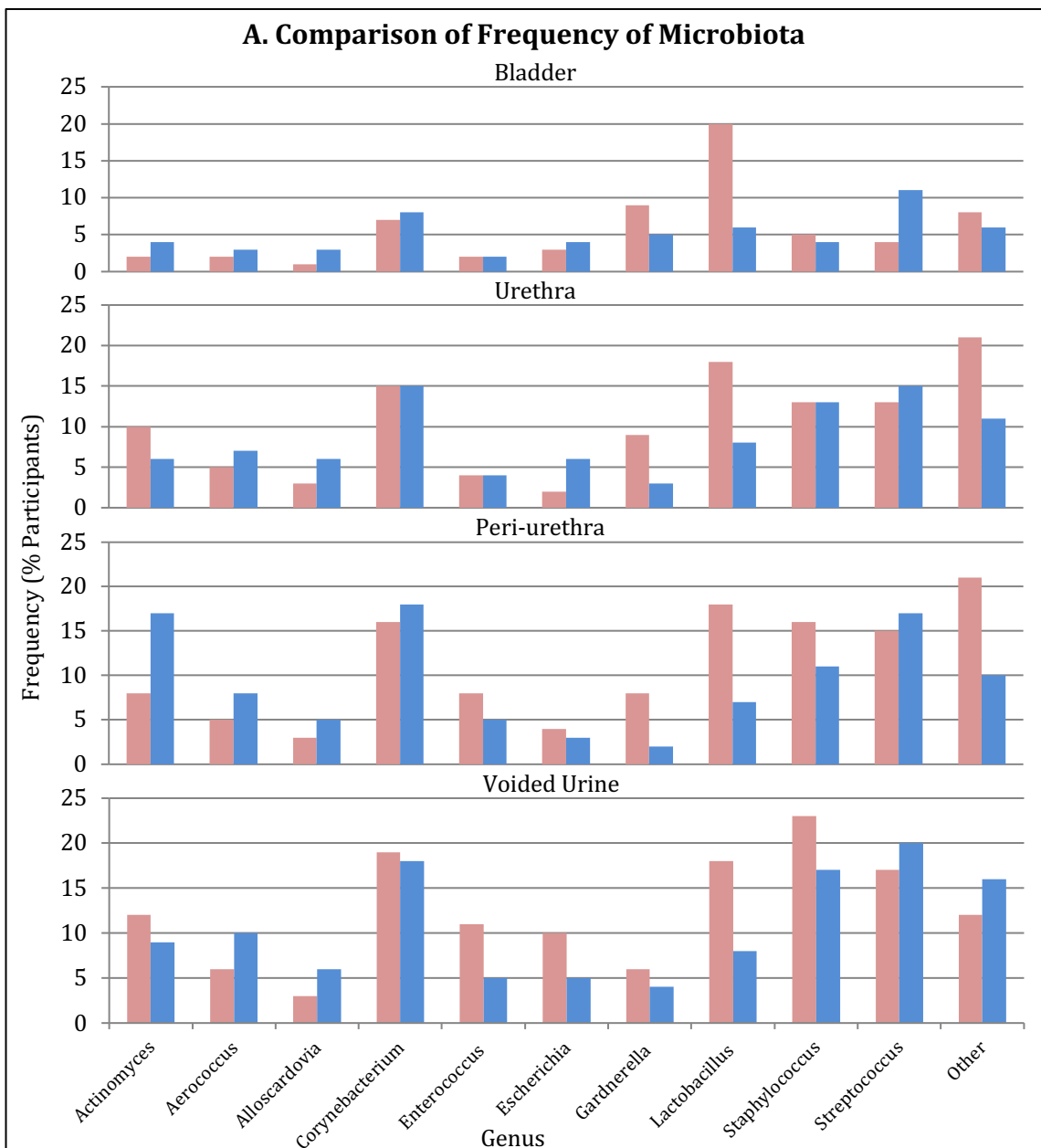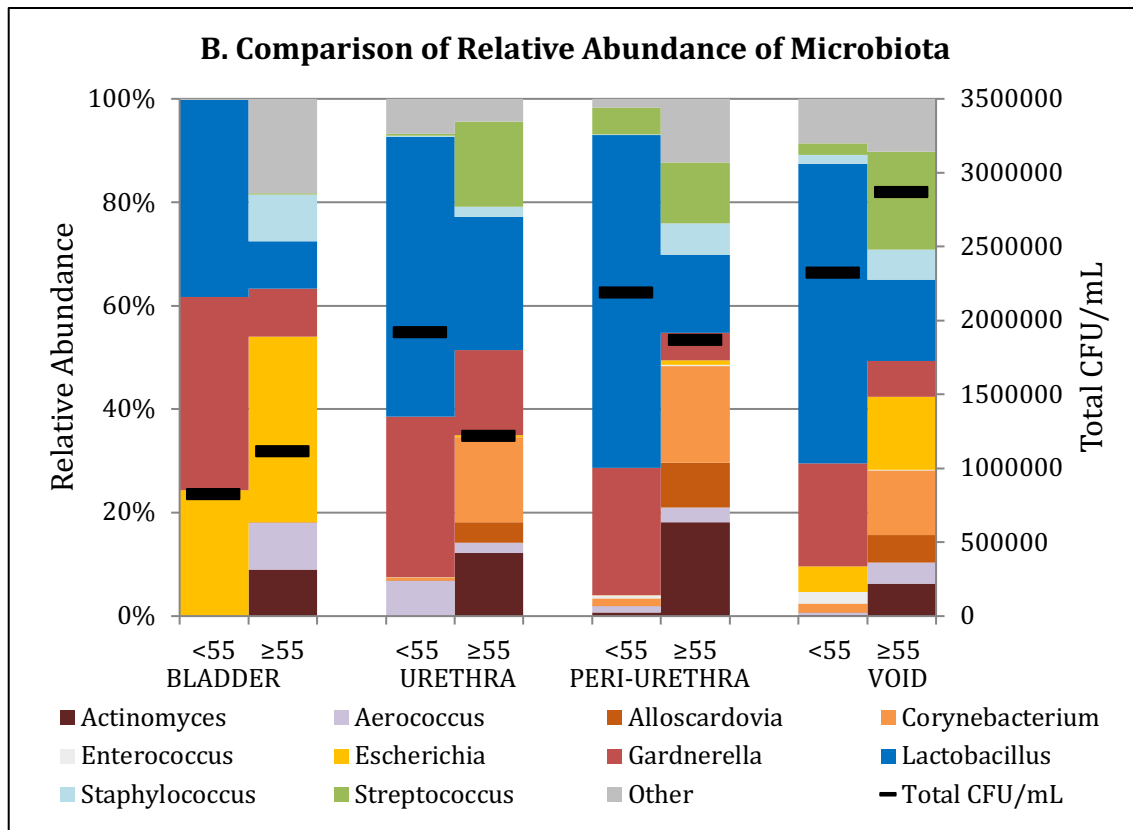
Figure 9. Distribution of Microbiota Based on Demographics. A) Frequency of most prevalent genera across LUT samples compared between demographic cohorts. Chi-square and Fisher's exact tests used to test for significance. B) Relative abundance of microbiota across LUT samples compared between demographic cohorts. Kruskal-Wallis test used to test for significance.

Different Demographics Are Responsible for Trends of Different Genera

Linear regression analysis was performed to identify the demographic factor (age, menopausal status, or sexual activity status) that was most influential for each observed microbiota trend. The abundance of *Lactobacillus* in the urethra and peri-urethra seems to be affected equally by age (AGE), menopausal status (MENO), and sexual activity status (SAS), but none of these demographic factors is solely responsible for TUC urine trends (**Table 5**). This means that younger, pre-menopausal, sexually active women have increased urethral and peri-urethral *Lactobacillus*. Significant demographic affects for abundance of *Corynebacterium* and *Gardnerella* species also can be seen in the urethra and peri-urethra microbiota. In the urethra,

Corynebacterium and *Gardnerella* are increased in younger, sexually active women, but these genera appear to be unaffected by menopausal status. Furthermore, the peri-urethral abundance of these genera is affected only by age.

Despite significant differences demonstrated by direct CFU comparison, no significant demographic effects were seen in TUC urine samples. This is most likely due to A) a combined effect of all three demographics and B) low biomass of TUC urine, resulting in sample sizes too low for this analytic method.

Table 5. Effect of Individual Demographic Factors on LUT Microbiota

TUC		Corynebacterium	Gardnerella	Lactobacillus
SAS	R^2	-0.02	-0.021	-0.021
	P	0.78	0.92	0.93
AGE	R^2	-0.001	-0.013	-0.016
	P	0.34	0.56	0.61
MENO	R^2	-0.019	0.012	-0.021
	P	0.73	0.22	0.92

URETHRA		Corynebacterium	Gardnerella	Lactobacillus
SAS	R^2	0.045	0.065	0.094
	P	**0.08**	**0.04**	**0.02**
AGE	R^2	0.05	0.043	0.09
	P	**0.06**	**0.08**	**0.02**
MENO	R^2	-0.003	0.018	0.113
	P	0.36	0.17	**0.01**

PERI-URETHRA		Corynebacterium	Gardnerella	Lactobacillus
SAS	R^2	-0.02	-0.012	0.096
	P	0.86	0.52	**0.02**
AGE	R^2	0.064	0.064	0.18
	P	**0.04**	**0.04**	**0.0015**
MENO	R^2	0.0076	0.024	0.25
	P	0.25	0.15	**0.0002**

Discussion

In terms of species presence and abundance, the female bladder and urethra represent distinct niches within the lower urinary tract. Furthermore, abundance data provides evidence that some genera are specialists, residing preferentially within the lower urinary tract (*Escherichia* and *Corynebacterium*), while others are generalists (*Gardnerella*), residing at similar levels throughout the lower urinary tract.

Analysis by demographics shows that younger, premenopausal women have a higher prevalence of *Lactobacillus* and *Gardnerella* throughout the lower urinary tract. Women who are older and post-menopausal display a loss of *Lactobacillus* and *Gardnerella* species accompanied by an increased diversity of other species belonging to other genera, such as *Actinomyces*, *Corynebacterium*, and *Streptococcus*. Despite these significant differences between the two cohorts, the previously mentioned trends of generalists and specialists throughout the lower urinary tract was still evident.

These data show that voided urine is more representative of peri-urethral skin than the bladder or urethra. Voided urine has a markedly higher abundance of microbiota than the bladder, suggesting that the biomass of bladder urine increases as the urine comes into contact with higher biomass locations, such as the urethra and peri-urethra. Due to the species overlap seen within these locations, it is improbable that we can determine the origin of lower urinary tract microbiota. Thus, our data support other studies that show that subtractive methods cannot permit interpretation of voided urine in terms of the bladder microbiota. Methods to reduce vulvovaginal contribution in voided samples should be developed and validated.

CHAPTER FIVE

IDENTIFYING A "CLEANER" CATCH METHOD FOR VOIDED URINE

Rationale

The current common practice in clinics is to collect midstream-voided urine using the SCC method, but this method has been shown to contain high amounts of vulvovaginal contribution when cultured, regardless of modification (Immergut et al 1981, Lifshitz et al 2000, Baerheim et al 1992). Since there is much overlap between vaginal and urinary microbiota (Thomas-White et al 2018), many individuals and companies see the need to develop a "cleaner" catch method to ensure accurate depiction of the urinary microbiota.

For this reason, the efficacy of the Peezy Midstream Collection Device (Peezy) by Forte Medical has been tested previously; however, these studies comparing voided urines collected by SCC and Peezy have been performed using SUC for analysis and have lacked appropriate internal controls. Since EQUC has been used to successfully characterize the urinary microbiota of healthy females (Hilt et al 2014), culturing urines collected by SCC and Peezy via EQUC and comparing the urinary microbiota of each participant to her vulvovaginal microbiota would produce more conclusive results regarding the efficacy of Peezy. If Peezy were capable of producing cleaner voided urines, it could be used in longitudinal and population urobiome studies.

Experimental Design

For this study, we recruited 83 asymptomatic female participants that we divided into three cohorts (**Figure 10**): standard clean catch (SCC), modified clean catch (MCC), Peezy with wipe (PZW), or Peezy without wipe (PZ). After receiving video instruction on sample collection,

each participant contributed voided urine and a peri-urethral swab. These samples were cultured by EQUC and bacterial isolates were identified with MALDI-TOF MS.

```
┌─────────────────────────────────────────┐
│  Participants (N=62) confirmed asymptomatic │
│     with UTISA and PFDI questionnaires    │
│                                           │
│   Watched video for specimen collection   │
└─────────────────────────────────────────┘
```

| PZW
N=21 | PZ
N=21 | SCC
N=20 | MCC
N=21 |

```
┌─────────────────────────────────────────────────────┐
│  Voided urines and peri-urethral swabs cultured by EQUC, │
│       bacterial isolates identified by MALDI-TOF MS      │
│                                                         │
│     Analyzed by diversity measures and CFU comparison    │
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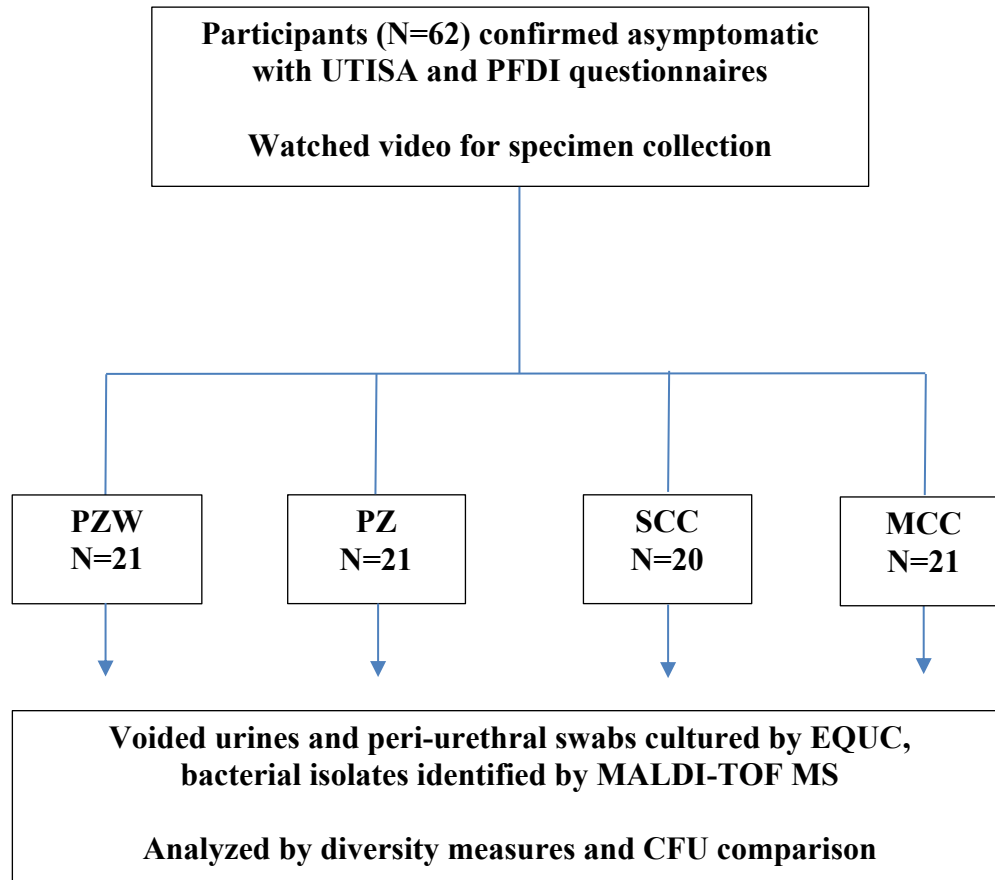
Figure 10. "Cleaner" Catch Method Experimental Design.

Results

Peezy Urines Have Significantly Different Bacterial Profiles than SCC Voided Urines

The 83 participants had a mean age of 28.8 years (range 22 -52), were predominantly Caucasian (65%), and a mean BMI of 25.7 kg/m^2 (17.2-47.2 kg/m^2). The cohorts did not differ significantly in their demographics, PFDI, or UTISA symptoms scores (**Table 6**).

To determine differences within the microbial communities sampled by each collection method, EQUC results were analyzed by various alpha diversity measures. The voided urines and peri-urethral specimens of both Peezy cohorts differed significantly in Pielou, Shannon, and

Simpson indices with higher diversity values in the peri-urethral specimens (**Figure 11A-C**). In contrast, for these three indices, there was no significant statistical difference between the SCC urine and paired peri-urethral specimens. The only diversity measure in which the SCC voided urines and the paired peri-urethral swabs significantly differed was Fisher's Diversity, which measures relative abundance (**Figure 11D**). The abundance values for urine and peri-urethral specimens for both Peezy cohorts and MCC did not differ significantly; however, the urine samples of both Peezy cohorts combined had significantly lower alpha diversity values compared to the SCC urine specimen, suggesting that significance for the individual cohorts may be reached with higher sample sizes. These data suggest that the Peezy device produces urines with significantly different microbiota community structure than SCC or MCC methods.

Table 6 – "Cleaner" Catch Method Study Participant Profile

Participant Profile (N=62)					
Cohort (N)	SCC (N=20)	MCC (N=21)	PZW (N=21)	PZ (N=21)	*P*-value
Average age (Range)	31.9 (21-49)	32 (21-48)	28.4 (23-52)	27.2 (22-43)	0.41
Average BMI (Range)	24.8 kg/m² (24-42 kg/m²)	25.8 kg/m² (18-35kg/m²)	24.7kg/m² (20-29 kg/m²)	23.6kg/m² (18-32 kg/m²)	0.59
Ethnicity: Caucasian	65%	62%	67%	48%	0.57
Asian	15%	4%	14%	33%	0.11
Hispanic	15%	14%	5%	14%	0.73
African American	5%	23%	14%	10%	0.65
Other	0%	0%	5%	5%	1.00
UTISA score, Average (range) 1. Urination regularity	0.0 (0.1-0.6)	0.0 (0.1-0.3)	0 (0)	0.0 (0.2)	0.15
2. Problems with urination	0.0 (0.1)	0.0 (0.3)	0 (0)	0 (0)	0.57
3. Pain associated with UTI	0 (0)	0 (0)	0 (0)	0 (0)	-
4.Blood in urine	0 (0)	0 (0)	0 (0)	0 (0)	-
PFDI-20 score, Average (range) 1. Total (out of 300)	3.7 (3.12-20.8)	2.7 (4.2-20.8)	3.5 (4.2-22.9)	4.2 (4.2-28.1)	0.90
2. POPDI-6	0.4 (1.86)	0.6 (12.5)	0.6 (1.99)	0 (0)	0.11
3.CRAD-8	0.0 (3.1)	0.9 (6.3-12.5)	1.5 (6.3-12.5)	2.4 (15.6-18.8)	0.79
4. UDI-6	3.1 (8.3-20.8)	1.8 (4.2-16.7)	1.4 (4.2-8.3)	1.8 (4.2-12.5)	0.84
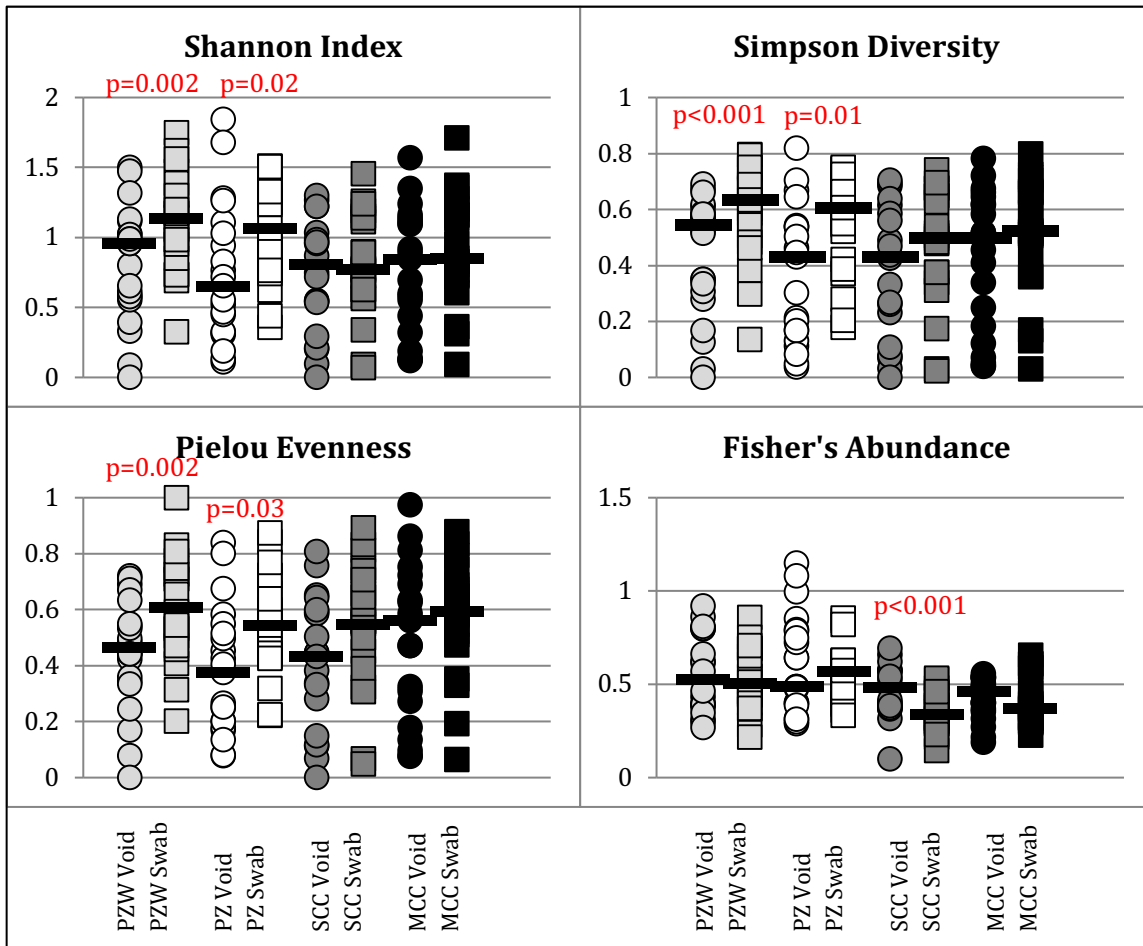
Figure 11. Differences in Alpha Diversity Between Sample Collection Techniques.
Voided urines and peri-urethral swabs are denoted by circles and squares, respectively. The
Kruskal-Wallis test was used to test for significance. Significant *p*-values are denoted in red.

Peezy Urines Possess Lower Total Bacterial Abundance

Because SCC urines displayed a significantly higher Fisher's Diversity index value than

their paired peri-urethral swabs and the Peezy cohorts did not, these data suggest that the SCC

voided urine sample has increased biomass compared to urine collected by Peezy. To test this

hypothesis, the CFU/ml for each urine type was compared to that of the paired peri-urethral swab

(**Figure 12**). The median voided urine CFU/ml was significantly lower than median peri-urethral

swab CFU/ml for each Peezy cohort (PZW p<0.001; PZ p=0.005). In contrast, there was no

significant difference in the mean CFU/ml for SCC or MCC voided urine and peri-urethral swabs

(p=0.27 and p=0.29, respectively). Since there was no statistical difference between swab

CFU/ml for the four cohorts (p=0.19), these data demonstrate that the Peezy device produces urines with lower bacterial loads than SCC or MCC methods.
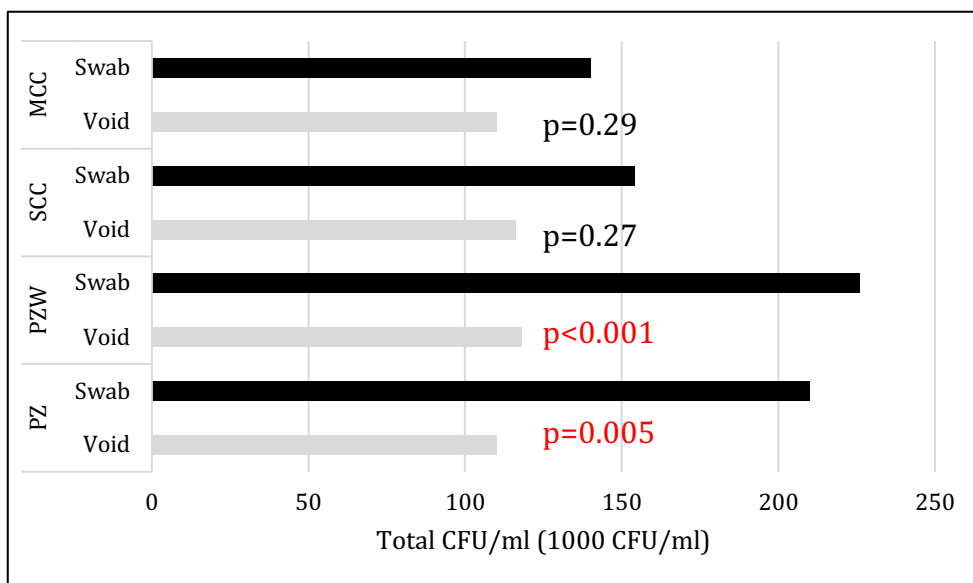
Figure 12. Comparison of Median Swab and Void CFU/ml. Median CFU/ml values for voided urines and peri-urethral swabs for each cohort. *P*-values were obtained by Kruskal-Wallis test for significance.

Peezy Urines Did Not Statistically Differ Based on Peri-urethral Wipe Usage

To compare the efficacy of the peri-urethral wipe, variance between PZW and PZ cohorts was assessed. Principal component analysis (PCA) was conducted to quantify any variance between the two cohorts (**Figure 13**). Considerable overlap between PZW and PZ urines was observed, meaning no statistical differences existed between the urines. Ultimately, the use of a sterilizing peri-urethral wipe does not contribute to or reduce microbiota diversity in Peezy urines.
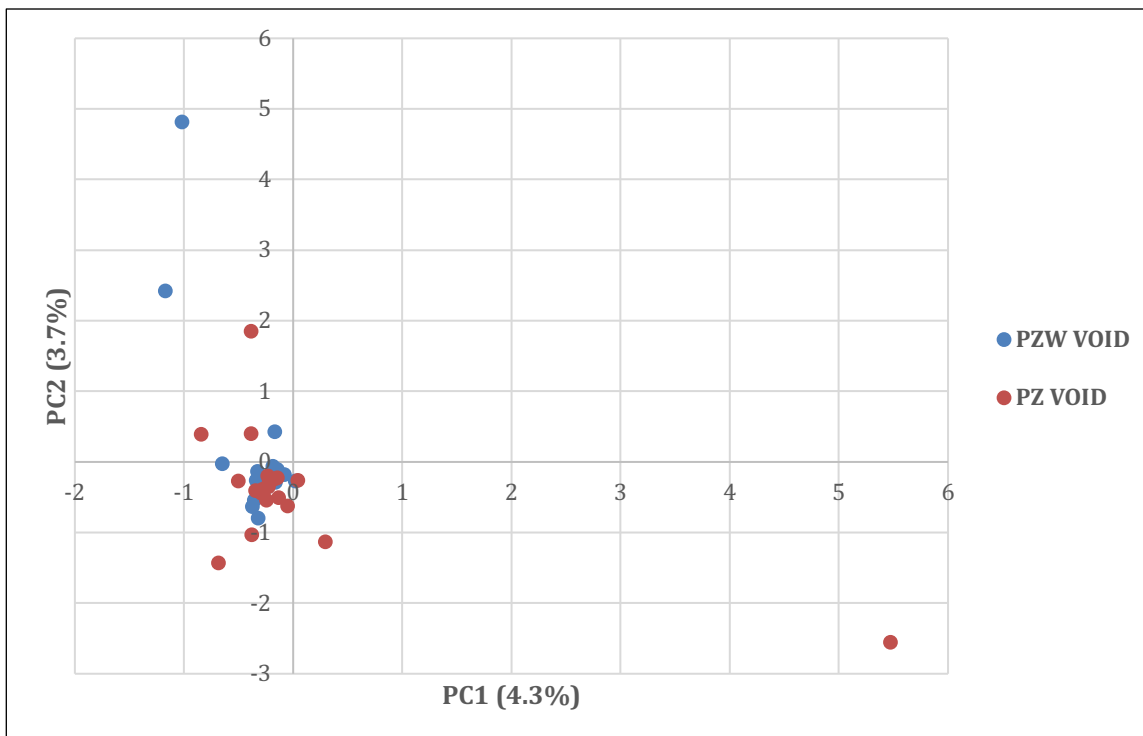
Figure 13. PCA Analysis of PZW and PZ Voided Urines. PCA analysis of PZW and PZ voided urine. Principal components (PC) 1 and 2 are shown on the X and Y axis, respectively. The proportion of variance encompassed by each respective PC is denoted in parentheses.

Discussion

Use of the Peezy device produces midstream voided urines with significantly different bacterial composition than that of paired peri-urethral specimens and of voided urines obtained by SCC and MCC. Peezy voided urines also had decreased bacterial abundance compared to voided urines collected by SCC. These data support the hypothesis that the Peezy device is capable of producing voided urines with reduced vulvovaginal contribution. However, there was no significant difference in bacterial composition of urine obtained by the Peezy device with or without a peri-urethral wipe, suggesting that the Peezy device is unaffected by the use of such wipes before urine collection. Once verified by larger cohort sizes, the Peezy device should be used in place of SCC for urobiome research.

CHAPTER SIX:

DISCUSSION

The utilization of TUC urine has allowed for the successful characterization of the female urobiome; however, this invasive urine collection method limits study participants to patient populations. A critical next step in urobiome research must be to generalize participant populations and perform longitudinal studies, which must be done by collecting urine in a non-invasive manner. Currently, studies of this nature require the use of voided urine. By addressing common issues with voided urine, the three studies described above provide insights crucial to advancing female urobiome research.

First, the knowledge that EQUC outperforms SUC on voided urine will allow researchers to obtain a more complete picture of the urobiome for future studies. Comparison of the microbiota in TUC and mid-stream voided urine samples from RUTI patients cultured via both culture methods allowed us to determine that EQUC detects more uropathogens and commensal organisms than SUC, regardless of urine collection method. This more accurate characterization of RUTI microbiota led to the finding that the microbiota associated with this condition differ greatly from the microbiota associated with acute, uncomplicated UTI. This insight may lead to better understanding of RUTI disease mechanisms, ultimately resulting in better treatment options.

Culturing paired TUC and voided urines from each RUTI participant via both EQUC and SUC not only allowed us to directly compare discrepancies between these culture methods, but it also allowed us to visualize differences in LUT microbiota composition. Many of these women had uropathogens present in midstream voided urine that were not detected in TUC urine, a result that suggests the possibility of a urethral infection or dysbiosis. This observation, combined with the finding that *E. faecalis* is the most common uropathogen associated with UTI

symptoms in this cohort, should instigate researchers to reevaluate how they define UTI (Price et al 2018). A substantial weakness associated with this study was the small cohort size. The associations uncovered in this study must be confirmed with larger sample sizes.

Second, the characterization of bladder, urethral and peri-urethral microbiota demonstrated differences in microbial composition within the LUT. Whilst the bladder microbiota were distinct from the other specimen types, the urethral and peri-urethral microbiota were similar in community structure and abundance. These results support the hypothesis that the urethra and bladder represent distinct niches within the LUT. It is reasonable to hypothesize that these differences are due to the different types of epithelium within the LUT, which range from urothelium to pseudostratified columnar (Hickling et al 2016).

Direct comparison of bladder, urethral, and peri-urethral specimens also provided evidence that some microbiota, such as *Corynebacterium* species and *E. coli*, reside preferentially in certain niches within the LUT. *Corynebacterium* species are commonly associated with the skin (Cogen et al 2008), which shares similar histology with the distal urethra and vulvovaginal skin (Hickling et al 2016). *E. coli*, a known bladder organism, has previously been shown to associate with urothelium of the bladder (Linder et al 1988). Conversely, other microbiota, such as *Lactobacillus* and *Gardnerella* species, were found at similar levels throughout the LUT, suggesting that these genera do not rely on specific epithelium types for successful establishment, but are perhaps more influenced by host factors.

The presence and abundance of these genera in LUT samples seems to be affected by hormonal status. Menopausal status has been reported to affect *Lactobacillus* prevalence in the vagina due to decreased vaginal secretions, which provide the amylase activity necessary to depolymerize glycogen (Nunn et al 2016, Shen et al 2016, Alvisi et al 2017). Since it has been shown that estrogen increases *Lactobacillus* bladder abundance in UUI patients (Thomas-White 2016, Dissertation), it is possible that a similar glycogen-degradation activity may be found throughout the LUT of pre-menopausal women.

Furthermore, characterization of LUT microbiota provides insight into how voided urines might be interpreted. This study shows that voided urine is more representative of peri-urethral skin than the LUT and demonstrates that voided urine generally reports on high biomass species present in any of these anatomical niches. Given this observation, one wonders how researchers can use voided urine to report on the bladder microbiota? Some groups have attempted to use subtractive methods to answer this question. Although sophisticated bioinformatic techniques (e.g. SourceTracker, Decontam) successfully identify sources of contamination in biological samples, they were designed to analyze high biomass specimens (Knights et al 2011, Karstens et al 2018). The low biomass of bladder urine combined with the high species overlap within the more abundant urethra, peri-urethra and vaginal communities diminishes the value of subtractive bioinformatics methods. Variance in LUT microbiota across demographics further complicates this issue, as extensive demographic information must also be considered.

The specimens for this study were collected by healthcare professionals, strengthening our findings by standardizing collection procedure and increasing specimen accuracy. However, our study is substantially weakened by the use of the SCC method for voided urine collection. Further analysis of these niches should be performed with a better voided urine collection technique, such as Peezy, to make every attempt to reduce vulvovaginal contamination. It is possible that, with the implementation of a cleaner catch method, voided urine may more closely resemble the microbiota of the LUT, if not the bladder. This would provide more reliable interpretation of voided urine for urobiome studies. Future studies also should investigate if the proximal urethra, which is histologically distinct from the distal urethra (Carlile et al 1987), houses contrasting microbiota to the distal urethra.

Third, the identification of a "cleaner" catch method makes using voided urine to study the bladder microbiota more feasible. Use of the Peezy device produced midstream voided urines with significantly different bacterial composition than that of paired peri-urethral specimens and of voided urines obtained by SCC of MCC. These differences in bacterial composition most likely result from standardization of the procedure provided by the use of Peezy; this device

consistently discards the same volume of the initial urine stream, which has been shown to have higher biomass than TUC urine and resemble urethral contamination (Dong et al 2011). We hypothesize that the observed decrease in abundance and diversity of Peezy urines results from the removal of bacteria that originate from the urethra and surrounding vulvo-vaginal skin before collection of the lower biomass midstream urine. Future studies should test this hypothesis.

This study was strengthened by the use of peri-urethral swabs as internal controls. Comparing each participant's voided urine to her peri-urethral microbiota allowed us to better assess vulvovaginal contribution to urine. In contrast, this study was limited by the lack of a TUC control. Although we were able to conclude that, in terms of the microbiota detected, Peezy urines resemble vulvo-vaginal skin less than they do SCC urines, we cannot relate the microbiota detected in voided urines to those present in bladder urine. Although the Peezy device reduces the bacterial abundance in voided urines, it does not produce culture results one would expect from TUC urine. Most telling is the frequency of EQUC-negative results; nearly half of all TUC urine samples obtained from asymptomatic women were EQUC-negative (Pearce et al 2014), whereas voided urines are almost always culture-positive, likely due to their higher biomass (Aisen et al 2018). Since all Peezy urines were culture positive, voided urines obtained by Peezy cannot be considered to represent the bladder microbiota, but rather the lower urinary tract microbiota. Further analysis, such as the comparison of urines obtained by TUC or Peezy and determination of Peezy's efficacy in symptomatic populations, is necessary before this device can be implemented in female urobiome research.

Combined, these studies highlight the importance of carefully selecting the methodologies used to conduct urobiome research. Collection of voided urine via a method shown to reduce post-bladder contribution, such as the Peezy device, and utilizing EQUC to culture these urines alongside appropriate negative and positive controls allows for a more accurate depiction of urinary microbiota. However, voided urine is not directly representative of the bladder, but contains significant microbial contribution from the urethra and vulvovaginal skin. Whilst efforts should be made to develop non-invasive means of bladder urine collection,

researchers must take into consideration that they are not only capturing bladder urine when interpreting voided urine data.

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