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The Contribution of the Female Urinary Microbiota to Lower Urinary Tract Symptoms

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LOYOLA UNIVERSITY CHICAGO

THE CONTRIBUTION OF THE FEMALE URINARY MICROBIOTA
TO LOWER URINARY TRACT SYMPTOMS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE
PROGRAM IN MICROBIOLOGY & IMMUNOLOGY

BY

TRAVIS K. PRICE

CHICAGO, IL

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LIST OF ABBREVIATIONS

BAP	Blood Agar Plate
BV	Bacterial Vaginosis
CFU	Colony Forming Units
CNA	Colistin and Nalidixic Acid
ENS	Effective Number of Species
EPEC/UPEC	Enteropathogenic/Uropathogenic <i>Escherichia coli</i>
EtBr	Ethidium Bromide
EQUC	Extended Quantitative Urine Culture
FBS	Fetal Bovine Serum
FUM	Female Urinary Microbiota
HPLC	High Performance Liquid Chromatography
IRB	Institutional Review Board
LB	Luria Broth
LUTS	Lower Urinary Tract Symptoms

MALDI-TOF MS	Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectroscopy
MIC	Minimum Inhibitory Concentration
MRS	de Man, Rogosa, Sharpe
OAB	Over Active Bladder syndrome
PCA	Principle Components Analysis
SCS	Spent Culture Supernatant
SPA	Suprapubic Aspirate
SUI	Stress Urinary Incontinence
TSA/TSB	Tryptic Soy Agar/Broth
TUC	Transurethral Catheter
UTI	Urinary Tract Infection
UTISA	Urinary Tract Infection Symptom Assessment
UUI	Urgency Urinary Incontinence
WGS	Whole Genome Sequencing

ABSTRACT

The recent finding that urine is not sterile raises a lot of questions: first and foremost, what microorganisms are present, and are these microorganisms correlated with clinical urinary symptoms? Recent work on the female urinary microbiota (FUM) found that the communities of microorganisms differ between women with no lower urinary tract symptoms (LUTS) and those with Urgency Urinary Incontinence (UUI) a form of over-active bladder syndrome. Specifically, a diverse or dysbiotic urinary microbiota was found to be associated with symptomatic women. This suggests the possibility that the FUM can be contributing to urinary symptoms. Conversely, it could also suggest that the FUM is simply a result of the physical environment of the urogenital tract and plays no active role in health. I hypothesize that the FUM is influencing LUTS, both as causative and preventative agents towards the clinical urinary symptoms. To test this, I first looked at the FUM of women with other urinary disorders: Stress Urinary Incontinence (SUI) and women with urinary tract infection (UTI)-like symptoms. Since many common urinary disorders share significant symptom overlap, I also assessed how the FUM correlates to individual symptoms. These data support the hypothesis that these microorganisms are able to influence LUTS. One specific organisms of the FUM, *Lactobacillus crispatus*, is able to exert a beneficial effect on LUTS, by inhibiting the growth and colonization of Uropathogenic *Escherichia coli* (UPEC), the most common cause of UTIs. The next step

is to assess these potential interactions in an *in vivo* setting. With that collective knowledge, LUTS and UTI's can be clinically treated with better effectiveness.

CHAPTER I

INTRODUCTION

A History of the Urinary Microbiome & Microbiota

Microorganisms resident in and on the human body have long been overlooked with respect to their ability to influence human health. A recent effort, through the Human Microbiome Project, has attempted to shed some light on this subject by cataloging the microbial composition of select anatomical sites of the human body, including the gastrointestinal tract, the skin, the respiratory tract, and the vagina (Turnbaugh *et al.*, 2007). This work was done largely through the use of 16S rRNA gene sequencing, which identifies microbial communities based on genomic DNA. This approach, however, has several limitations. The process of sequencing may create biases depending on the specific primers used, causing some taxonomic groups to be largely overlooked and others to be overrepresented. Also, the data are not readily quantifiable. But most importantly, it is unclear whether the identified microorganisms are actually viable. Unfortunately, this problem is exacerbated by the fact that the majority of microorganisms are not cultivatable using today's methods. Indeed, several body sites were inadvertently ignored with respect to their microbial composition due to outdated culture information. One of these sites is the bladder.

The long-standing belief that urine is sterile resulted in the Human Microbiome Project overlooking the bladder. Clinical urinary dogma relates the presence of any bacteria

with an inflammatory response to a urinary tract infection (UTI), while bacteria found in the absence of inflammation or clinical symptoms are termed asymptomatic bacteriuria (ABU). Since the 1950's, the clinical practice for detection of infection in the bladder has been based on the threshold of greater than or equal to 10^5 colony forming units per mL urine (CFU/mL) of a bacterial uropathogen present in mid-stream urine. However, this threshold of $\geq 10^5$ CFU/mL was originally intended to be used to detect kidney infection or pyelonephritis (Kass, 1956). The idea was to screen a patient for pyelonephritis with this assay before initiating kidney surgery to prevent post-operative sepsis (Kass, 1956, Kass, 1957). This threshold was later equated to all urinary infections and the assay is still used today as the method of detection for lower urinary tract infections. Several studies over the past 30 years have provided evidence that this threshold level is insufficient for the detection of clinical UTIs (Stamm *et al.*, 1982, Lipsky *et al.*, 1987, Maskell, 2010, Khasriya *et al.*, 2013). There have even been efforts to assert that urine is not sterile in the absence of clinically relevant infection (for a review, see Maskell, 2010). In more recent years, the push has become even stronger to both re-evaluate this long standing belief and to change urine cultivation protocols (Hooton *et al.*, 2013).

There is now mounting concrete scientific evidence to verify the claim the urine isn't actually sterile. Evidence from both DNA sequencing and culture indicate the presence of urinary microbial communities in urine samples obtained by voided midstream, transurethral catheter and suprapubic aspirate methods (Siddiqui *et al.*, 2011, Dong *et al.*, 2011, Fouts *et al.*, 2012, Nelson *et al.*, 2012, Wolfe *et al.*, 2012, Lewis *et al.*, 2013, Hilt *et al.*, 2014). 16S rRNA gene sequencing shows that a diverse group of bacteria reside in the

urine of both men and women (Fouts *et al.*, 2012, Nelson *et al.*, 2012, Hilt *et al.*, 2014). This was identified through the comparisons of DNA sequences obtained from midstream voided urine, transurethral catheter urine (TUC), and suprapubic aspirates (SPA) in women with and without LUTS. These findings show that the DNA detected from voided urine samples comes in part from the bladder (Wolfe *et al.*, 2012). Use of an extended quantitative urine culture (EQUC) protocol on TUC samples from women demonstrated that a portion of the detected DNA comes from viable microorganisms that have been overlooked by the use of the standard urine culture protocol (Hilt *et al.*, 2014). Therefore, female urinary microbiota (FUM) exist. Urinary microorganisms have also been found in men after cultivation of centrifuged voided urine samples (Khasriya, *et al.*, 2013, Nelson *et al.*, 2012, Dong *et al.*, 2011). Thus, unique urinary microbiota (population of live cultivatable microorganisms in a particular environment) and urinary microbiomes (collective genetic material present in a particular environment) exist. Whether these microorganisms represent a basal microflora of the bladder or are just transiently found between various urogenital sites is unclear.

Interactions between the Urinary Microbiota & the Host

The urothelium is a stratified layer of epithelial cells lining the bladder wall. It is known that several uropathogens (microorganisms known to be associated with inflammation and symptoms) can associate with the urothelium (Coker *et al.*, 2000, Struve *et al.*, 2008). Some organisms (e.g, *Escherichia coli*) can become intracellular with the use of type 1 fimbriae (allows for attachment to uroplakin on the superficial layer of the urothelium), and form intracellular bacterial communities that are resistant to antimicrobials and the host immune

response (Anderson *et al.*, 2003, Struve *et al.*, 2008). Certain non-uropathogenic bacterial species also are found associated with shed urothelial cells in voided urine (Khasriya *et al.*, 2013). Thus, it is expected that a large portion of the urinary microbiota exist attached to the urothelium.

Because these urinary microorganisms are likely in direct contact with the host cells, they could exert some influence on the host and vice versa. This is the case for many known and well-studied uropathogens. This contact is primarily what drives the infection and resulting symptoms of UTIs. Using uropathogenic *Escherichia coli* (UPEC) as an example, there are a multitude of encoded virulence factors that enable bacteria to colonize the urinary tract and persist (in both humans and mice). The presence of adhesins and fimbriae on the bacterial cell surface allows UPEC to trigger host and bacterial signaling pathways, deliver bacterial products into the host tissue, and promote bacterial invasion and biofilm growth (Mulvey, 2002). Other surface-associated virulence factors include lipopolysaccharide (LPS), which can trigger a proinflammatory immune response through induction of nitric oxide and cytokine release (Backhed *et al.*, 2001), as well as flagella, which allow for more effective colonization and motility throughout the upper and lower urinary tract (Pichon *et al.*, 2009). Additionally, UPEC can produce several toxins that result in inflammatory responses. α -haemolysin (HlyA) is a pore-forming lipoprotein that acts on epithelial cells throughout the urinary tract (Bhakdi *et al.*, 1986). Cytotoxic necrotizing factor 1 (CNF1) is produced by several strains of pyelonephritic strains of UPEC and helps facilitate intracellular invasion and protection against immune phagocytosis (Rycke *et al.*, 1999, Mills *et al.*, 2000). TIR domain-containing protein (Tcp)

allows for subversion of TLR signaling, permitting enhanced survival of the bacteria (Cirl, *et al.*, 2008). Conversely, diverse host defenses limit the colonization of UPEC. The low pH and presence of salts, urea, and organic acids in the urine can all reduce UPEC survival within the urinary tract (Sobel, 1997). The Tamm-Horsfall glycoprotein acts as an anti-adhesive factor by binding the type 1 fimbriae of UPEC to prevent proper adherence to the urothelium (Leeker *et al.*, 1997). Bacterial cells that can bind to the urothelium and/or invade, can be subjected to exfoliation of the superficial urothelial cells (Mysorekar *et al.*, 2002). Upon adherence to the urothelium, UPEC elicits an innate immune response characterized by the local production of a number of proinflammatory cytokines and chemokines, as well as apoptosis of the infected eukaryotic cells (Schilling, 2001). This complex cross-talk between UPEC and the host is not unique to this microorganism; other uropathogens behave similarly and evoke similar host responses. Whether these characteristics apply to the entire urinary microbiota is unclear. It is probable that some species of the microbiota communicate with the host in less harmful ways, which allows them to persist as normal members of the bladder flora, similar to what occurs in the GI tract. An additional layer of complexity to understanding the microbial interactions, exists in that the microorganisms likely communicate with one another and this communication can be either inter- or intra-species.

The Urinary Microbiota & Urinary Health

One piece of evidence that supports the claim that the non-uropathogens of the urinary microbiota influence the host comes from Pearce *et al.* (2014). The authors of this manuscript showed that the FUM is distinct in both composition and alpha-diversity

between women with urgency urinary incontinence (UUI) and women without any LUTS (Pearce *et al.*, 2014).

LUTS refers to a group of urinary symptoms including filling or irritative symptoms (frequency, urgency, dysuria, nocturia, and urinary incontinence) and voiding or obstructive symptoms (hesitancy, weak stream, incomplete voiding, and urinary retention) (Abrams, 2011). UUI is a form of over active bladder syndrome (OAB) that encompasses the feelings of urgency with involuntary leakage (Stewart *et al.*, 2003). Current prevalence estimates for urinary incontinence in middle-aged and older women in the general population range from 30-60% (Danforth *et al.*, 2006). Treatment and therapies for incontinence result in the spending of more than \$20 billion per year in the United States. The understood etiology of UUI is that of a neurological disorder resulting in hyperactivity of the detrusor muscle surrounding the bladder (Michel *et al.*, 2009). However, treatment to block this misfiring is only successful in about 50-60% of individuals (Santos *et al.*, 2010). This knowledge, combined with the knowledge from Pearce *et al.* (2014), suggests that an additional etiology of UUI, and possibly OAB, in women, is the microbiota. One could imagine that the interplay between the bacteria and the host tissue could result in downstream signaling events in the urothelial cells leading to inappropriate detrusor muscle function. For example, human urothelial cells express carnitine acetyltransferase (CarAT), an enzyme that catalyzes the synthesis of acetylcholine (Le *et al.*, 2014). Acetylcholine binds to the muscarinic receptors present on the detrusor muscle activating muscle contraction. Therefore, if the FUM induces upregulation of CarAT by the urothelium, then this shows that the microbiota can cause

UI and OAB. If this is indeed the case, then it is possible that the FUM is involved in several other urinary symptoms, as major or minor contributors.

Additional evidence in support of the possibility that the FUM contributes to urinary health and symptoms comes from data showing that about 50% of women experiencing UTI-like symptoms (frequency and urgency of urination, and dysuria) have no detectable bacteria present by use of the standard urine culture (Najar *et al.*, 2009, Franz *et al.*, 1999). Since EQUIC is able to detect urinary microbiota in about 90% of urine samples deemed, “no growth”, by the standard urine culture (Hilt *et al.*, 2014), a majority of the women experiencing UTI-like symptoms (despite a negative standard urine culture) may have bacteria present. These microorganisms could be accounting for the urinary symptoms. Therefore, using the techniques used in Hilt *et al.* (2013) and Pearce *et al.* (2014), I will assess if the previously unidentified FUM is responsible for these symptoms.

***Lactobacillus crispatus*: An Overview**

Lactobacillus is an abundant group of Gram-positive lactic acid-producing bacteria commonly residing in the oral cavity, gastrointestinal (GI) tract, and the urogenital tract of mammals. Many species of Lactobacilli are associated with food production due to their preservative action (Bernardeau *et al.*, 2006). Some Lactobacilli in the GI tract are commonly considered to be probiotics because they confer a health benefit to the host (Goldin *et al.*, 1992). These benefits include prevention of intestinal infections, diarrhea, and hypercholesterolemia, as well as maintenance of gut barrier function (Kailasapathy *et al.*, 2000). These probiotic bacteria exert their effects by activating both non-specific and specific host immune responses (Gill, 1998, Kaila *et al.*, 1992, Schiffrin *et al.*, 1995). They

also can alter the microbial balance of commensal enteric microorganisms (Kailasaparthi *et al.*, 2000). A similar protection is believed to occur in the healthy human vagina, where colonization with Lactobacilli is correlated with the absence of numerous urogenital conditions (Uehara *et al.*, 2006, Stapleton *et al.*, 2011, Hemmerling *et al.*, 2009).

In many women, a healthy vagina is predominantly colonized by *Lactobacillus*. Alternatively, some women may be prominently colonized by other anaerobes, but much of these microorganisms also fall into the group of lactic acid-producing bacteria, demonstrating an important role for lactic acid in this environment (Ravel *et al.*, 2010).

Lactobacillus crispatus is one of four main species of *Lactobacillus* that tends to dominate the vaginal microflora, where it can account for upwards of 80% of all vaginal bacteria in a single individual (Ravel *et al.*, 2010). *L. crispatus* contributes to the stability of normal vaginal microbiota. Its absence is linked to a variety of vaginal abnormalities, including BV (Fredricks *et al.*, 2007). Interestingly, these highly supportive actions of *L. crispatus* seem to be unique to this species, as other dominating Lactobacilli like *L. gasseri* and *L. iners*, tend to be more conducive to the occurrence of abnormal vaginal microflora as suggested by a longitudinal analysis in pregnant women (Verstraelen *et al.*, 2009). In regards to BV, it has been shown that *L. crispatus* can competitively exclude *G. vaginalis*, promoting a healthy vaginal environment (Aroutcheva *et al.*, 2001, Castro *et al.*, 2013). Not only can this occur through steric hindrance, but new data suggest that *L. crispatus* can act to directly disrupt the pilus-mediated attachment process used by *G. vaginalis* to adhere to the epithelium (Ojala *et al.*, 2014). Additionally, certain strains of *L. crispatus*, particularly strain CTV-05, are being tested as biotherapeutics for treatment of recurrent

UTIs in women (Stapleton *et al.*, 2011, Hemmerling *et al.*, 2009, Antonio *et al.*, 2009, Kwok *et al.*, 2006). The modest success of this strategy is likely due to the ability of *L. crispatus* to inhibit uropathogenic *E. coli* (UPEC), the most common causative agent of UTI, through the production of lactic acid and H₂O₂, competitive exclusion, and possibly through targeted antimicrobial peptide production.

The genome sequences of ten *L. crispatus* strains are currently available (Nelson *et al.*, 2010, Salvetti *et al.*, 2012). The genomes range in size from 2.0-2.7 Mb, and are predicted to encode 2,022-2,643 proteins. Genome annotation data suggest that these strains are able to produce lactic acid, H₂O₂, bacteriocins, and adhesion proteins, which could be used in competitive adhesion to epithelial cells. Nine out of ten strains are vaginal isolates; the one remaining strain is a chicken-isolated strain, ST1 (Nelson *et al.*, 2010, Ojala *et al.*, 2010). Phylogenetic analysis of *L. crispatus* shows that this species shares the most recent common ancestor with *Lactobacillus helveticus* and is also closely related to *Lactobacillus acidophilus*. Among the *L. crispatus* cluster, the ST1 strain branches off first from the vaginal isolates (Ojala *et al.*, 2014, Kant *et al.*, 2011).

Adhesion to tissue surfaces is necessary for long-term colonization and persistence. In the vagina, strong adhesion to the epithelium by the commensal microbiota is protective against pathogens through competitive exclusion. All ten strains of *L. crispatus* contain several predicted adhesion proteins including mucus-binding and fibrinogen-binding proteins (Ojala *et al.*, 2014). *L. crispatus* strain ST1 was also recently found to produce a *Lactobacillus* epithelium adhesion (LEA) protein that allows for binding to both chicken crop epithelium and vaginal epithelial cells (Edelman *et al.*, 2012). The adhesion provided

by this protein and others allows ST1 to competitively exclude *E. coli* and *Salmonella* (Edelman *et al.*, 2003). *L. crispatus* also contains several putative S-layer proteins and protein-encoding genes, some of which are involved in adhesion. S-layer proteins are paracrystalline surface protein arrays commonly found in *Lactobacillus* and many other bacteria and archaea. These proteins usually share very little homology. Typically, S-layer proteins are around 45 kDa in size and consist of a moderately conserved C-terminus and a strongly variable N-terminus (Sillanpaa *et al.*, 2000). The function of these proteins can vary from providing stability to the outer membrane, protection from bacteriophages, resistance to pH, and adhesion (Sleytr *et al.*, 1997). Many of the *L. crispatus* strains use these S-layer proteins for binding to collagen (Toba *et al.*, 1995, Sun *et al.*, 2013). These proteins may also be involved in the ability of *L. crispatus* to competitively exclude pathogens (Chen *et al.*, 2007). Additionally, *L. crispatus* is able to rapidly modify its surface properties in response to changes in pH (Antikaninen *et al.*, 2007).

L. crispatus strains contain several putative bacteriocin genes including ones similar to enterolysin A, helveticin J, and pediocin (Ojala *et al.*, 2014). Some strains also contained genes for gassericin A, a bacteriocin commonly found in *L. gasseri* (Stoyacheva *et al.*, 2014). Recently, cervicovaginal lavage (CVL) from healthy women was shown to contain proteins that are bactericidal towards *E. coli*. Several of these proteins correspond to *L. crispatus* (Kalyoussef *et al.*, 2012). Additionally, it is known that a *L. crispatus* dominant vaginal microbiome correlates with an inhibitory effect against *E. coli* from vaginal secretions (Ghartey *et al.*, 2014). The bacteriocin, crispacin A, is produced by *L.*

crispatus and shares sequence homology with other bacteriocins from lactic-acid bacteria, but direct targets of this protein have not been identified (Tahara *et al.*, 2006).

Thus, *L. crispatus* is clearly an important member of the commensal vaginal flora and its probiotic characteristics have garnered much recent attention; however, the nature of these characteristics remains poorly understood.

CHAPTER II

MATERIALS & METHODS

Patients and Sample Collection

Following Loyola institutional review board (IRB) approval for all phases of this project, participants gave verbal and written consent for the collection and analysis of their urine for research purposes. Participants were women with and without either subtype of urinary incontinence (*Chapter III*) and women with and without UTI symptoms (*Chapter IV*). Participants' symptoms were characterized with the Pelvic Floor Distress Inventory (PFDI), a self-completed, validated symptom questionnaire (Due *et al.*, 2013) (*Chapter III*), or by a validated UTI Symptom Assessment (UTISA) questionnaire (Clayson *et al.*, 2005) (*Chapter IV*). All urinary incontinence participants were without clinical evidence of UTI (i.e., standard urine culture negative and absence of clinical UTI diagnosis). Urine was collected via transurethral catheterization from participants for the period December 2013 to May 2015 at the Female Pelvic Medicine and Reconstructive Surgery center of Loyola University Medical Center. A portion of each urine sample was placed in a BD Vacutainer Plus C&S Preservative Tube (Becton Dickinson and Co; Franklin Lakes, NJ) and sent to the clinical microbiology laboratory for Standard Urine Culture. A separate portion of the urine sample was placed at 4°C for no more than 4 h following collection, to

be used for sequencing (*Chapter III*). To this portion, 10% AssayAssure (Thermo Scientific; Waltham, MA) was added before freezing at -80°C .

Urine Culture Protocols

Standard & Modified Urine Culture.

The standard urine culture protocol involves the inoculation of 1 μL of urine onto 5% sheep blood agar (BAP) and MacConkey agar (BD BBL™ Prepared Plated Media, Becton Dickinson and 94 Co; Sparks, MD) using a quantitative pinwheel streak. These plates are incubated aerobically at 35°C for 24 hours (**Table 1**). This plating method preferentially allows for the growth and quantification of rapidly growing Gram-negative obligate aerobes at concentrations $\geq 10^3$ CFU/mL. Most well-studied uropathogens will be identified under these conditions.

The modified urine culture protocol is a revised version of the standard culture. Instead of incubation under aerobic conditions, the BAP and MacConkey agar plates are incubated in 5% CO_2 . Additionally, if no growth is detected at 24 hours, they are held in the same conditions for a further 24 hours (**Table 1**). This plating method will allow for the identification of slower growing microorganisms, including facultative anaerobes and microaerophiles.

Standard culture is the typical method used by clinical microbiology laboratories for the identification of UTIs. A positive urine culture, indicative of infection, is the growth of any microorganism at $\geq 10^5$ CFU/mL, though this threshold may vary based upon the

patient population, type of urine specimen, and standard practice of the laboratory. The modified culture was implemented by the clinical microbiology laboratory at Loyola University Medical Center in December, 2014. All of the urine samples used in the later described studies were also processed by the clinical microbiology laboratory staff. Thus, data comparing the Extended Quantitative Urine Culture (EQUC) (*see **Extended Quantitative Urine Culture***) to routine culture may represent data from use of either the Standard or Modified Cultures. These distinctions are indicated.

Extended Quantitative Urine Culture.

The EQUC protocol was used on TUC urine samples to identify the FUM (Hilt *et al.*, 2014). This protocol involves the inoculation of 100 μ L of urine onto BAP, chocolate agar, and colistin and nalidixic acid (CNA) agar (BD BBL™ Prepared Plated Media) incubated in 5% CO₂ at 35°C for 48 hours. Chocolate agar contains lysed red blood cells and supports the growth of fastidious microorganisms. CNA agar is a selective medium for Gram-positive bacteria. Additionally, 100 μ L of urine is inoculated onto BAP and incubated aerobically at 35°C for 48 hours. Finally, 100 μ L of urine is inoculated onto CDC anaerobe 5% sheep blood agar plate (BD BBL™ Prepared Plated Media) and incubated under anaerobic conditions at 35°C for 48 hours (**Table 1**). All urine is plated using a quantitative pinwheel streak. Collectively, this protocol will allow for the growth and identification of rapid and slow growing Gram-negative and Gram-positive microorganisms with a wide range of oxygen tolerances.

Protocol (Volume)	Media	Condition	Identification
Standard (1µL)	Blood, MacConkey	Aerobic 35° C	24 hr
Modified (1µL)	Blood, MacConkey	5% CO ₂ 35° C	24hr 48hr
EQUC (100µL)	Blood, Chocolate, CNA	5% CO ₂ 35° C	48 hr
	Blood	Aerobic 35° C	
	Anaerobic	Anaerobic 35° C	
Expanded EQUC (1µL, 10µL, 100µL)	Blood, Chocolate, CNA	5% CO ₂ 35° C	24hr 48hr
	Blood, MacConkey	Aerobic 35° C	24hr 48hr
	Anaerobic	Anaerobic 35° C	48hr
	Anaerobic	Campy gas mixture (5% O ₂ , 10% CO ₂ , 85% N) 35° C	48hr

Table 1. Summary of Urine Culture Protocols. The modified culture was implemented at the Loyola University Medical Center Clinical Microbiology Laboratory on December 17, 2014.

The expanded EQUIC protocol is a modified version of the original EQUIC. This protocol uses two additional conditions: MacConkey agar, incubated aerobically at 35°C for 48 hours and CDC anaerobe 5% sheep blood agar, incubated in a Campy gas mixture (5% O₂, 10% CO₂, 85% N) at 35°C for 48 hours. The Campy condition provides an optimal environment for the isolation of microaerophilic fecal microorganisms. Additionally, the expanded EQUIC uses three different volumes of urine: 1, 10, and 100 µL. Thus, a total of 21 plates are used for this protocol (**Table 1**).

Isolation, Identification, and Storage of Microorganisms

All morphologies and counts of colonies are recorded and documented for each individual plate per culture protocol. Each unique morphology (per plate) is then sub-cultured to new media and incubated in the appropriate conditions for 48 hours.

Once a pure culture is obtained, the microorganism is identified using Matrix assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). This instrument uses mass spectrometry to analyze the bacterial proteins, allowing for identification to the species level. A small portion of a single isolated colony is applied to a 96-spot, polished, stainless steel target plate (Bruker Daltonik GmbH, Leipzig, Germany). 1 µL of 70% formic acid is applied to each sample and allowed to dry at room temperature. 1 µL of the matrix solution is then added to each sample and allowed to dry. This solution is comprised of saturated α -cyano-4-hydrocinnamic acid (Bruker Daltonik) in an organic solvent (High-Pressure Liquid Chromatography-Mass Spectrometry [HPLC-MS]-grade water, 100% Trifluoroacetic Acid, and Acetonitrile; Fluka). The target plate is

then placed in the MicroFlex LT mass spectrometer (Bruker Daltonik), and the results are analyzed using MALDI Biotyper 3.0 software (Bruker Daltonik). A bacterial quality control strain of DH5 α *Escherichia coli* is included in each analysis. Each identification is given a confidence score. A score between 2.000 and 3.000 is confident to the species level; a score between 1.700 and 1.999 is confident to the genus level; a score below 1.700 is not reliable. Samples with scores below 2.000 are repeated twice. If no confident score (≥ 2.000) is obtained, the sample is re-sub-cultured for 48 hours and assessed by MALDI-TOF MS again. If still no confident score is obtained, the sample is not included in the data analysis for that urine sample, and is Gram stained and classified as an “unknown”.

Each unique species per urine sample is inoculated into a vial of Brucella Broth with 10% Glycerol (CryoSavers, Hardy Diagnostics) and stored at -80°C for future use.

A major limitation of these methods are that culture-based methods are biased by the specific physiological conditions used. Though EQUIC has been used with success (Hilt *et al.*, 2014, Pearce *et al.*, 2014), it still has bias. Additionally, identification of unique species based solely off of morphological characteristics may not always delineate species accurately. Finally, MALDI-TOF MS can only identify a bacterial species if that bacterial profile is already present in the computer database. Therefore, any unclassified or less-than-confident classifications will remain unidentified and not included in any study analyses in this thesis.

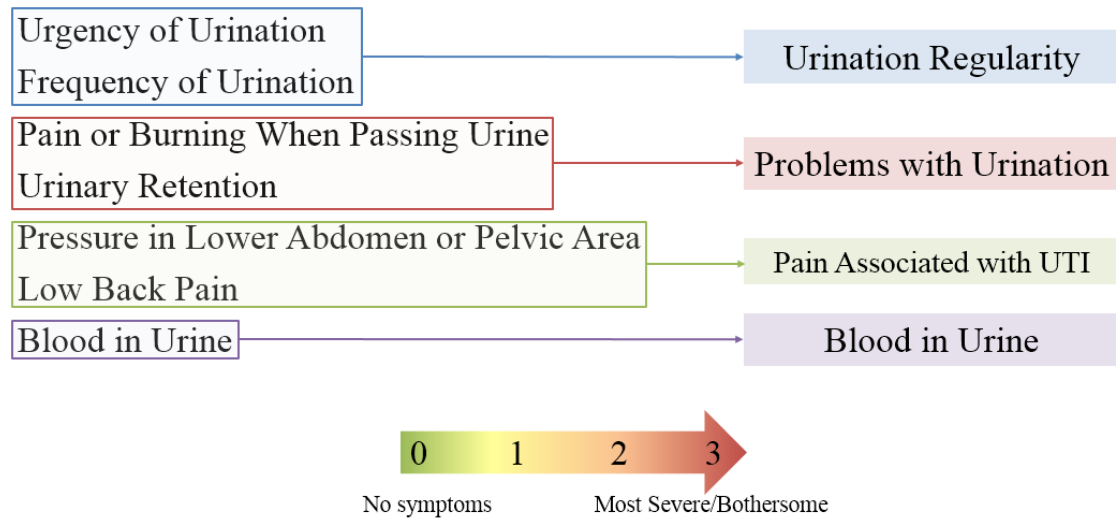


Figure 1. Overview of the UTISA Questionnaire. The validated UTISA questionnaire asks the participant to score the degree of severity and bother on a scale of 0-3 for seven common UTI symptoms: Urgency of Urination, Frequency of Urination, Pain or Burning when Passing Urine, Urinary Retention, Pressure in the Lower Abdomen or Pelvic Area, Lower Back Pain, and Blood in the Urine. A score of 0 indicated no symptom present, while a score of 3 indicated most severe/bothersome. On the left is a grouping of the seven symptoms into four domains (Clayson *et al.*, 2005).

UTI Symptom Assessment Questionnaire

The UTI Symptom Assessment (UTISA) Questionnaire (Bayer Pharmaceuticals Corp., Global Health Economics and Outcomes, Research Department, West Haven CT. 2005) is a validated questionnaire that is used to assess various common clinical symptoms of UTIs (Clayson *et al.*, 2005). This self-administered questionnaire asks the participant to rate the degree of severity and bother on a scale of 0 to 3 for seven common UTI symptoms: frequency of urination, urgency of urination, incomplete bladder emptying (urinary retention), pain or burning during urination (dysuria), lower abdominal discomfort or pain/pelvic pressure, lower back pain, and blood in the urine (hematuria). A score of 0 corresponds to no symptom present, whereas a score of 3 indicates high severity or bother of the symptom. The seven UTISA symptoms can be clustered into four symptom domains (Clayson, *et al.*, 2005). A schematic outline of the questionnaire is seen in **Figure 1**.

16S rRNA and Whole Genome Sequencing

To further characterize selected urinary isolates, genomic sequencing was used to determine closely related strains, as well as to verify the taxonomic assignment by MALDI-TOF MS. To begin the process, pure bacterial cultures were taken from the isolate collection (Brucella Broth vials stored at -80°C) and grown in their optimal environment for 48 hours. Pure colonies were transferred to broth (*Gardnerella* – Tryptic Soy Broth (TSB) supplemented with 10% Fetal Bovine Serum (FBS); *Lactobacillus* – de Man, Rogosa, and Sharpe (MRS) broth) and grown in the same environment for 48 hours. To isolate the genomic DNA, the urinary organism was centrifuged for 2 minutes at 13,000

RPM, the supernatant liquid was decanted and the pellet was subjected to a phenol-chloroform extraction protocol.

The genomic DNA was used for both Sanger Sequencing and Whole Genome Sequencing (WGS). The Sanger Sequencing method used the incorporation of fluorescently tagged chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication (Sanger *et al.*, 1975, Smith *et al.*, 1986). The bacterial genomic DNA was used as a template to amplify the 16S rRNA gene using universal primers (11F and 1492R) and Polymerase Chain Reaction (PCR). The 1.5 kb gene was cloned into a pCR2.1-TOPO plasmid (Invitrogen™, Thermo Fisher Scientific Inc.) and introduced into Ca²⁺-competent DH5α *E. coli* via transformation. The insertion of the PCR product into the plasmid interrupted the *lacZα* gene. The full length LacZα subunit is required to form functional β-galactosidase. The presence of active β-galactosidase was assayed by growing the transformed *E. coli* on Luria-Bertani (LB) media with X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma-Aldrich, St. Louis, MO) (200μL of 20mg/mL X-GAL into dimethylformamide (DMF)) . Formation of a blue-colored product, precipitated within the cells causing the colonies to appear blue, was indicative of active β-galactosidase. Thus, white colonies were screened (indicative of a disrupted *lacZα* gene and successful insertion of the bacterial PCR product). The pCR2.1-TOPO plasmid with the 16S rRNA insert was purified using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The isolated plasmid was sent for Sanger Sequencing by GenScript (GenScript USA

Inc., Piscataway, NJ). The resultant sequence was analyzed using the program LaserGene (DNASTAR Inc., Madison, WI).

WGS was performed using the MiSeq Illumina desktop sequencer (Illumina, San Diego, CA). Briefly, this method involved the random fragmentation of the full bacterial genome, followed by ligation of oligonucleotides of known sequence onto either end of each fragment. These fragments were amplified and then sequenced. The sequencing involved the repetitive addition of four reversible terminator nucleotide bases, each of which was fluorescently labeled. Once the non-incorporated nucleotides were washed away, a camera imaged the incorporated fluorescent nucleotide. The nucleotides were then chemically removed and the next cycle began. Raw sequences were processed using the open-source program Mothur, v1.31.2 (Kozich *et al.*, 2013). Paired ends were joined and contigs of incorrect length (<285 bp or >300 bp) and/or contigs that contained ambiguous bases were removed. Sequences were aligned using the SILVA database, and chimeric sequences were removed with UCHIME (Edgar *et al.*, 2011). These sequences, as well as the full 16S rRNA gene sequences from the Sanger Sequencing, were compared to an online database of known bacterial sequences (Basic Local Alignment Search Tool – BLAST) (NCBI) to look for any homology greater than 97%, which is the typical threshold for interspecies variability.

Urothelial Cell Invasion Assay

A non-malignant urothelial cell line (HURO23A) established by Dr. Phong Le (Le *et al.*, 2014) was used to assess the ability of urinary isolates to prevent the invasion of

Uropathogenic *E. coli* (UPEC) into the urothelial cells. UPEC strain *NU14* was plated onto LB agar and incubated aerobically overnight at 37°C. A single colony of UPEC was inoculated into 5mL of LB broth and incubated in the same conditions. Several subcultures of UPEC were made into new LB media (1:1000) to ensure purity. The urinary isolates (*Lactobacillus crispatus*) were grown in MRS broth and incubated in 5% CO₂ at 37°C for 48 hours. The urothelial cells were counted with a hemocytometer and diluted to 10⁵ cells/mL in tissue culture media (DMEM/F-12/5% FBS). 1 mL aliquots of urothelial cells were placed into a 24 well plate in triplicate and incubated overnight in 5% CO₂ at 37°C to promote adherence of the cells.

The details of the invasion assay protocol are found in **Figure 2**. Two experimental conditions were used. The first was a two-hour pretreatment of 10⁷ CFU/mL of the urinary isolates prior to addition of an equal concentration of UPEC. The other was a simultaneous addition of equal concentrations of the urinary isolates and UPEC. The controls were the addition of no bacteria and the addition of UPEC alone, which can invade the cells normally. After addition of the bacteria, the 24 well plate was centrifuged at 1400 RMP for 5 minutes to initiate bacteria-urothelial cell contact. The plate was incubated in 5% CO₂ at 37°C for 2 hours. The cells were then treated with 200 µg/mL Gentamicin for 2 hours to remove extracellular bacteria. Next, the cells were washed with 1 mL PBS and then treated with 1 mL 0.1% Triton-X-100 in PBS for 5 minutes. The lysed cells were serially diluted and plated on LB agar to assess the average intracellular count per well of UPEC. This

experimental setup was loosely based on a previously published protocol (Edelman *et al.*, 2003).

Competition Assays

Lawn Assay.

To assess the interactions between two microorganisms, a lawn assay was performed. An overnight culture of UPEC (strain *NU14*) was grown in TSB aerobically at 37°C. This was diluted to an OD₆₀₀ of 1.00. 1 mL of the culture was spun down at 13,000 RPM for 2 minutes, and the pellet was re-suspended in 100 µL to create a 10X concentrated culture. 50 µL of the 10X UPEC culture was added to 700 µL fresh TSB and plated on Tryptic Soy Agar (TSA). Once the lawn was completely dry, the second microorganism was spotted. The urinary isolates (*Lactobacillus*) were grown for 48 hours in MRS broth in 5% CO₂ at 37°C. A 10X concentrated culture was made in the same manner as described above. 5 µL of the 10X urinary isolate culture was added to the dry lawn of UPEC. The plates were then incubated for 24 hours in 5% CO₂. A variation of this protocol was 5 µL of the un-concentrated supernatants of the urinary isolates (**Figure 3**).

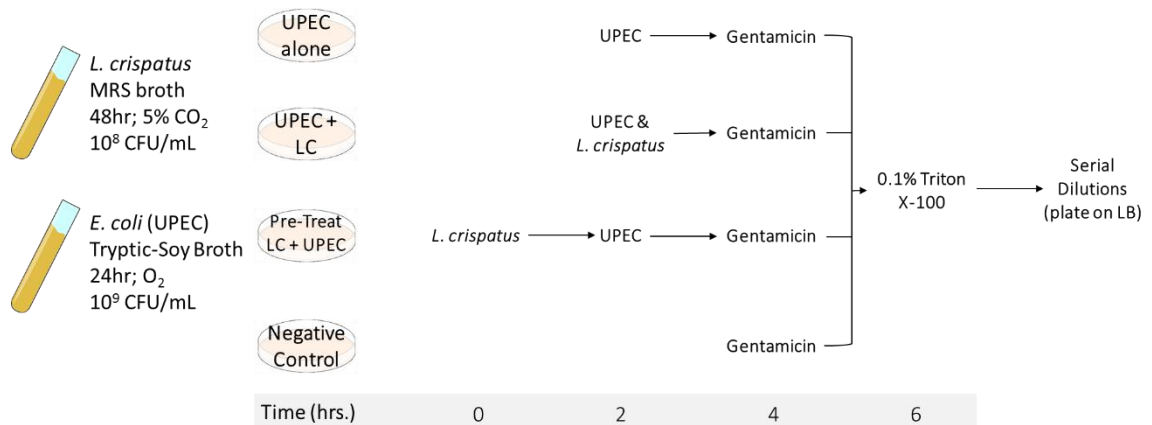


Figure 2. Protocol for the Urothelial Invasion Assay. The assay used the non-malignant urothelial cell line, HURO23A and Uropathogenic *E. coli* (UPEC), strain *NU14* and a urinary isolate of *Lactobacillus crispatus*. Four different experimental conditions were used: a positive control, UPEC alone condition; a negative control, no bacteria added condition; a pre-treatment condition with *L. crispatus* (2 hours); and a co-inoculation treatment (*L. crispatus* and *E. coli* added together). 100 µg/mL of Gentamicin was used to wash off unbound bacteria, and 0.1% Triton-X 100 was used to lyse the urothelial cells to assess internalized bacteria. Lysed cells were serially diluted and plated on LB plates and incubated aerobically for 24 hours.

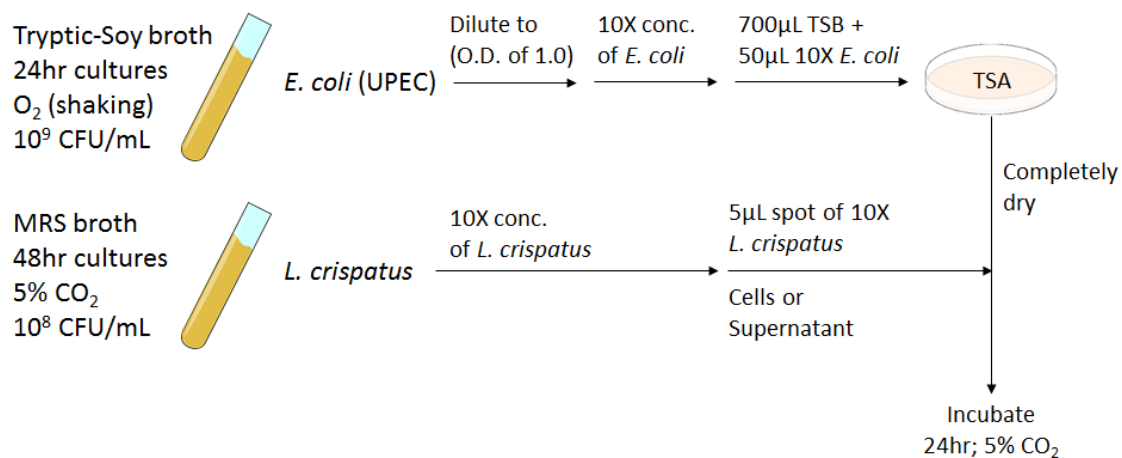


Figure 3. Protocol for the Lawn Assay. UPEC was grown in Tryptic Soy Broth (TSB) for 24 hours in aerobic-shaking conditions. The UPEC culture was diluted to an OD₆₀₀ of 1.00. 1 mL of the UPEC culture was centrifuged (10,000 RPM for 4 min), the supernatant was removed, and the pellet was resuspended in 100 μL of fresh TSB (10X concentrated solution). 50 μL of the concentrated UPEC solution was added to a Tryptic Soy Agar (TSA) plate with 700 μL fresh TSB and evenly spread on the plate. Once dry, 5 μL of a 10X concentrated solution of the urinary isolate (*L. crispatus* depicted) (10X concentration was obtained through the same method as UPEC) was spotted on to the lawn. A variation of this protocol was to use the supernatant of the urinary isolate culture. The plates were incubated in 5% CO₂ for 24 hours.

Growth Inhibition Assay.

To assess the interactions between two microorganisms in liquid culture, a growth inhibition assay was performed. An overnight culture of UPEC (strain *NU14*) was grown in TSB aerobically at 37°C. Various spent culture supernatants (SCS) of urinary isolates (*Lactobacillus* and *Gardnerella*) were filter sterilized using a Nucleopore filter with a pore size of 0.2 µm. These supernatants were added in a 1:1 ratio to the overnight cultures of UPEC and incubated aerobically at 37°C shaking. At 0, 2, and 24 hours, 1 mL of the cultures were collected, serially diluted in PBS, and were plated on TSA to assess the average CFU/mL of UPEC in response to the various supernatants. (**Figure 4**). This assay was performed in triplicate using three different strains of the various urinary isolates.

Ethidium Bromide Assay

To assess the effects of the SCS on UPEC, an Ethidium Bromide (EtBr) assay was performed. The concept behind this assay is that EtBr will bind to DNA and will fluoresce under UV light. Thus, use of EtBr can be used to assess the integrity of the envelope including the cytoplasmic membrane. If the SCS induces lysis or acts to form a pore in the membrane of UPEC, then the EtBr would be able to bind the genomic DNA and this binding can be detected via UV light. The SCS were added in a 1:1 ratio to an overnight culture of UPEC and then EtBr was added to the tube at a final concentration of 100 µmol/L for 5 minutes before washing twice with equivalent volume of PBS. As a positive control, UPEC was heat-lysed at 100°C for an equivalent amount of time as the SCS experimental

condition. As a negative control, the UPEC was exposed to the media control. (**Figure 5**). This protocol was based on the one used in Turovskiy *et al.*, 2009.

Statistical Analysis

Statistical analyses were performed using SAS software version 9.3 and SYSTAT software version 13.1. Pearson Chi-Square tests were used to compare frequency data between cohorts (**Chapters III & IV**). The Pearson Chi-Square test was used on unpaired large sample size data sets to evaluate the likelihood that an observed difference arose from random chance. Student's t-tests for independent samples (two-tailed) were performed on data from the competition assays (**Chapter V**). The Student's t-test was used on normally distributed data sets to determine if the sets are statistically different from one another. Pearson's Correlation Coefficient (Pearson's r) was used to assess correlations between the FUM and various urinary symptoms. This is a measure of the linear correlation between two variables. The Shannon Diversity Index and Simpson's Index were used to assess differences in biodiversity (alpha-diversity) between microorganism communities associated with different cohorts (**Chapters III & IV**). The Shannon Diversity Index is a measure of uncertainty in selecting the next randomly chosen microorganism. A higher Shannon Diversity score corresponds to more uncertainty, and thus higher biodiversity. The effective number of species (ENS) was calculated by taking e^x of the Shannon Diversity scores. The ENS value refers to the number of species that are equally distributed in the population. The Simpson's Index is a measure of the chance that two randomly selected microorganisms are the same species. These values can range from 0 to 1, where

a value of 0 corresponds to no diversity (a population of all the same species) and a value of 1 corresponds to infinite diversity.

Graphs were generated using Microsoft Excel 2013, Plotly™ 2015, SAS 9.3, and SYSTAT 13.1.

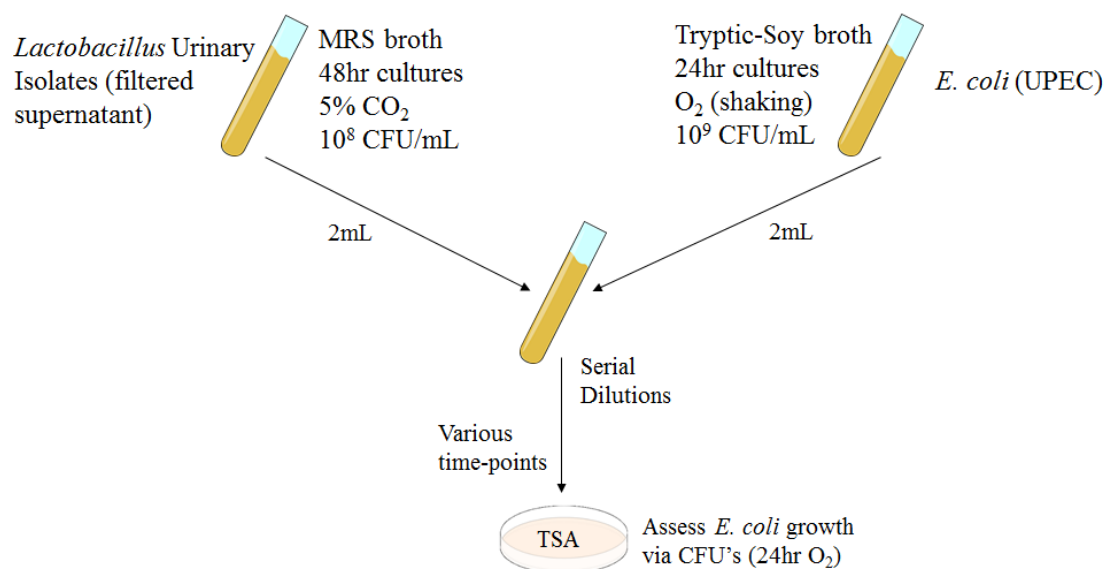


Figure 4. Protocol for the Growth Inhibition Assay. UPEC was grown in Tryptic Soy Broth (TSB) for 24 hours in aerobic-shaking conditions. The urinary isolates were grown in MRS broth for 48 hours in 5% CO₂ conditions. 2 mL of the UPEC culture were added to 2mL of the filtered supernatants of the urinary isolates and incubated in aerobic-shaking conditions for various timepoints. At each timepoint, serial dilutions were made and plated on Tryptic Soy Agar (TSA) plates. The plates were incubated in aerobic conditions for 24 hours and then assessed for UPEC growth/viability.

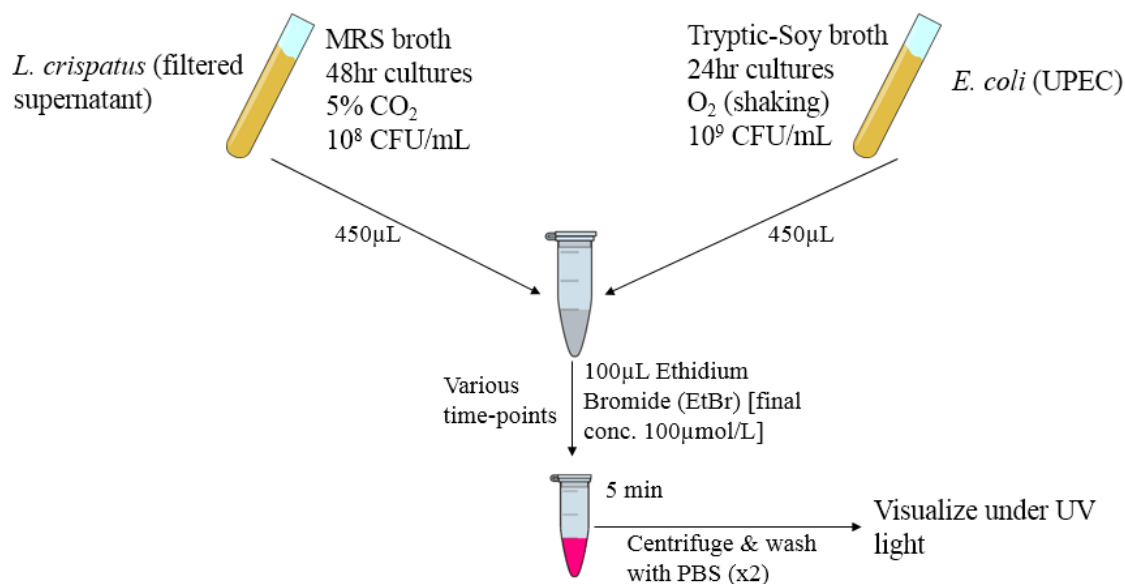


Figure 5. Protocol for the EtBr Susceptibility Assay. The figure depicts the protocol for the Ethidium Bromide (EtBr) Assay. The assay assesses Uropathogenic *E. coli* (UPEC), strain *NU14* and a urinary isolate of *Lactobacillus crispatus*. An overnight culture of UPEC was added in a 1:1 ratio with the filtered supernatant of *L. crispatus*. 100μL of EtBr (final concentration of 100μmole/L) was added to assess the integrity of the UPEC cytoplasmic membrane and visualized under UV light. As a positive control, UPEC was added in a 1:1 ratio with Tryptic-Soy Broth (TSB) and heat-lysed at 100°C. As a negative control, UPEC was added in a 1:1 ratio with the media control from *L. crispatus*, MRS broth.

CHAPTER III

THE FUM OF WOMEN WITH URINARY INCONTINENCE

Introduction & Rationale

Urinary incontinence refers to the involuntary leakage of urine. Urgency urinary incontinence (UUI) is a form of overactive bladder syndrome (OAB) that encompasses both the feelings of urgency along with involuntary leakage (Stewart *et al.*, 2003). It represents one of two subcategories of urinary incontinence; the other being Stress Urinary Incontinence (SUI), which is not a form of OAB, but rather involuntary leakage during physical exertion, such as coughing, sneezing, laughing, or exercise. While it is common for adult women affected by incontinence to have elements of both subtypes (mixed urinary incontinence), typically one subtype predominates. The etiology of SUI is understood to be a sphincteric deficiency, which likely occurs with age and can be treated effectively with surgery (Cundiff, 2004). The etiology of UUI is related to that of OAB, which is thought to be related to abnormalities in detrusor muscle regulation. One possible cause is a neurological disorder due to increased release of the neurotransmitter acetylcholine, which binds to the muscarinic receptor on the detrusor muscle surrounding the bladder, causing contractions and the feelings of urgency (Michel *et al.*, 2009). Therefore, treatment of OAB, with or without incontinence (i.e., UUI), is often via administration of anti-muscarinic drugs (Michel *et al.*, 2009). However, current UUI therapy is suboptimal, a

many affected patients have persistent symptoms despite treatment with medications and/or other UUI therapies (Santos *et al.*, 2010). This suggests that there exist etiologies outside of neruo-muscular dysfunction. Recently, our group demonstrated a correlation between the FUM and presentation of OAB symptoms with urinary incontinence (Pearce *et al.*, 2014). The FUM of UUI and non-LUTS women are distinct in species richness and abundance, as well as composition (Pearce *et al.*, 2014). Thus, it may be possible to direct therapies towards the bladder microbiota, which could in turn affect the efficacy of UUI treatment, or the symptoms themselves.

To understand the role of the FUM in LUTS, EQUC was used to assess the microbiota composition of women with the various forms of urinary incontinence, with the hypothesis that women with SUI, a physical abnormality affecting the urogenital tract, should have a FUM that is different from UUI, which is characterized by specific urinary symptoms. Thus, the FUM of women with SUI would be likely to be more similar to that of continent women than to that of women with UUI. Additionally, the FUM of women with urinary incontinence (regardless of subtype) is likely different from that of continent women. The act of excessive involuntary loss of urine creates a distinct physical urogenital environment that differs dramatically from women without urinary incontinence.

Urinary Incontinence Study

Overview of Study.

A prospective study with IRB approval was conducted between 2013 and 2015 in which women presenting to the urogynecology clinic were grouped into three categories:

UUI, SUI, and Continence. Participants' symptoms were characterized with the Pelvic Floor Distress Inventory (PFDI), a self-completed, validated symptom questionnaire (Due *et al.*, 2013). Participants also completed the Pelvic Organ Prolapse Quantification (POPQ) assessment, to quantify the degree of organ prolapse. TUC urine samples were collected from all three patient groups.

Patient recruitment for this study will ultimately total 150 women. There will be 50 women recruited into the UUI cohort, 50 women recruited into the SUI cohort, and 50 women recruited into the Continence cohort. Exclusion criteria include: women who cannot communicate or read in English, women under the age of 18, pregnant women, and women with an indwelling catheter.

The TUC urine specimens were assessed for bacterial growth using the standard urine culture protocol as well as the EQUC protocol (**Table 1**) (Hilt *et al.*, 2014). The standard urine cultures were performed by the Clinical Microbiology Laboratory staff at Loyola University Medical Center. All patient care was a consequence of the results of the standard culture alone; the EQUC results were been reported and thus did not impact patient care. In December 2014, the Clinical Microbiology Laboratory began using the modified urine culture protocol (**Table 1**). This change affected patients with the study ID's UUI50, SUI39-50, and C016-24 (patients 102-124).

Comparison of the FUM among Incontinence Subtypes.

At the time of writing (May 2015), recruitment for this study has not been completed. All data, calculations, and conclusions for this study are based on the

recruitment of the first 124 participants. These include all 50 women in the UUI cohort, all 50 women in the SUI cohort, and 24 women in the Continence cohort. This may create a bias in the data since the three cohorts are not equal.

Of the 124 TUC urine samples, 97 (78.2%) had bacterial growth. The median number of unique bacterial species per urine sample was one (IQR=1-4) for the UUI cohort, three (IQR=1-4) for the SUI cohort, and two (IQR=0-3.5) for the Continence cohort [(UUI & SUI [p=0.256], UUI & Continence [p=0.984], SUI & Continence [p=0.353]). The interquartile range (IQR) is a measure of data dispersion. Thus, in general, the women in the SUI cohort had a more diverse urinary microbiota than those in the UUI cohort. These data are summarized in **Table 2**.

Amongst the 124 urine samples, 77 different species of microorganisms were identified using MALDI-TOF MS. These species came from 5 different phyla (Actinobacteria, Firmicutes, Proteobacteria, Ascomycota, and Bacteroidetes) and 31 different genera.

The overall false negative rate for standard/modified urine culture was 85.71%. 83 of the 97 urine samples that had bacterial growth were deemed “no growth” using the routine cultures.

	UII	SUI	Continence	TOTAL
Number of samples collected	50	50	24	124
% of samples with bacterial growth	76.00%	86.00%	70.83%	78.20%
Number of unique species (cohort)	57	53	28	77
Average number of unique species (per urine)	2.6	3.3	2.6	2.9
Median number of unique species (per urine)	1	3	2	2
IQR - number of unique species (per urine)	1-4	1-4	0-3.5	1-4

Table 2. Overview of Urine Cultivation for Incontinence Study. The table lists various descriptive values for each cohort. The TOTAL column lists values for all 124 samples combined.

In terms of diversity, as previously mentioned, the SUI cohort appeared to have a more diverse FUM than the UUI cohort, with the continence cohort falling in between. However, when this is depicted graphically (**Figure 6A**), in terms of a rarefaction curve, there did not appear to be a difference between the UUI and SUI cohorts in terms of diversity. Rarefaction is a technique used to assess species richness for a group of individual samples. The curve plots the number of unique species as a function of the number of samples (patients recruited). As the curve plateaus, this corresponds to the saturation of the given population. A difference in diversity would be represented by two curves with different plateaus or trajectories.

The UUI cohort contained 57 unique species, the SUI cohort contained 53 unique species, and the Continence cohort contained 28 unique species. The curves for UUI and SUI appeared very similar, while the curve for the Continence cohort may be plateauing at a lower number of unique species, but it is not yet clear from the existing data. Statistical analyses of these data support these claims. The Shannon Diversity Index for the UUI cohort was 3.686, for the SUI cohort it was 3.670, and for the Continence cohort it was 3.126. The effective number of species for the UUI cohort was 40, for the SUI cohort it was 39, and for the Continence cohort it was 23. Together, these data show that the Continence cohort was comprised of a statistically less diverse FUM than either Incontinence cohort. The UUI and SUI cohorts appeared very similar in terms of FUM diversity.

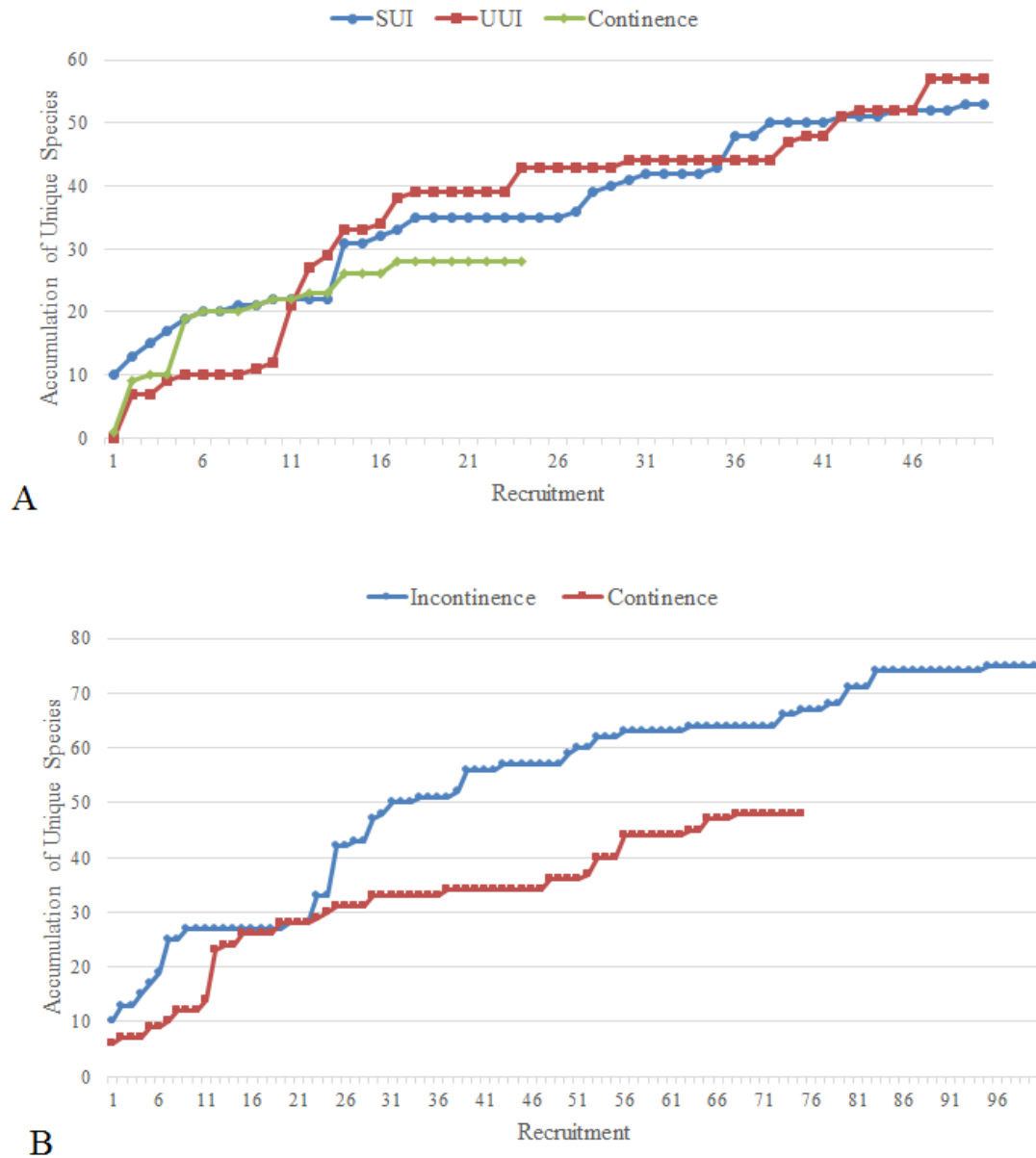


Figure 6. Rarefaction Analysis of Incontinence & Continence Cohorts. (A) The SUI cohort (N=50) contains 53 unique species and is depicted by the blue circles. The UUI cohort (N=50) contains 57 unique species and is depicted by the red squares. The Continence cohort (N=24) contains 28 unique species and is depicted by the green diamonds. (B) The Incontinence cohort (N=100) contains 75 unique species and is depicted by the blue circles. The Continence cohort (N=75) contains 48 unique species and is depicted by the red squares. The Continence cohort combines the Continence cohort from this study (N=24) and from Pearce *et al.*, 2014 (N=51).

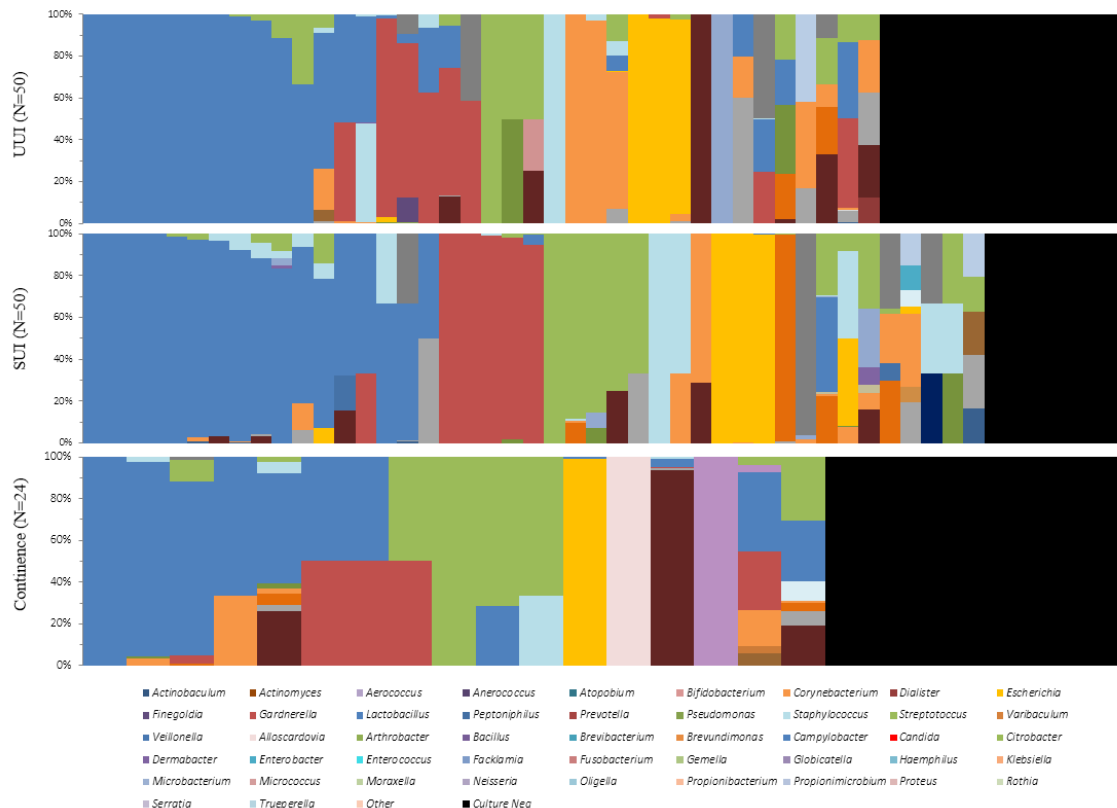


Figure 7. Microbiota Profiles of Incontinence & Continence Cohorts. Depicts the genus-level microbiota composition based on percent CFU/mL urine for the given patient urine sample. The UUI cohort (*top*) contains 50 urine samples, the SUI cohort (*middle*) contains 50 urine samples, and the Continence cohort (*bottom*) contains 24 urine samples. The figures have been stretched to be equivalent sizes for better qualitative comparisons.

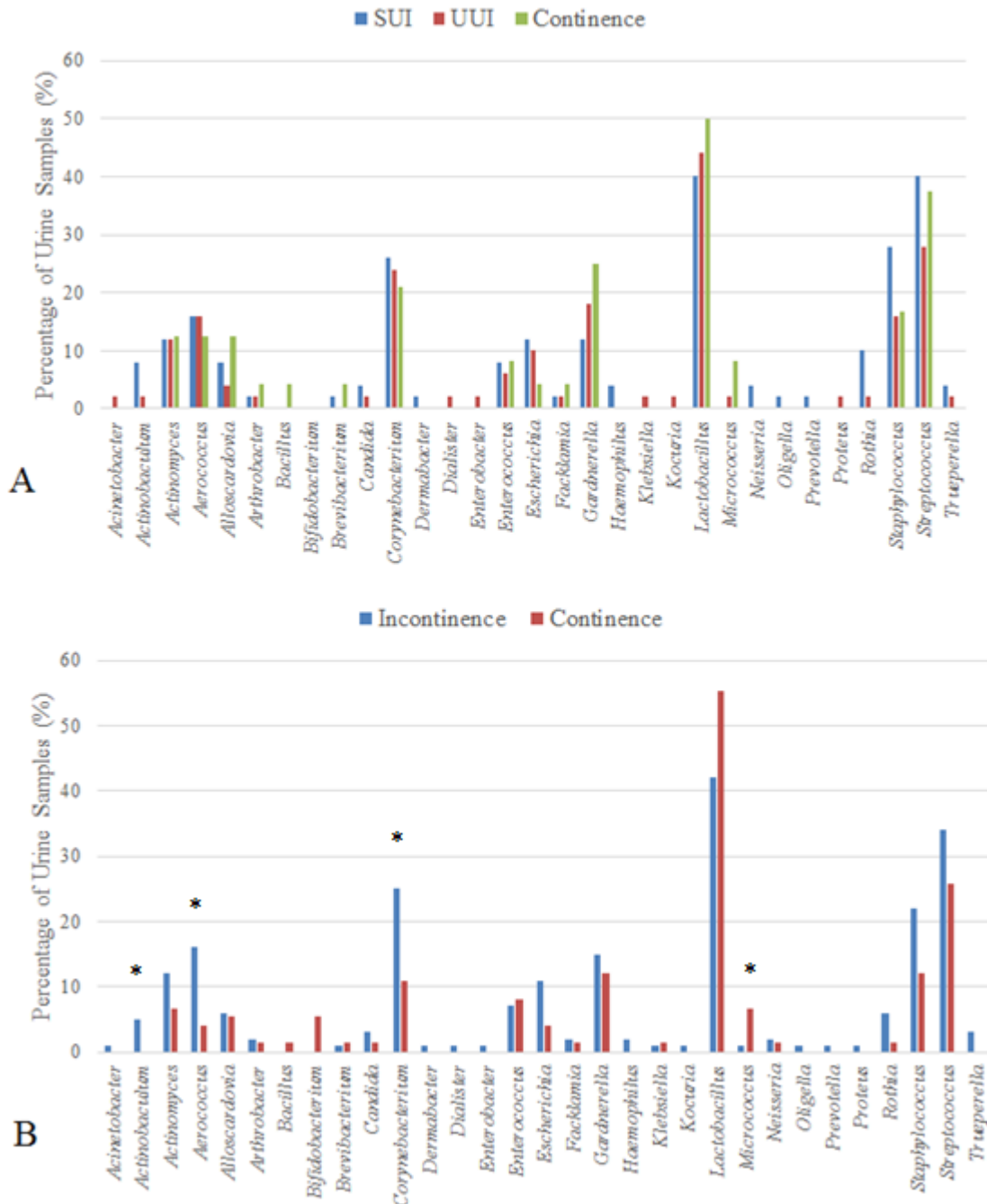


Figure 8. Frequency of Genera in Incontinence & Continence Cohort. (A) The SUI cohort (N=50) is depicted by the blue bars, the UII cohort (N=50) is depicted by the red bars, and the Continence Cohort (N=24) is depicted by the green bars. (B) The Incontinence cohort (N=100) is depicted by the blue bars, and the Continence cohort (N=75) is depicted by the red bars. Denoted are the genera that are statistically associated with either cohort, as determined by a Pearson Chi-square test for significance (* $p < 0.05$; ** $p < 0.01$)

When assessing the composition of the FUM among the cohorts, there were several noticeable differences. The microbiota profiles for the three cohorts are seen in **Figure 7**. These are graphical representations of the genus-level composition based on the CFU/mL obtained for each genus. There were more culture negative samples in the Continence cohort than either Incontinence group. The SUI cohort had the least relative amount of culture negative samples. The number of diverse samples (more than one or two dominant genera) was also greater in both Incontinence groups than the Continence cohort. There were more *Corynebacterium*-dominant samples in the UUI cohort than either the SUI or Continence cohorts. There were also no *Staphylococcus*-dominant samples in the Continence cohort. Finally, *Bacillus* and *Micrococcus*-dominant samples were only found in the Continence cohort.

In terms of frequency of detection among the urine samples of the three cohorts, there were also several trends (**Figure 8A**). At the genus level, there were higher levels of *Micrococcus*, *Lactobacillus*, and *Gardnerella* in the Continence cohort, whereas there were higher levels of *Actinobaculum*, *Staphylococcus*, *Streptococcus*, and *Rothia* in the SUI cohort. *Rothia* was noticeably absent from the Continence cohort. At the species level, *Corynebacterium minutissimum*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Micrococcus luteus* were all associated with the Continence cohort. *Corynebacterium aurimucosum*, *Actinobaculum schaalii*, *Rothia dentocariosa*, and *Rothia mucilaginosa* were all associated with the SUI cohort. Finally, *Actinomyces turicensis* was associated with the UUI cohort (**Figure S2**).

Together, these data show that the FUM is moderately distinct between all three cohorts, which suggests that certain bacterial communities correspond to specific lower urinary tract disorders or lack thereof. However, contrary to one of the initial hypotheses, the SUI cohort did not appear similar to the Continence cohort. If anything, SUI looked more like UII, especially in terms of diversity. This suggests that the FUM is distinct between urinary incontinence and continence.

Comparison of the FUM between Incontinence and Continence.

By combining the UII and SUI cohorts into a larger “Incontinence” cohort, there appeared to be larger distinctions from the Continence cohort; more so than either incontinence subgroup individually. The Incontinence cohort was comprised of 100 patients, while the Continence cohort only contained 24. To reduce bias, we balanced these numbers by adding the Continence cohort from Pearce *et al.* (2014) to these 24 patients. The 51 continent patients from Pearce *et al.* (2014) were recruited, sampled, and assessed in an identical manner as the patients from this study. The diversity between the continence groups was comparable. The Shannon Diversity Indices were 3.126 (Present Study) and 2.998 (Pearce *et al.*, 2014). **Figure 9** shows that the microbiota profiles for the two groups were also vastly similar. The relative amounts of *Lactobacillus*, *Gardnerella*, *Streptococcus*, and *Escherichia* dominant samples, as well as the amounts of culture negative samples, were nearly identical, although there were slight differences in the more diverse samples. For example, the Continence cohort in Pearce *et al.* (2014) contained *Enterococcus*, *Candida*, *Alloscardovia*, and *Micrococcus*-dominant samples, while the Continence cohort in this study contained *Bacillus* and *Micrococcus*-dominant samples.

Generally, the two groups were very comparable and were thus combined into a larger Continence cohort (N=75).

When comparing the Incontinence cohort (N=100) with the Continence cohort (N=75), we observed large differences in diversity. The median number of unique species per urine sample for the Incontinence cohort was two (IQR=1-4), while the median for the Continence cohort was one (IQR=0-2) [p=0.012]. These differences are also seen in **Figure 6B**, which depicts the data as rarefaction curves. The Incontinence cohort contained 75 unique species, whereas the Continence cohort contained 48 unique species. The curve for the Continence cohort plateaued at a much lower number of unique species than the Incontinence curve. Statistical analyses of these data supported these claims. The Shannon Diversity Index for the microorganism communities of the Incontinence cohort was 3.833, and for the Continence cohort 3.149. The ENS for the Incontinence cohort was 46 and for the Continence cohort it was 23.

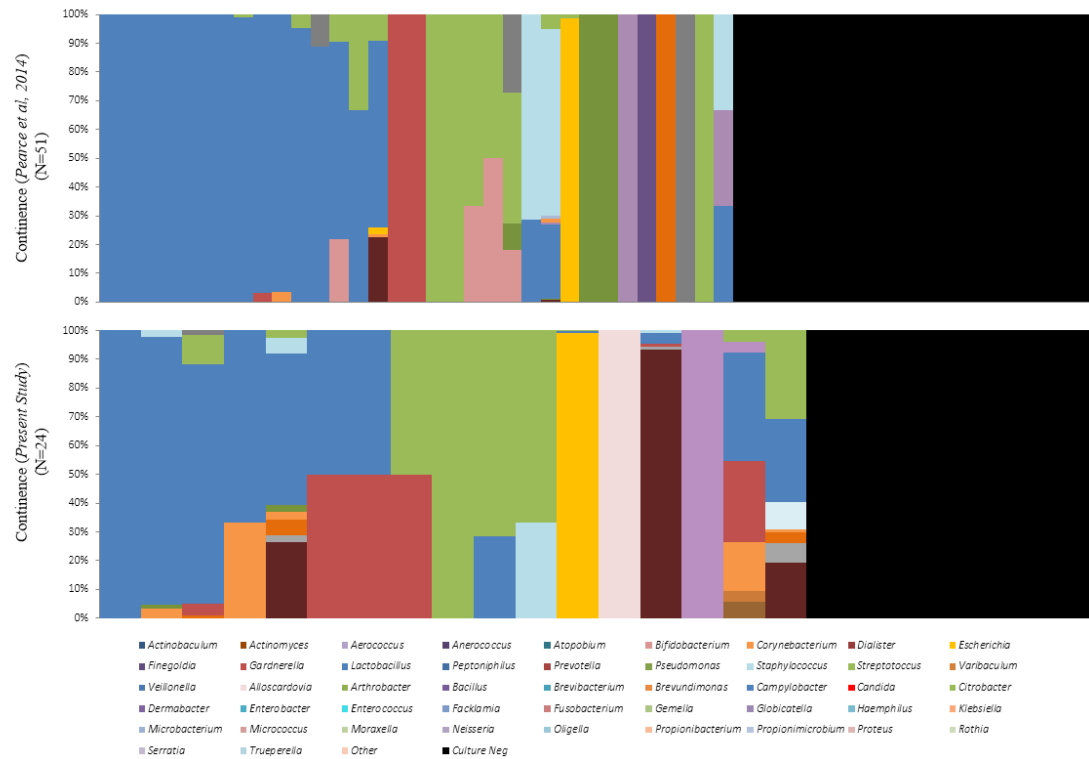


Figure 9. Microbiota Profiles of Continent Controls. The figure depicts the genus-level microbiota composition based on percent CFU/mL urine for the given patient urine sample. The Continence cohort from Pearce *et al.*, 2014 (*top*) contains 51 urine samples and the Continence cohort from the current study (*bottom*) contains 24 urine samples. The figures have been stretched to be equivalent sizes for better qualitative comparisons.

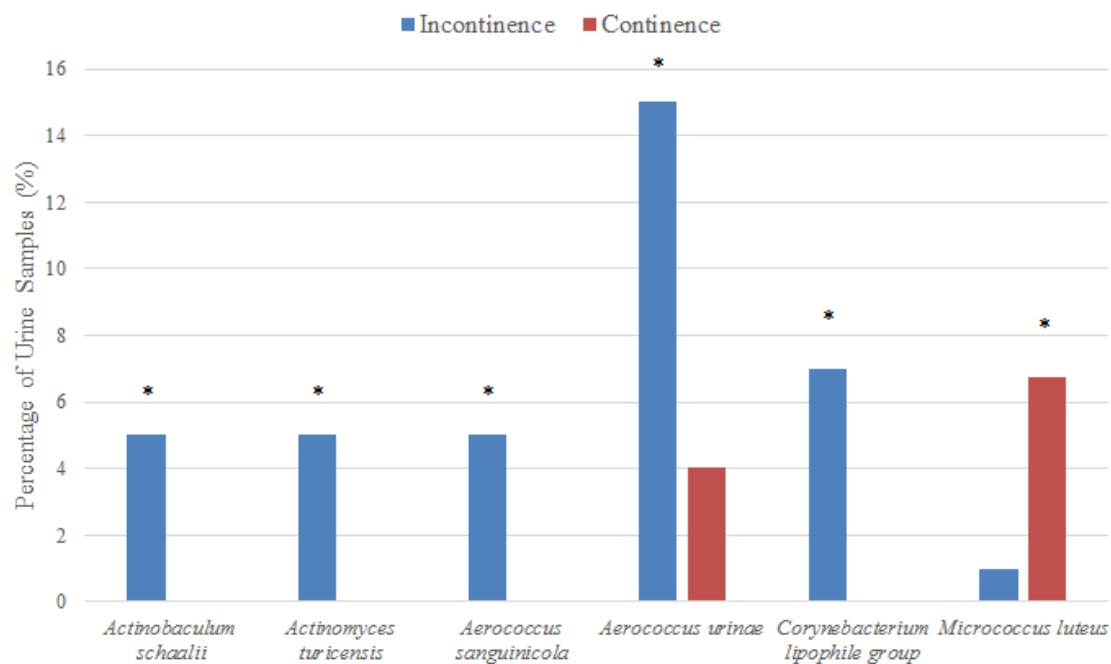


Figure 10. Frequency of Significant Species between Incontinence & Continence Cohorts. The Incontinence cohort (N=100) is depicted by the blue bars, and the Continence cohort (N=75) is depicted by the red bars. Depicted are the species that are statistically associated with either cohort. The degree of association is denoted. The values are determined by a Pearson Chi-square test for significance (* $p < 0.05$; ** $p < 0.01$).

At the genus level, in terms of microbiota profiles, the Continence and Incontinence cohorts looked quite different (**Figure S3**). About one-third of the samples in the Continence cohort were EQUC-negative, while only about 10% of the samples in the Incontinence cohort were negative. Additionally, the profiles highlighted the large differences in diversity. Generally, samples in the Incontinence cohort contained three to four dominant genera, while the Continence cohort was dominated by just one or two. There were also fewer *Gardnerella*-dominant samples in the Continence cohort, and no *Corynebacterium*-dominant samples. Interestingly, there were about equivalent numbers of *Lactobacillus*, *Streptococcus*, and *Staphylococcus*-dominant samples between the two cohorts. In terms of frequency of association, four genera were statistically associated with either cohort. The three genera that were associated with the Incontinence cohort were: *Aerococcus* [p=0.01], *Corynebacterium* [p=0.02], and *Actinobaculum* [p=0.05] (**Figure 8B**). The one genus that was associated with the Continence cohort was *Micrococcus* [p=0.04]. At the species level, six microorganisms were statistically associated with either cohort. The five microorganisms associated with the Incontinence cohort were: *Actinobaculum schaalii* [p=0.05], *Actinomyces turicensis* [p=0.05], *Aerococcus sanguinicola* [p=0.05], *Aerococcus urinae* [p=0.02], and *Corynebacterium lipophile group* [p=0.02] (**Figure 10**). MALDI-TOF MS cannot distinguish between the various species of *Corynebacterium* that fall under the category “lipophile group”. The one microorganism associated with the Continence cohort was *Micrococcus luteus* [p=0.04] (**Figure 10**). A chart of the frequencies of all the species between the cohorts is depicted in **Figure S5**. *A. schaalii*, *A. urinae*, and *A. sanguinicola* are emerging uropathogens that are commonly

associated with infection, but have not been studied extensively (Bank *et al.*, 2010, Senneby *et al.*, 2014). *A. turicensis* is commonly found on mucosal membranes and has been associated with vaginal and urinary infections (Chudackova *et al.*, 2010). Thus, it is very intriguing that many of the microorganisms associated with the incontinence group have pathogenic capabilities. Therefore, the hypothesis that these bacteria contribute to incontinence is not far-fetched. *M. luteus*, the one microorganism that is associated with the Continence cohort, may have probiotic characteristics. Metabolites produced by environmental isolates of *M. luteus* have been found to be inhibitory towards a wide range of pathogens, including several known uropathogens (Akbar *et al.*, 2014).

Overall, it seems that the FUM of women with incontinence is significantly different from continent women. The implication of this are that the microbiota may have a role in etiologies of incontinence. However, considering that there is not a dramatic difference in the FUM (in terms of diversity and composition) between the different subgroups of urinary incontinence (UUI & SUI), it may in fact be that the FUM is more of a result of the physical environment of the urogenital tract. Women with urinary incontinence are continually leaking urine, creating a wet environment that may be more suitable to the growth and colonization of a distinct group of microorganisms compared to those associated with healthy women. Whether these communities of microorganisms could then act in turn to exacerbate the urinary symptoms.

CHAPTER IV

ASSESSING THE RELATIONSHIP BETWEEN
THE FUM AND URINARY SYMPTOMS

Introduction & Rationale

With the knowledge that the FUM is significantly distinct between incontinent and continent women (this study), as well as between women with UUI and continent women (Pearce *et al.*, 2014), it is reasonable to hypothesize that these microorganisms, as a single entity, or as a complex community, can contribute to these etiologies. However, the findings that the FUM of women with UUI or SUI are rather indistinguishable (*Chapter III*) could suggest the opposite; that the FUM are more a result of the physical environment: dry or moist. Thus, these data would suggest that the FUM does not cause the various disorders, but rather is a consequence of them. Since both theories are entirely plausible, the FUM of one more group of women was assessed: women with and without UTIs. UTIs can manifest themselves through a variety of symptoms, and are known to be a direct result of the presence of microorganisms. Thus, this group of women present an ideal opportunity to test if the FUM has any correlation with specific urinary symptoms outside of current definitions for urinary infection, as opposed to simply being a consequence of a urinary disorder.

Urinary Tract Infections

Most uncomplicated UTIs occur in women (Wien, 2007). At least 50% of women will have at least one infection during their lifetime, making UTIs a significant public health concern (Wien, 2007). Furthermore, UTIs are the most common nosocomial infections, annually costing \$1.6 billion in the United States alone (Foxman, 2002). Typically, patients with an uncomplicated UTI present with symptoms that can include, but are not limited to, one or more of the following: urinary frequency and urgency, painful urination (dysuria), lower pelvic pain and/or lower back pain, new or increased urinary incontinence, malodorous urine, and blood in the urine (hematuria) (Wien, 2007, Clayson *et al.*, 2005). These symptoms, in addition to a positive standard urine culture of $\geq 10^5$ CFU/mL of known uropathogens, usually leads clinicians to treat the patient with an appropriate antibiotic regimen. Notably, some patients are given antibiotics prior to culture results based on their symptoms alone.

UTI Study

Overview of Study.

A prospective study with IRB approval was conducted between 2014 and 2015, in which women presenting to the urogynecology clinic were asked if they felt they had UTI symptoms and were separated into two groups based on their responses: “YES” and “NO”. Both patient populations completed a brief demographics questionnaire, as well as the validated UTISA questionnaire (Clayson *et al.*, 2005), to assess the degree of severity and bother of various common UTI symptoms. TUC urine samples were collected from both

groups. Additionally, the YES cohort was asked to complete a follow-up UTISA questionnaire by phone, 1 to 2 weeks after the initial visit, as an assessment of whether their clinical treatment resolved, or did not resolve, their symptoms. A schematic outline of the study design is depicted in **Figure 11**.

Patient recruitment for this study will be a total of 150 women. There will be 75 women recruited into the YES cohort and 75 women recruited into the NO cohort. Exclusion criteria include: women who cannot communicate or read in English, women under the age of 18, pregnant women, and women with an indwelling catheter.

The TUC urine specimens that are collected from all 150 women will be assessed for bacterial growth using the standard urine culture protocol as well as the expanded EQUIC protocol (**Table 1**). The expanded EQUIC protocol adds MacConkey agar and Campy gas mixture to the original EQUIC protocol (Hilt *et al.*, 2014). MacConkey agar is ideal for the growth of typical uropathogens, and the Campy gas mixture provides an optimal environment for the isolation of fecal contaminant microorganisms.

The standard urine culture was performed by the Clinical Microbiology Laboratory staff at Loyola University Medical Center. All patient care was a consequence of the results of the standard culture alone; the expanded EQUIC results have not been reported and thus have not impacted patient care. In December 2014, the Clinical Microbiology Laboratory began using the modified urine culture protocol (**Table 1**). This change has affected patients with study IDs 108-118.

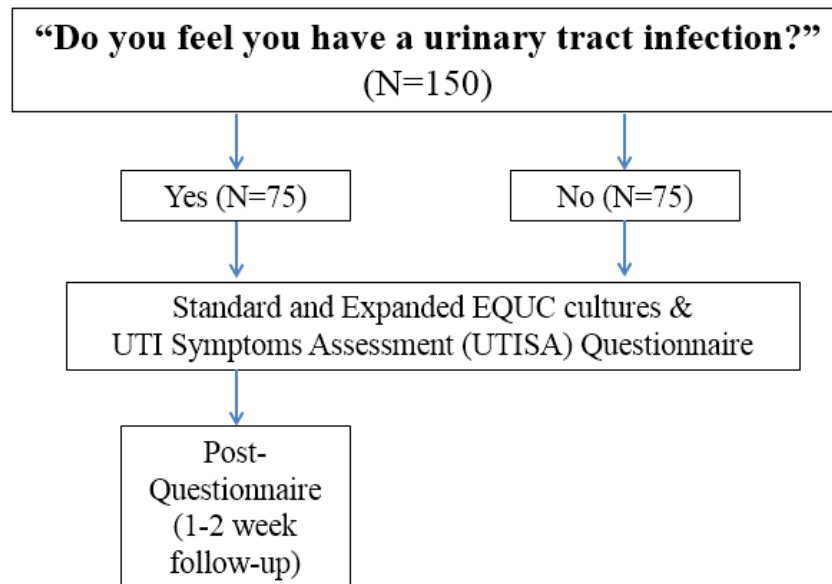


Figure 11. Flow Chart for the UTI Study. Schematic outline of the study design.

This study serves an additional purpose, which is documented in *Appendix A*. The use of three volumes of urine for the expanded EQUUC is intended to determine an optimal urine culture protocol for recommendation to the clinical microbiology community as a better method for detecting clinically relevant microorganisms (uropathogens). Thus, for this study, the data obtained from all three urine volumes of the expanded EQUUC protocol were combined. Additionally, all reported data were taken strictly from the 48 hour timepoint.

After 48 hours of incubation in the respective environmental conditions, the various plates were documented for morphologies and sub-cultured. The pure cultures were identified using MALDI-TOF MS, and were stored at -80°C for future study.

Comparison of the YES and NO Cohorts.

At the time of writing (May 2015), recruitment for this study has not completed. Thus, all data, calculations, and conclusions for this study are based on the recruitment of the first 118 women. This recruitment includes all 75 women in the NO cohort and 43 women in the YES cohort. This may create a bias in the data since the two cohorts are not equal.

Of the 118 TUC urine samples, 108 (91.5%) contained viable bacteria. The median number of unique bacterial species per urine sample was two (IQR=1-3) for the YES cohort, and three (IQR=1-5) for the NO cohort [$p=0.067$]; thus, on average, the women in the YES cohort had a less diverse urinary microbiota than those in the NO cohort. These data are summarized in **Table 3**.

	YES	NO	TOTAL
Number of samples collected	43	75	118
% of samples with bacterial growth	93.02%	89.33%	91.50%
Number of unique species (cohort)	42	75	84
Average number of unique species (per urine)	2.5	3.5	3.1
Median number of unique species (per urine)	2	3	2
IQR - number of unique species (per urine)	1-3	1-5	1-4

Table 3. Overview of Urine Cultivation for the UTI Study. The table lists various descriptive values for each cohort. The TOTAL column lists values for all 118 samples combined.

Amongst the 118 urine samples, 84 different species of microorganisms were identified using MALDI-TOF MS. These species represent 4 different phyla (Actinobacteria, Firmicutes, Proteobacteria, and Ascomycota) and 31 different genera.

The overall false-negative rate for standard/modified urine culture was 70.37%. 76 of the 108 urine samples that had bacterial growth were deemed “no growth” by the standard culture protocol. For the YES cohort alone, the false-negative rate was 35.00%; for the NO cohort, it was 92.65%.

The diversity, genus level microbiota composition, and species level composition differ greatly between the YES and NO cohorts. As previously mentioned, the YES cohort appeared to have a less diverse FUM than the NO cohort. This is depicted graphically in **Figure 12** in the form of a rarefaction curve. This analysis assesses the species richness for a group of individual samples, and the data are plotted as the number of unique species as a function of the number of patients recruited. The plateau of the curve corresponds to the saturation of the given population. A difference in diversity would be represented by two curves with different plateaus. This appears to occur for these data. The NO cohort contained a bacterial population of 75 unique species and the YES cohort contained 42 unique species. The YES cohort appeared to plateau at a lower number of unique species than the NO cohort, inferring less diversity. Statistical analyses of these data support the claim of different diversities. The Shannon Diversity Index for the YES cohort was 3.339 and for the NO cohort it was 3.892. The ENS for the YES cohort was 28, and for the NO cohort it was 49. Thus, the bacterial population of the NO cohort was comprised of a more diverse FUM than the YES cohort and this difference was statistically significant.

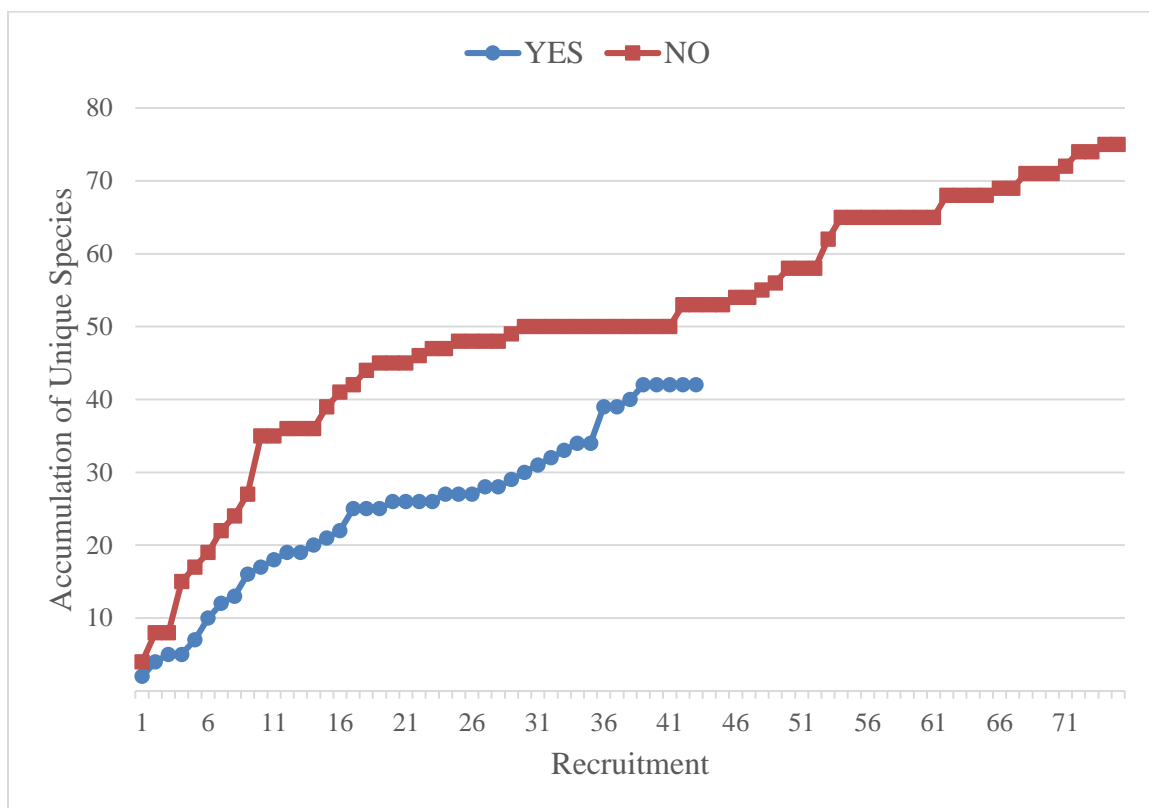


Figure 12. Rarefaction Analysis of YES & NO cohorts. The YES cohort (N=43) contains 42 unique species and is depicted by the blue circles. The NO cohort (N=75) contains 75 unique species and is depicted by the red squares.

In addition to diversity, the YES and NO cohorts also differed in their microorganism composition. The microbiota profiles for the two groups are seen in **Figure 13**. These are graphical representations of the genus-level composition based off of the CFU/mL of each genus. The NO cohort contained more *Lactobacillus*, *Corynebacterium*, and *Streptococcus*-dominant samples, while the YES cohort contained more *Escherichia* and *Klebsiella*-dominant samples, and notably no *Gardnerella*-dominant samples. The NO cohort, however, contained more EQU-negative dominant samples. Additionally, the microbiota profiles highlight the difference in overall diversity between the two cohorts. On average, samples in the NO cohort contained 3 to 4 dominant genera per urine sample, while samples in the YES cohort only contained 1 to 2.

In terms of frequency of detection among urine samples, *Streptococcus* [p=0.038] and *Gardnerella* [p=0.047] were statistically associated with the NO cohort, while *Escherichia* [p<0.001] was strongly statistically associated with the YES cohort. These frequencies are depicted in **Figure 14**. Other trends, though non-statistically significant, included: *Actinobaculum* [p=0.084], *Corynebacterium* [p=0.103], and *Actinomyces* [p=0.128] associated with the NO cohort, and *Klebsiella* [p=0.113] associated with the YES cohort. These frequencies represent similar conclusions made by assessment of the microbiota profiles in **Figure 13**.

Four species were statistically associated with either cohort. The three species that were associated with the NO cohort were: *Gardnerella vaginalis* [p=0.047], *Streptococcus parasanguinis* [p=0.027], and *Streptococcus mitis/oralis/pneumoniae* [p=0.041]. MALDI-TOF MS cannot distinguish between the species *S. mitis*, *S. oralis*, and *S. pneumoniae*

confidently, and were thus grouped together for these analyses. The one species associated with the YES cohort was *Escherichia coli* [$p < 0.001$]. These values are depicted in **Figure 15**. A chart of the frequencies of all the species between the cohorts is depicted in **Figure S7**. It is not surprising that *Escherichia coli* was strongly associated with the YES cohort as it is the most common cause of UTIs (Nicolle, 2008). *Streptococcus parasanguinis* is associated with healthy microflora in the oral cavity (Corby *et al.*, 2005). *Streptococcus pneumoniae* is the causative agent of Pneumococcal Infection, which is responsible for a large percentage of community-acquired pneumonia as well as bacteremia and bacterial meningitis (Verma *et al.*, 2012). Again, whether the isolates found in these urine sample are *S. pneumoniae* is unclear. *Streptococcus mitis* and *oralis* are commonly found in the oral cavity and have a slight association with endocarditis (Lamas *et al.*, 2003, Marsh *et al.*, 1999). It is interesting that *Gardnerella vaginalis* was associated with the NO cohort, as it is a known biomarker for Bacterial Vaginosis (BV), a dysbiosis of the vaginal tract (Eschenback, 2007). This findings reinforce that the women in the NO cohort are not entirely devoid of lower urinary tract symptoms. It is possible that the role of *G. vaginalis* in the bladder may be distinct from its role in the vagina. For example, *G. vaginalis* was found to be statistically associated with women with UUI (Pearce *et al.*, 2014), and many of the women in the NO cohort have incontinence.

Taken together, these data further support the claims made in **Chapter III** that the FUM is distinct between various LUTS. It seems that the FUM is not only distinct in terms of diversity and composition between women with and without incontinence, but also between women who do and do not feel they have a UTI based upon their symptoms.



Figure 13. Microbiota Profiles of YES & NO Cohorts. Depicts the genus level microbiota composition based on percent CFU/mL urine for the given patient urine sample. The NO cohort (*top*) contains 75 urine samples and the YES cohort (*bottom*) contains 43 urine samples. The figures have been stretched to be equivalent sizes for better qualitative comparisons.

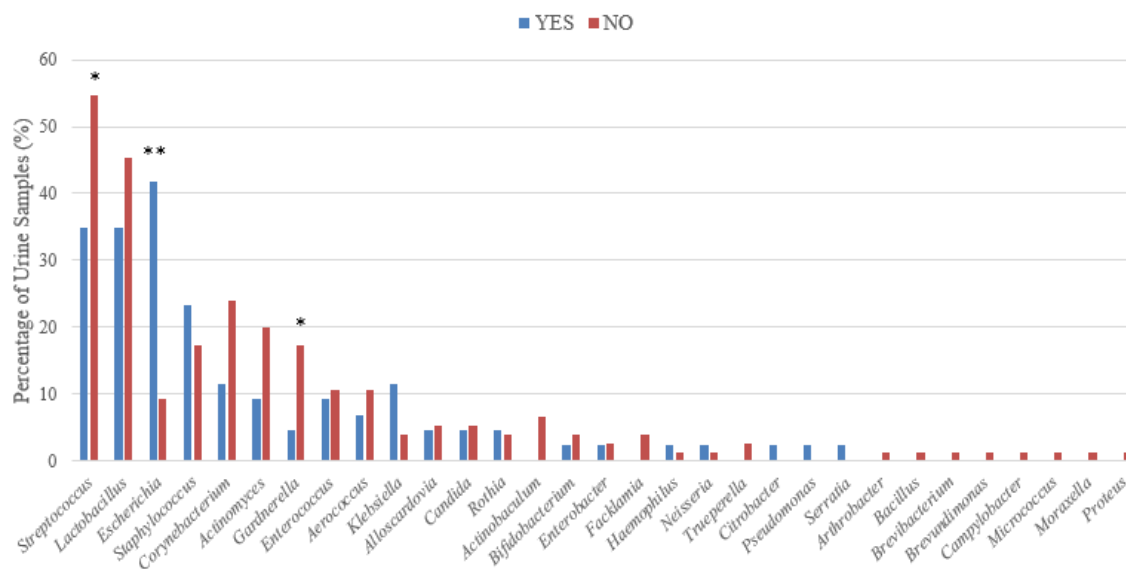


Figure 14. Frequency of Genera in YES & NO Cohorts. The YES cohort (N=43) is depicted by the blue bars, and the NO cohort (N=75) is depicted by the red bars. Denoted are the genera that are statistically associated with either cohort, as determined by a Pearson Chi-square test for significance (* $p < 0.05$; ** $p < 0.01$).

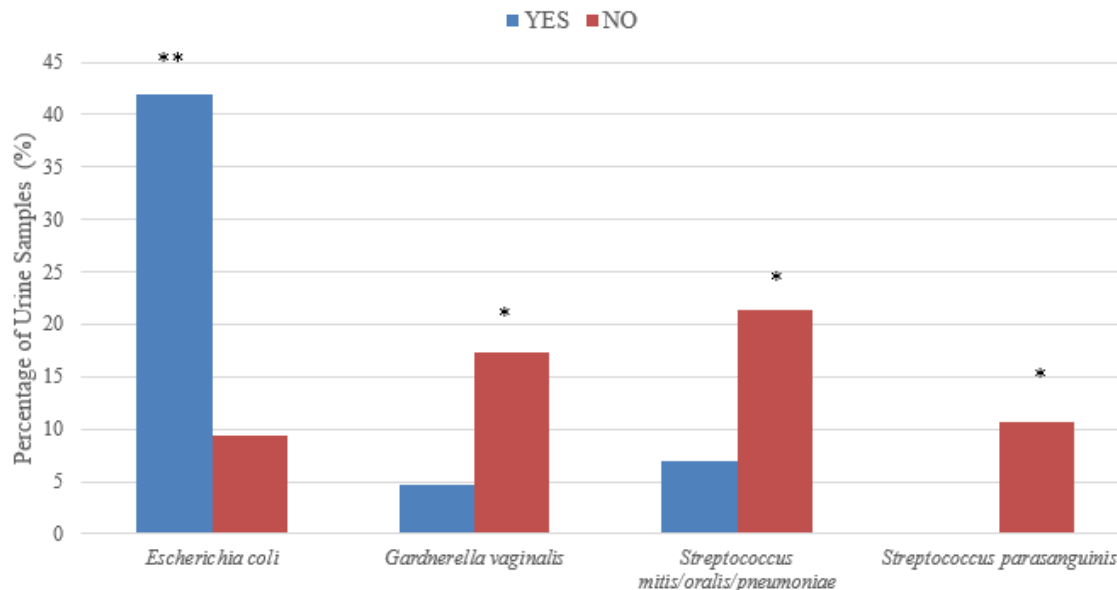


Figure 15. Frequency of Significant Species between YES & NO Cohorts. The YES cohort (N=43) is depicted by the blue bars, and the NO cohort (N=75) is depicted by the red bars. Depicted are the species that are statistically associated with either cohort. The degree of association is denoted. The values are determined by a Pearson Chi-square test for significance (* $p < 0.05$; ** $p < 0.01$).

FUM and Symptom Correlations.

The validated UTISA questionnaire is a self-administered questionnaire that asks the patient to rate the degree of severity and bothersome on a scale of 0 to 3, for seven classic UTI symptoms. These seven symptoms can be grouped into four broader symptom domains termed urination regularity, problems with urination, pain associated with UTI, and blood in the urine. The average domain scores can range from 0 to 12, except Blood in the Urine, which can range from 0 to 6. These groupings are detailed in **Figure 1**.

To make appropriate correlations between the UTISA symptoms and the FUM, we must first verify that the NO cohort is acting as an appropriate negative control. We would expect that the women in the YES cohort give higher scores on the UTISA questionnaire than the NO cohort, which is indeed the case (**Figure 16**). The average scores for urination regularity were: YES cohort – 8.07 and NO cohort – 5.35 [$p=0.0008$]. The averages for problems with urination were: YES cohort – 5.37 and NO cohort – 1.99 [$p<0.00001$]. The averages for pain associated with UTI were: YES cohort – 4.28 and NO cohort – 2.13 [$p=0.0012$]. The average scores for blood in the urine were: YES cohort – 0.35 and NO cohort – 0.11 [$p=0.088$]. The UTISA scores for three of the four domains were statistically significant ($p<0.05$) using a two-tailed t-test for independent samples. These scores were then compared to the average scores from the original UTISA validation study (Clayson *et al.*, 2005) (**Figure 16**). This study validated the UTISA questionnaire on 267 women, all of whom had a UTI at greater than 10^3 CFU/mL of a uropathogen(s) (Clayson *et al.*, 2005). If the current study is similarly valid, then the scores for the YES cohort should be much similar to the scores of Clayson *et al.*, 2005, while the scores for the NO cohort should be

much different. **Figure 16** shows that this is the case. Therefore, the NO cohort acts as an appropriate control for this study.

To assess whether there are correlations between the FUM and individual urinary symptoms, the data were analyzed for differences in diversity, genus-level composition, species-level composition, and community structure. **Figure 17** is an assessment of the difference in diversity of the urinary microbiota among the seven urinary symptoms. A patient is included in the symptom grouping if she gave a non-zero severity or bother score for that symptom. The Frequency of Urination cohort (N=90) contains 78 unique species, the Urgency of Urination cohort (N=89) contains 75 unique species, the Pain or Burning During Urination cohort (N=37) contains 52 unique species, the Urinary Retention cohort (N=61) contains 68 unique species, the Pressure in Lower Abdomen cohort (N=52) contains 64 unique species, the Low Back Pain cohort (N=40) contains 50 unique species, and the Blood in Urine cohort (N=10) contains 20 unique species. There appear to be no differences in diversity between any of the seven symptoms, as their trajectories appear similar in **Figure 17**.

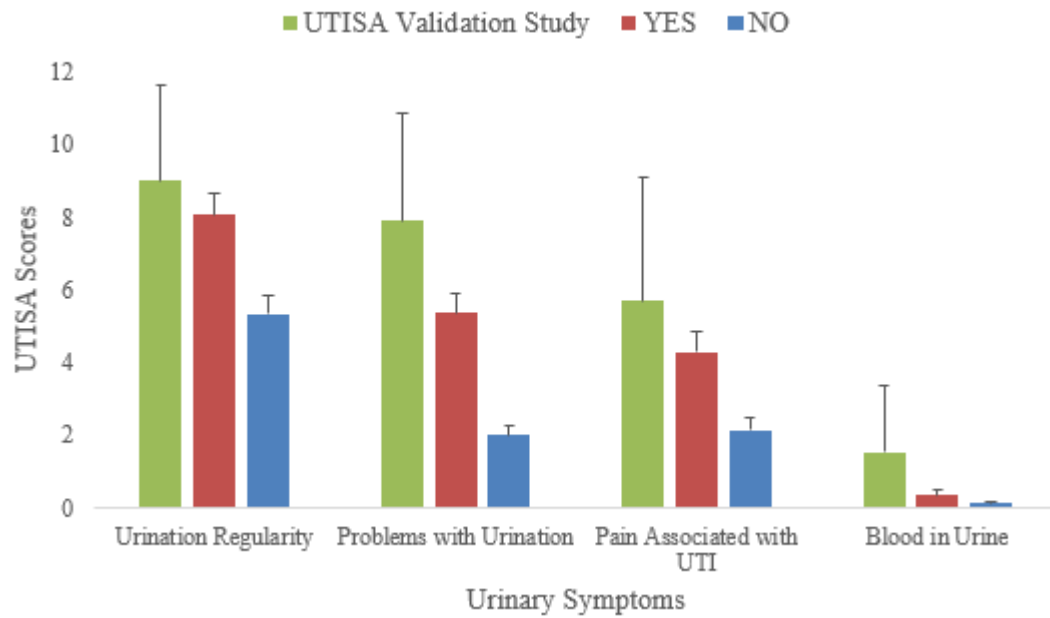


Figure 16. Average UTISA Scores for YES & NO Cohorts. The average scores for the four symptom domains for the YES (red bars) and NO (blue bars) cohorts, compared to those from the UTISA Validation Study (Clayson *et al.*, 2005) (green bars). Scores for Urination Regularity, Problems with Urination, and Pain Associated with UTI range from 0-12. Scores for Blood in the Urine range from 0-6.

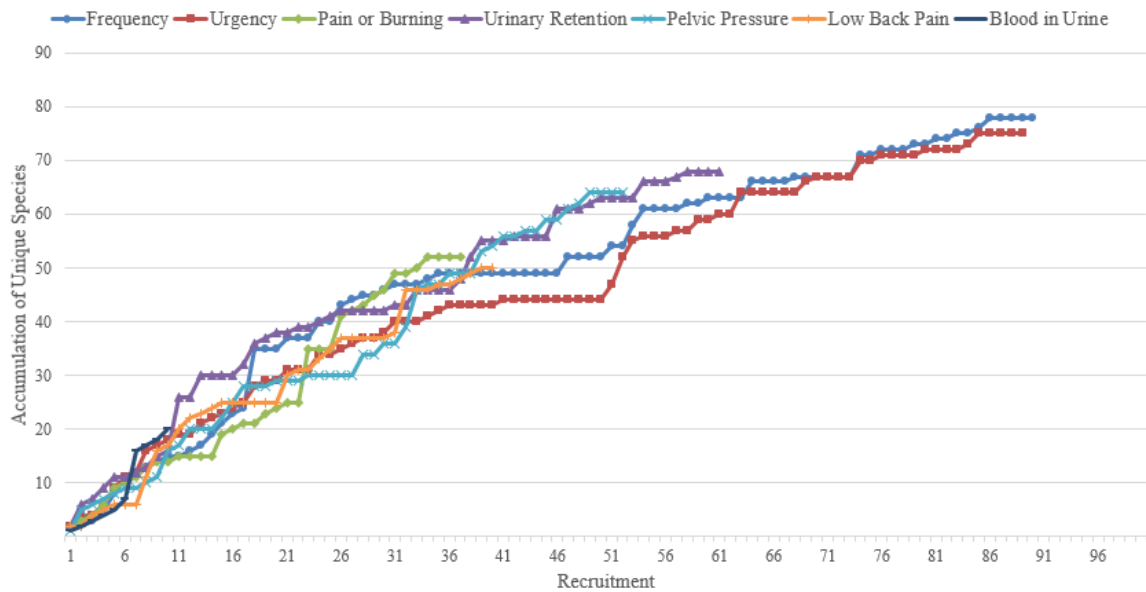


Figure 17. Rarefaction Analysis of UTISA Symptoms. The Frequency of Urination cohort (N=90) contains 78 unique species and is depicted by the blue circles. The Urgency of Urination cohort (N=89) contains 75 unique species and is depicted by the red squares. The Pain or Burning during Urination cohort (N=37) contains 52 unique species and is depicted by the green diamonds. The Urinary Retention cohort (N=61) contains 68 unique species and is depicted by the purple triangles. The Pelvic Pressure cohort (N=52) contains 64 unique species and is depicted by the blue x's. The Low Back Pain cohort (N=40) contains 50 unique species and is depicted by the orange pluses. The Blood in Urine cohort (N=10) contains 20 unique species and is depicted by the dark blue dashes.

Statistical analyses of these data support the claim of no difference in diversity. The Shannon Diversity Index for Frequency was 3.858, Urgency was 3.833, Pain and Burning was 3.678, Urinary Retention was 3.855, Pelvic Pressure was 3.847, Low Back Pain was 3.567, and Blood in Urine was 2.932. Thus, there appear to be no large differences in the diversity in the FUM of women presenting with each symptom. However, there are some limitations to this conclusion. First, the degree of severity and bother are not considered here, and neither is the CFU/mL of each cultured microorganism. The CFU/mL could play a large role in symptoms. This is discussed more in **Appendix A**, although the average total CFU/mL for urine samples included in each of the seven symptoms does not differ dramatically among the symptoms. Also, most women present with multiple symptoms at once; therefore, the values for diversity may be skewed as significant overlaps exist.

In terms of the composition of the urinary microbiota among the seven urinary symptoms, there did not appear to be any large differences in the genus-level composition of each symptom (**Figure 18**). These data are presented in terms of average relative abundance, which was calculated by averaging the percent CFU/mL for each genera across the seven symptom groups. These data are essentially a summary of the microbiota profiles. While no large differences were noticeable, there were some subtle differences between symptoms. For example, there were more *Escherichia* and *Klebsiella* in the Pain and Burning symptom group relative to the other six symptoms. There were also fewer *Lactobacillus* and *Gardnerella*. There were fewer *Staphylococcus* and more *Corynebacterium* in the Pelvic Pressure symptom group relative to the other symptoms.

Frequency, Urgency, and Urinary Retention looked similar in their average relative abundances of all the genera, while Blood in the Urine and Low Back Pain looked much different from the other symptoms. Overall, there were minor differences in the average relative abundances of the various genera among the seven symptoms, but no major distinctions.

At the species level, however, there were noticeable differences among the seven urinary symptoms. **Figure 19** shows that all seven symptoms had a unique microorganism profile associated with them. These data are presented in the form of a heat map with associations displayed as “degree of association”. Thus, **Figure 19** assesses how likely it is for a given microorganism to be cultured from the urine of someone with the corresponding urinary symptom. These data again do not take into consideration the degree of severity or bother. An “association” is interpreted to be any non-zero score for either severity or bother. In **Figure 19**, a degree of association of 1 (dark blue) is interpreted to mean that the microorganism is always cultured from the urine of someone with the corresponding symptom. A degree of association of 0 (pale yellow) infers the opposite. Both axes of the heat map are clustered using the appropriate Dendrogram, which is formed using Euclidean distance.

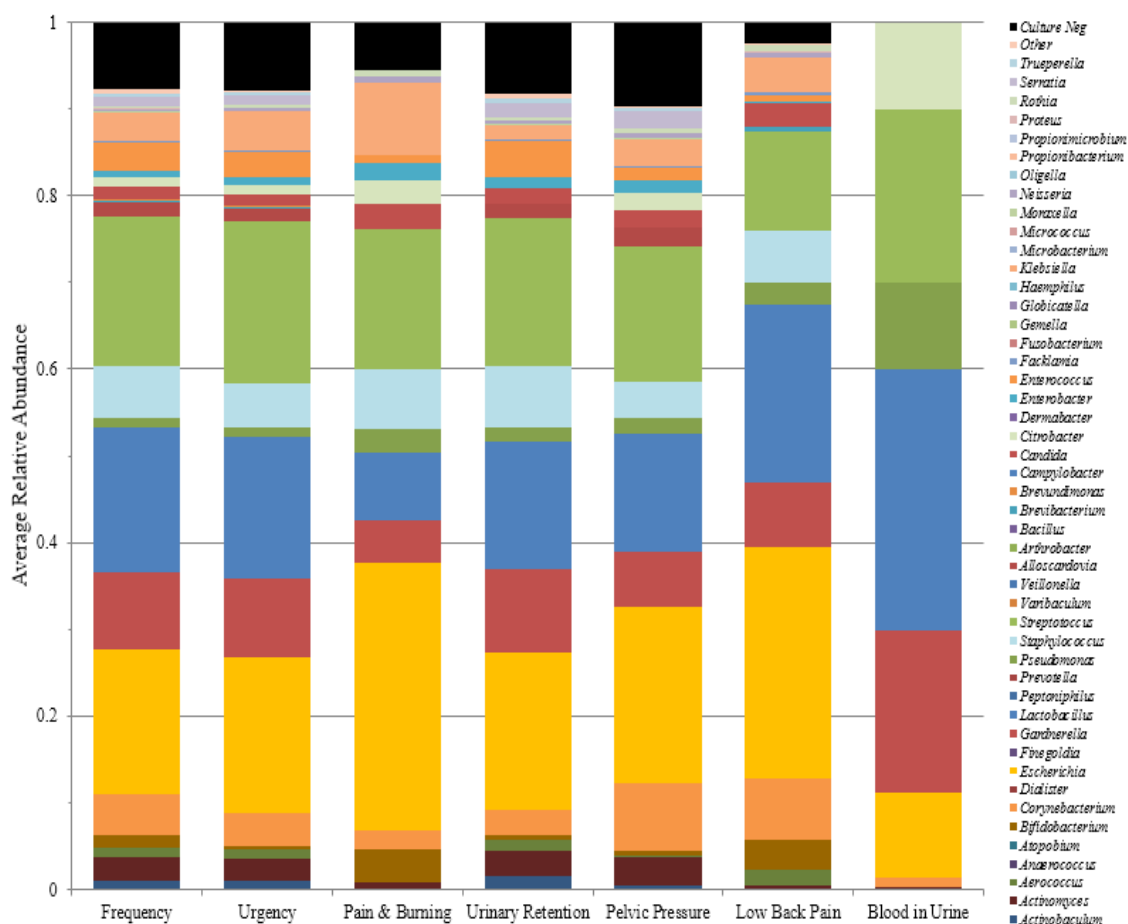


Figure 18. Average Relative Abundances of Genera for UTISA Symptoms. Average relative abundances are calculated by averaging the percent CFU/mL of each genera found in all patients included in that symptom group and dividing by 100. Frequency cohort (N=90), Urgency cohort (N=89), Pain & Burning cohort (N=37), Urinary Retention cohort (N=61), Pelvic Pressure (N=52), Blood in Urine (N=10).

From these data (**Figure 19**), three distinct populations appear. On the bottom section of the heat map are the microorganisms that are strongly associated with several symptoms. Many of these microorganisms include known uropathogens (e.g. *Citrobacter koseri*, *Pseudomonas aeruginosa*, *Candida albicans*), but other organisms are also included (e.g., *Bifidobacterium breve*). In the middle section of the heat map are the microorganisms that are moderately associated with several of the symptoms. These organisms (including *Escherichia coli*, *Gardnerella vaginalis* and several species of *Lactobacillus*) may be interpreted to be biomarkers for those symptoms, part of the normal flora, or actual causes of the symptoms. The role of these organisms is currently unclear. The top section of the heat map are the microorganisms that are strongly associated with a few symptoms. Many of these microorganisms are also known uropathogens, but several of them have also only been cultured once or twice. Therefore, for these organisms, the values for the degree of association may be skewed.

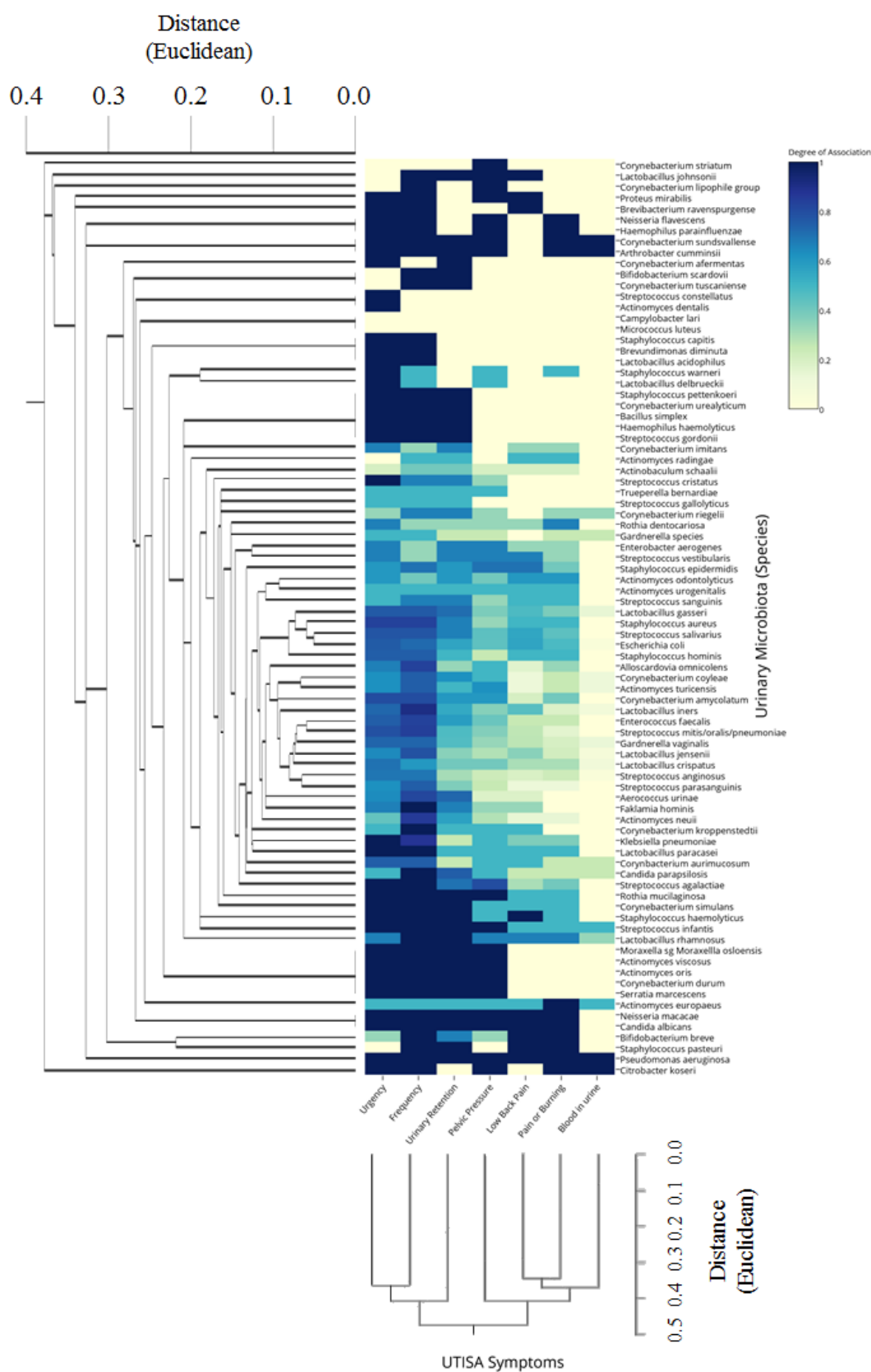


Figure 19. Clustered Heat Map of Associations between Urinary Microbiota and UTISA Symptoms. Associations are displayed as “degree of association” - how likely is it for a given organism to be cultured from the urine of someone with the corresponding urinary symptom (regardless of score). Both axes are clustered using the corresponding Dendrogram, which is measured in terms of Euclidean distance.

To help address some of the issues raised by **Figure 19**, **Figure 20** presents the same data in terms of correlation, also in the form of a heat map. A Pearson's r Correlation value was calculated for each microorganism-symptom pairing, and then clustered using a Dendrogram based off of Euclidean distance. These correlation values assess the linear correlation between presence of a particular urinary symptom and presence of the corresponding microorganism. The values can range from -1 to 1, where negative values denote negative correlations, and positive values denote positive correlations. For this data set, correlation values greater than 0.181 or less than -0.181 are statistically significant ($p < 0.05$). This figure is asking a slightly different question than **Figure 19**. In **Figure 19**, the associations were based off of the likelihood of someone with a particular *microorganism* in their urine sample having the corresponding *symptom*. Whereas **Figure 20** asks what is the likelihood (in terms of correlation) of someone with a particular *symptom* having the corresponding *microorganism* in their urine sample. Thus, the two figures are comparable as a whole, but individual values are not. The clustering of **Figure 20** also resulted in several distinct groupings. There are several organisms that appeared red or blue across all of the seven symptoms. These microorganisms were either positively (blue) or negatively (red) associated with the urinary symptoms as a whole; those that were positively associated with symptoms (red) could have a protective effect on urinary health,

whereas those negatively associated (blue) could cause symptoms or promote the colonization of microorganisms that cause symptoms. Several microorganisms appeared grey across the seven urinary symptoms. These microorganisms could be part of the normal flora of the bladder, but do not significantly influence symptoms. They are likely involved in maintaining a degree of homeostasis. Interestingly, not all of the uropathogens appeared entirely blue across the seven symptoms. Some, like *E. coli*, tended to have strong positive correlations with selected symptoms, while others (e.g. *Streptococcus anginosus*) actually appear to be negatively correlated with all symptoms.

A factor that may be impacting these results is the CFU/mL with which the microorganisms were cultured. For example, *Enterococcus faecalis* (a Gram-positive uropathogen) appeared to exhibit strong distinctions in the degree of associations with the various UTISA symptoms when the data is broken up in terms of CFU/mL (**Figure 21**). If the CFU data is broken up into four groups (10, 11-99, 100-999, and ≥ 1000), there is a trend that the higher the CFU/mL of *E. faecalis*, the greater the degree of association is with several of the symptoms. Thus, the data from **Figures 19 & 20** may be strongly impacted by considering the CFU data. However, this assumption is based solely upon the data for one of the 84 different microorganisms that have been cultured from this patient population.

A final way to assess the correlations between the FUM and the urinary symptoms is to mathematically analyze the variances among the symptoms. **Figure 22** is a principle component analysis (PCA) of the UTISA symptoms in terms of their degree of association with the FUM. These PCA plots are calculated by maximizing the potential variance

among the variables and then plotting these values. The various components represent the total possible variance for the group of variables. The first component, PC1, calculates the highest possible variance, while the second component, PC2, calculates the next highest possible variance, and so on. In **Figure 22A**, PC1 is plotted against PC2. PC1 accounted for 35.45% of possible variance, and PC2 accounted for 17.45% of possible variance, for a total of 51.80%. This plot allowed for mild separation of the data. In contrast, **Figure 22B**, which depicts PC2 plotted against PC3 (16.25%) for a total of 32.70% possible variance, showed a much larger separation of the data. This analysis shows that the urinary symptoms are distinct in their associations with the FUM, but the degree to which they are distinct can vary greatly. **Figure 22C** plots PC1, PC2, and PC3 for a three dimensional look at the variables. This plot accounted for 68.05% of the possible variance.

Collectively, these data are strongly suggestive that the species-level FUM is directly involved in these individual symptoms and thus may be involved in the etiologies of various lower urinary tract disorders that are defined by the particular symptoms.

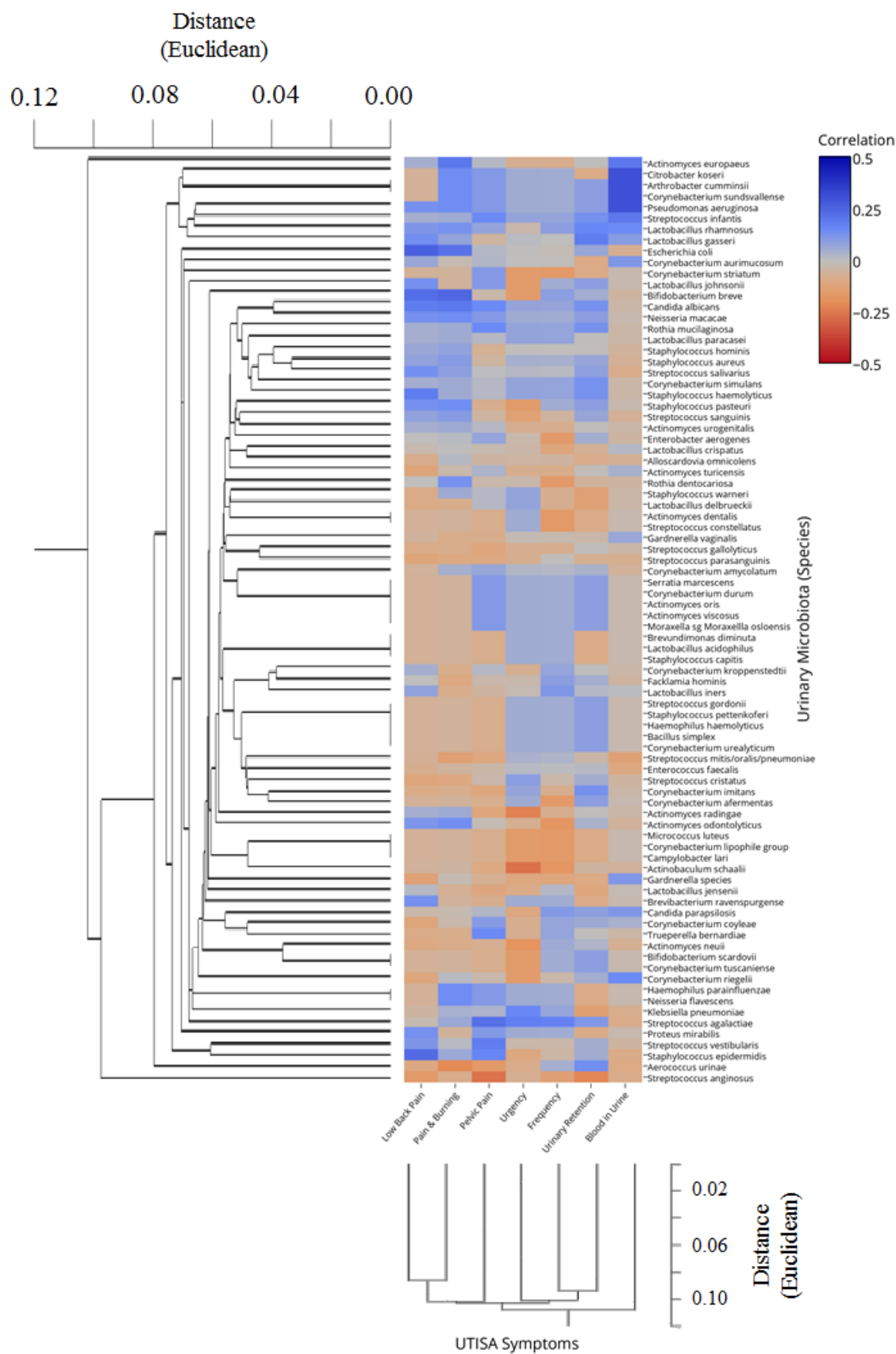


Figure 20. Clustered Heat Map of Correlations between Urinary Microbiota and UTISA Symptoms. Correlations are calculated using Pearson's R. Values are scaled from scores of 0.5 to -0.5. Both axes are clustered using the corresponding Dendrogram which is measured in terms of Euclidean distance. Values greater than 0.181 or less than -0.181 are significant ($p < 0.05$).

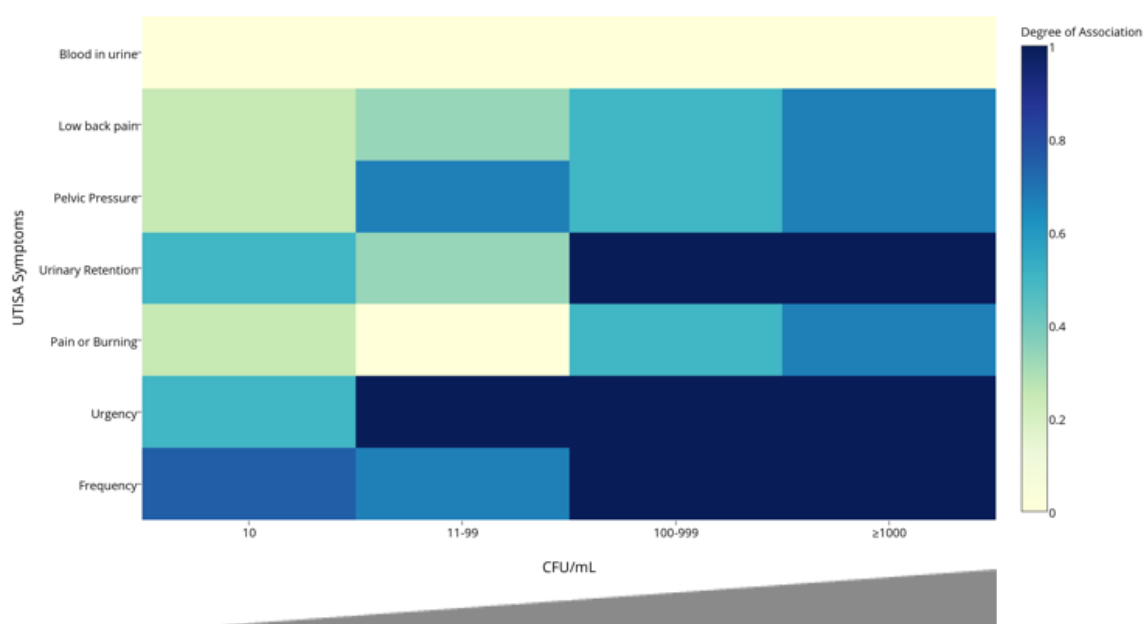
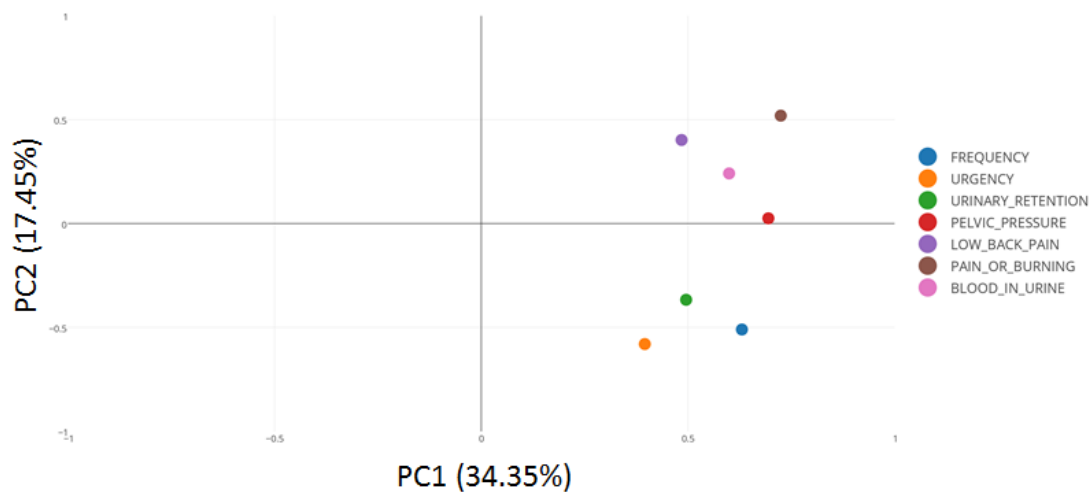


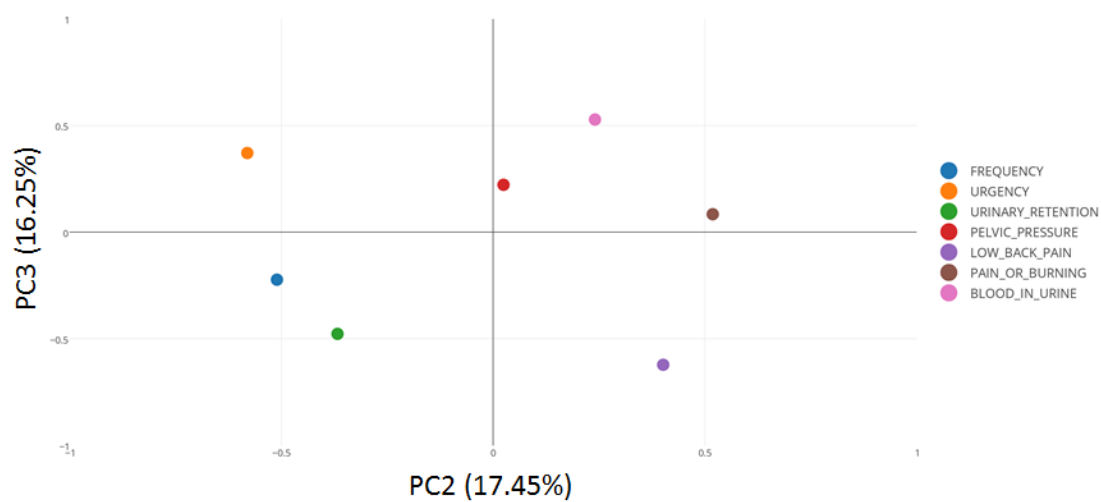
Figure 21. Heat Map of Associations between *Enterococcus faecalis* and UTISA Symptoms Grouped by CFU. Associations are displayed as “degree of association” - how likely is it for the organism to be cultured from the urine of someone with the corresponding urinary symptom (regardless of score). *E. faecalis* has been cultured a total of 12 times at CFU's ranging from 10 to 100,000 CFU/mL. The 10 CFU/mL group contains 4 samples, the 11-99 CFU/mL group contains 3 samples, the 100-999 CFU/mL group contains 2 samples, and the ≥1000 CFU/mL group contains 3 samples.

Principle Components Analysis (PC1 & PC2 – 51.80%)

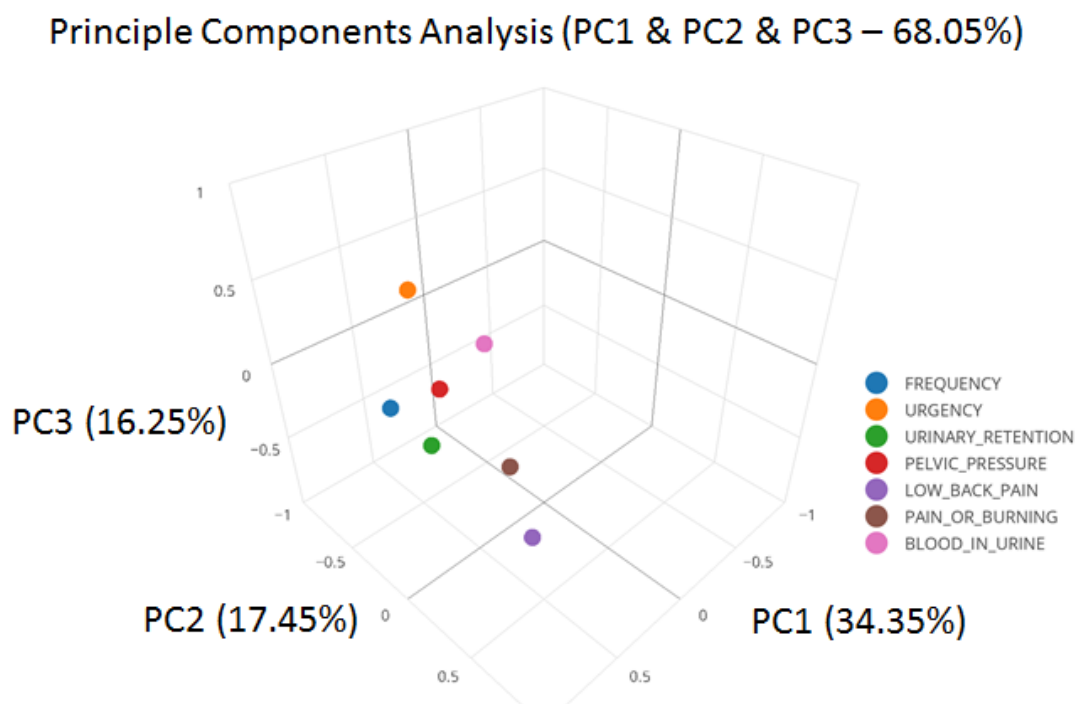


A.

Principle Components Analysis (PC2 & PC3 – 33.70%)



B.



C.

Figure 22. Principle Component Analyses of Associations between Urinary Microbiota and UTISA Symptoms. Figures depict principle component analysis (PCA) plots for components 1 and 2 (PC1 & PC2) (A), for components 2 and 3 (PC2 & PC3) (B), and for components 1, 2, and 3 (PC1 & PC2 & PC3) (C). The principle components were calculated based on degree of association between the urinary microbiota and the various symptoms (see **Figure 19**). The variance in the correlations between the symptoms is maximized through each principle component. PC1 accounts for 34.35% of the possible variance. PC2 accounts for 17.45% of the possible variance. PC3 accounts for 16.25% of the possible variance. Together, PC1, PC2, and PC3 account for 68.05% of the possible variance. The factor coefficients for the symptoms are: Frequency (0.262, -0.418, -0.196), Urgency (0.165, -0.475, 0.326), Urinary Retention (0.206, -0.300, -0.419), Pelvic Pressure (0.289, 0.021, 0.195), Low Back Pain (0.202, 0.329, -0.547), Pain or Burning (0.301, 0.425, 0.074), and Blood in the Urine (0.250, 0.198, 0.465).

CHAPTER V

CHARACTERIZING SELECT MICROORGANISMS
OF THE FUM

Introduction & Rationale

Knowing that the FUM is distinct between women with incontinence and the continent controls (*Chapter III*), and that certain urinary microbiota have strong associations to individual urinary symptoms (*Chapter IV*), the question arises: do these microorganisms cause these symptoms and contribute to etiologies, or are they a result of the urinary environment caused by the conditions? The data from *Chapter III* suggests that the FUM is a result of the physical environment of the urogenital tract, whereas the data from *Chapter IV* shows that the FUM is distinct across diverse unrelated urinary symptoms, suggesting the opposite - that these microorganisms contribute to the various symptoms. The most effective way to resolve this issue would be to introduce selected urinary bacterial isolates or communities of bacterial isolates into an *in vivo* animal model and see if these microorganisms alone induce symptoms. However, this approach would first require that we characterize the urinary microbiota of that animal model, a time-consuming procedure that we have just begun. It is also difficult to assess clinical urinary symptoms in an animal model. Thus, the next best approach is to take an *in vitro* look at the ability of these microorganisms to interact with one another and the host.

FUM Interactions

To begin to assess the interactions of the microorganisms of the FUM, we analyzed the likelihood that two microorganisms are co-cultured from the same urine sample (**Figure 23**). These data are presented in the form of a heat map. The microorganisms on both axes are ordered from most cultured (*S. anginosus* – N=118) to least cultured. Only microorganisms that have been cultured greater than or equal to 10 times are included in the figure. The values for each pairing are based on the frequency with which they are co-cultured together. The values on the x-axis (row) serve as the denominators for the frequency calculations, while the values on the y-axis (variable) act as the numerators. The data depicted come from a total of 497 EQUUC-assessed TUC urine samples. 230 samples come from Hilt *et al.*, 2014 and Pearce *et al.*, 2014. 124 samples come from **Chapter III** and 118 samples come from **Chapter IV**. The final 52 samples come from unpublished work on the maternal urinary microbiota. Of special interest are particular pairings with high frequency scores and ones with low scores. A high frequency pairing (like *Streptococcus parasanguinis* and *Streptococcus mitis/oralis/pneumoniae* – 86.67%) could infer that these microorganisms create a microenvironment that is suitable for one another. This could be important to know and understand because both microorganisms are also statistically associated with the NO cohort in **Chapter IV (Figure 15)**. Another interesting pairing is one with low frequency scores. For example, *E. coli* and *Lactobacillus crispatus* have a co-culture frequency score of just 3.17%. This could infer that these microorganisms fill the same niche or that one directly or indirectly inhibits the other. This is a striking relationship considering that *E. coli* is the most common cause of UTIs (Nicolle, 2008) and

is strongly statistically associated with the YES cohort in *Chapter IV* (**Figure 15**), while *L. crispatus* is statistically associated with healthy controls (Hilt *et al.*, 2014), and shows trending associations towards the continent controls in *Chapter III*. This suggests that there may be a clinical significance behind this pairing.

Figure 24 looks at the same data set as **Figure 23** but the values are represented as correlations. A Pearson's r Correlation value was calculated for each microorganism-microorganism pairing. These correlation values assess the linear correlation between the presence of one microorganism and the presence of the corresponding microorganism. The values can range from -1 to 1, where negative values denote negative correlations, and positive values denote the opposite. For this data set, correlation values greater than 0.088 or less than -0.088 are statistically significant ($p < 0.05$). Since correlations consider the total N for both microorganisms, the heat map of **Figure 24** is a mirror image across the left-to-right diagonal. The interesting pairing of *L. crispatus* and *E. coli* from **Figure 23** has an r -value of -0.0769 in **Figure 24**. This is one of the lowest correlations assessed in the entire figure, yet it is not statistically significant [$p = 0.0863$]. A major limitation of using correlation values is that they consider the entire ($N = 497$) data set. Consequently, for a pairing to be completely correlated ($r = 1$), both organisms would have to have been cultured in every urine sample. This is not biologically rational and thus skews all of the data closer to values of 0. Therefore, an r -score of -0.0769 (for the *L. crispatus* and *E. coli* pairing), likely has physiological significance despite its lack of statistical significance.

Co-Culture Frequencies of the Urinary Microbiota

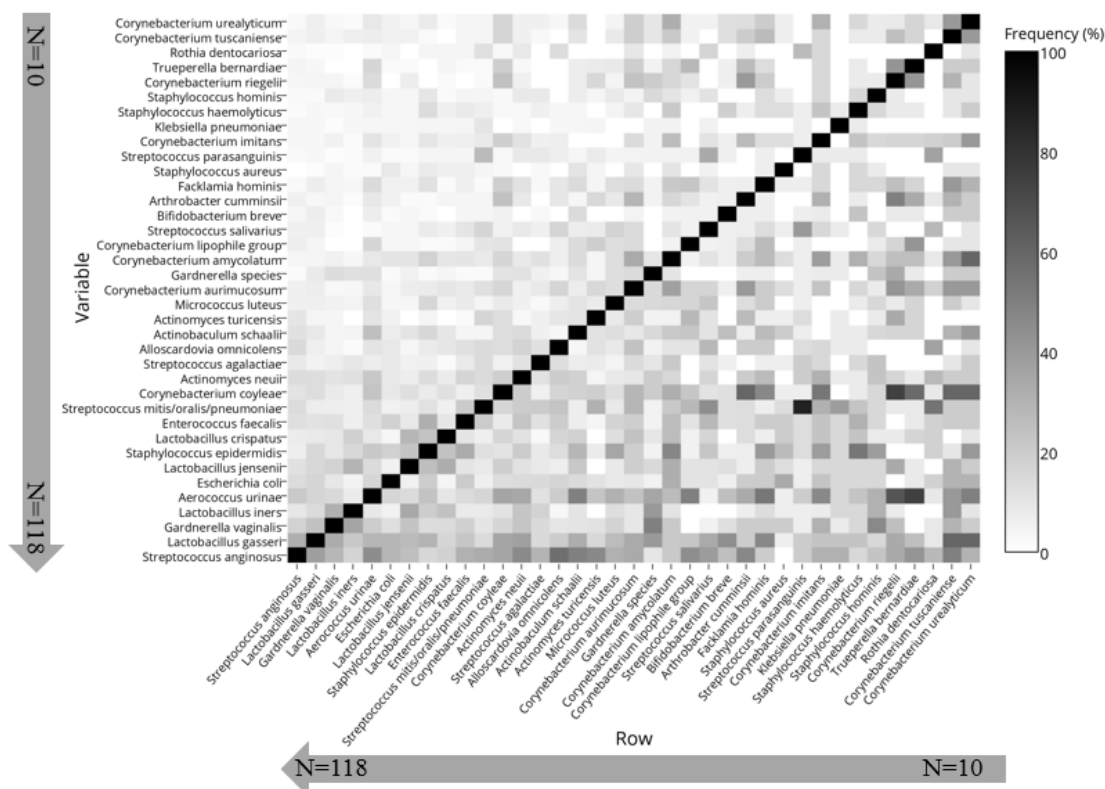


Figure 23. Heat Map of Co-Culture Frequencies of Urinary Microbiota Species. Co-culture associations are displayed as “frequency (%)” - percentage of the time when the microorganisms were culture, were they found together in the same sample. On both axes are listed all the microorganisms cultured from various urine samples listed in order from highest (bottom left) to lowest (top left, bottom right). Microorganisms cultured less than 10 times are not included. Frequencies are based on the number of times the microorganism on the x-axis (row) was cultured. Data comes from a total of 497 urine samples: 230 from Hilt *et al*, 2014, Pearce *et al*, 2014, unpublished work; 124 from **Chapter III**; 118 from **Chapter IV**; and 52 from unpublished work on the maternal urinary microbiota.

Correlation of the Co-culture of the Urinary Microbiota

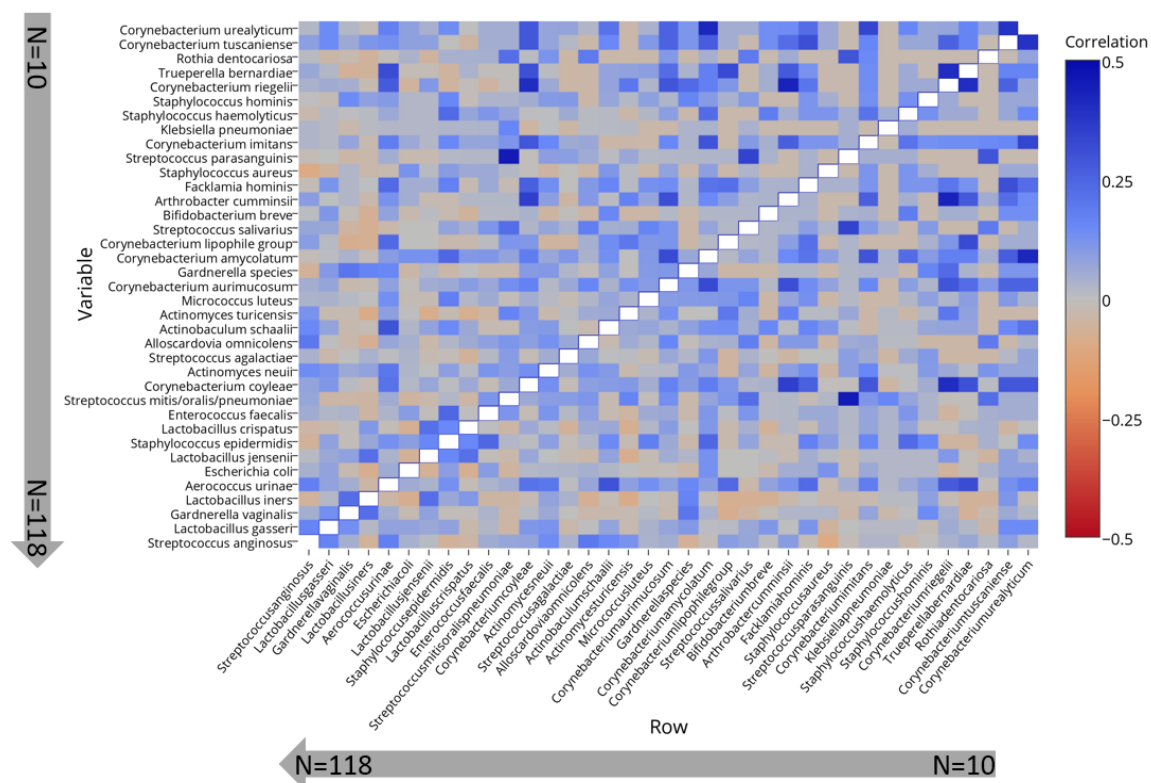


Figure 24. Heat Map of Co-Culture Correlations of Urinary Microbiota Species. Co-culture associations are displayed as “Correlations”. Correlations are calculated using Pearson’s R. Values are scaled from scores 0.5 to -0.5. On both axes are listed all the microorganisms cultured from various urine samples listed in order from highest (bottom left) to lowest (top left, bottom right). Microorganisms cultured less than 10 times are not included. Frequencies are based off of the number of times the microorganism on the x-axis (row) was cultured. Data comes from a total of 497 urine samples: 230 from Hilt *et al*, 2014, Pearce *et al*, 2014, unpublished work; 124 from **Chapter III**; 118 from **Chapter IV**; and 52 from unpublished work on the maternal urinary microbiota. Values greater than or less than 0.088 are significant ($p < 0.05$).

***Lactobacillus crispatus*: A Potential Probiotic for *E. coli* UTIs**

Lactobacilli are Gram-positive lactic acid-producing bacteria commonly residing in the oral cavity, gastrointestinal tract, and the urogenital tract of mammals. Four species tend to dominate the vaginal microbiome of young healthy women: *L. iners*, *L. crispatus*, *L. jensenii*, and *L. gasseri*. Colonization of these microorganisms generally leads to a protection from urogenital pathogens through a variety of known probiotic characteristics including the production of lactic acid and hydrogen peroxide, strong adhesion to epithelial cells, ability to modulate the host immune response, and production of bacteriocins and antimicrobials (Ravel *et al.*, 2010, Klebanoff *et al.*, 1991, Ojala *et al.*, 2014).

L. crispatus contributes to the stability of normal vaginal microbiota. Its absence is linked to a variety of vaginal abnormalities, including BV. Certain strains of *L. crispatus* are being tested as biotherapeutics for treatment of recurrent urinary tract infections (UTI) in women (Stapleton *et al.*, 2011). Additionally, cervicovaginal lavage (CVL) from healthy women was shown to contain proteins that are bactericidal towards *E. coli*. Several of these proteins correspond to *L. crispatus* (Kalyoussef *et al.*, 2012).

16S rRNA Sequencing.

16S rRNA gene sequencing was performed on 10 urinary isolates of *L. crispatus* to verify the MALDI-TOF MS identification as well as to determine the most closely related strain. 8 of the chosen urinary isolates were from patients without any LUTS (**Chapter III**) and Pearce *et al.*, 2014), and 2 were from patients with UUI (Hilt *et al.*, 2014, Pearce *et al.*, 2014). All 10 sequences were most closely related to strain *ST1*, one of 10 sequenced

strains of *L. crispatus* (Ojala *et al.*, 2014). Whereas 9 of the 10 sequenced strains are vaginal isolates, *ST1* is a GI isolate originally identified in an agricultural study of the crop (gut) of chickens. This phylogenetic relationship leads to the hypothesis that the urinary isolates detected by EQUIC are quite distinct from known vaginal isolates and thus likely have unique roles and characteristics.

In relation to *E. coli*, strain *ST1* plays a potentially beneficial role. Strain *ST1* has been shown to be able to prevent the adherence and invasion of Enteropathogenic *E. coli* (EPEC) to GI epithelial cells (Edelman *et al.*, 2003, Edelman *et al.*, 2012). This could explain why urinary *ST1*-like urinary isolates of *L. crispatus* are not co-cultured with *E. coli*.

Urothelial Cell Invasion Assay.

Using the protocol detailed in **Figure 2**, a *ST1*-like urinary isolate of *L. crispatus* was competed with UPEC for its ability to competitively exclude UPEC from urothelial cells. A non-malignant urothelial cell line (HURO23A) was used to assess binding. These urothelial cells possess characteristics of both immature basal cells and mature superficial urothelial cells (Le *et al.*, 2014). **Figure 25** shows that there was a significant drop in the ability of UPEC to invade these cells in the presence of *L. crispatus*, but this difference was only seen when the two microorganisms were added to the urothelial cells simultaneously. The pre-treatment condition (in which *L. crispatus* was added 2 hours prior to the UPEC) was not statistically different from the positive control. Thus, the urinary *ST1*-like isolates of *L. crispatus* may protect urothelial cells from UPEC in a manner similar to how the canonical *ST1* strains protect gut epithelial cells from EPEC. More urinary

strains of *L. crispatus* must be assessed to determine if this interaction is a general characteristic of these microorganisms.

Competition Assays.

With the knowledge that *L. crispatus* and *E. coli* are not frequently co-cultured (**Figure 23**), it is possible that there is an indirect or direct inhibition occurring between the microorganisms (in addition to competitive exclusion of UPEC by *L. crispatus*). Since *L. crispatus* is cultured more often in the non-LUTS control patients (**Chapter III**, Hilt *et al.*, 2014, Pearce *et al.*, 2014), and *E. coli* is cultured more often in the YES (UTI) cohort (**Chapter IV**), and with the knowledge that vaginal strains of *L. crispatus* may be protective against *E. coli* colonization, it is possible that *L. crispatus* produces something that is inhibitory towards *E. coli*.

To rapidly screen for inhibitory behavior, a lawn assay was performed (detailed in **Figure 3**). There are several possible outcomes of this lawn assay. First, the lawn microorganism might dominate. This would involve UPEC growing over the urinary isolate. Second is co-existence of both. This would involve both UPEC and the urinary isolate growing together. Last, the spotted organism might dominate. This could involve the urinary isolate preventing UPEC preventing growth. UPEC strain *CFT073* was used a positive control, as it inhibits the growth of UPEC strain *NU14*.

Figure 26 shows that when a lawn of UPEC (*NU14*) was created, and a spot of *L. crispatus* (cells or supernatant) was added, a zone of inhibition formed in which no UPEC grew. This cannot be attributed to lactic acid or hydrogen peroxide (common metabolites

of *Lactobacillus*) because when other species of *Lactobacillus* are assayed in the same manner, there is no observable inhibition of UPEC growth despite the cultures having identical pH (4.8-5.0). To verify this effect, spent culture supernatants (SCS) of various *Lactobacillus* urinary isolates were added to an overnight culture of UPEC (NU14) in a 1:1 ratio and then assessed for UPEC viability over time (detailed in **Figure 4**). **Figure 27** shows that the SCS of *L. crispatus* dramatically reduced UPEC viability within 2 hours of incubation, while even after 24 hours, the SCS of *L. gasseri* and *L. jensenii* resulted in only a mild reduction. As a control, the SCS of *Gardnerella vaginalis* (a non-lactic acid producer) was tested; it resulted in no reduction in UPEC viability. Thus, the mild reduction seen by *L. gasseri* and *L. jensenii* can likely be attributed to lactic-acid production, while the large reduction seen by *L. crispatus* cannot. Other urinary strains of *L. crispatus* have been assessed and they affect UPEC differently; some result in complete killing of UPEC by 2 hours, whereas others result in less killing.

Ethidium Bromide Assay.

To begin to assess how the *L. crispatus* SCS dramatically reduces the viability of UPEC, an EtBr assay was performed. If EtBr can permeate the cell membrane of UPEC, then it will bind the DNA, which can be detected by UV light. Thus, if a component of the *L. crispatus* SCS acts to disrupt the membrane of UPEC, the EtBr will bind the DNA (detailed in **Figure 5**). Indeed, **Figure 28** shows that, after just 20 minutes, the *L. crispatus* SCS induced UPEC EtBr binding at a level intermediate between the positive control (heat-killed UPEC) and the untreated negative control. Thus, it is likely that the inhibitory component of the *L. crispatus* SCS acts as a pore to reduce the UPEC viability.

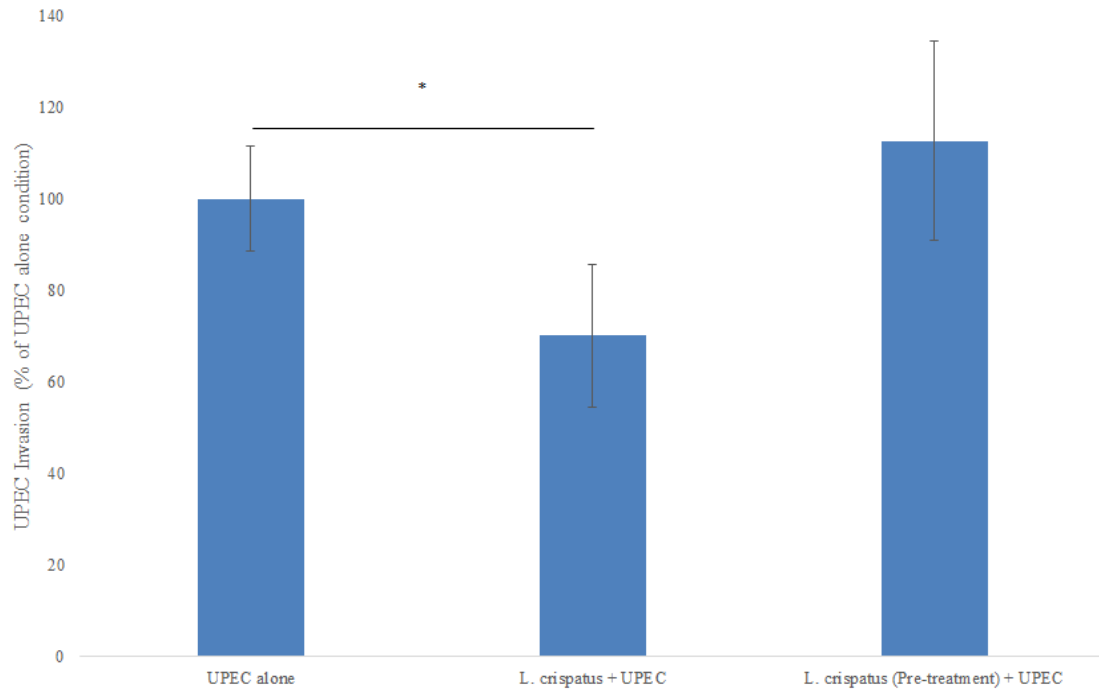


Figure 25. UPEC Urothelial Cell Invasion in the Presence of *L. crispatus* Urinary Isolates. The data are normalized to the amount of *E. coli* invasion in the UPEC alone condition. There is statistical difference in UPEC invasion between the UPEC alone condition and the *L. crispatus* + UPEC condition as assessed by a T-test for independent samples ($\alpha=0.05$) (* $p<0.05$; ** $p<0.01$).



L. crispatus



L. gasseri

Figure 26. UPEC Lawn Inhibition by *Lactobacillus* Urinary Isolates. (*Left*) Growth of *L. crispatus* on a lawn of UPEC (NU14) yields a distinct zone-of-clearance of the UPEC. (*Right*) Growth of *L. gasseri* on a lawn of UPEC (NU14) yields no distinct zone-of-clearance of the UPEC. Assay was performed on TSA at 24 hours post-incubation in 5% CO₂, 37°C.

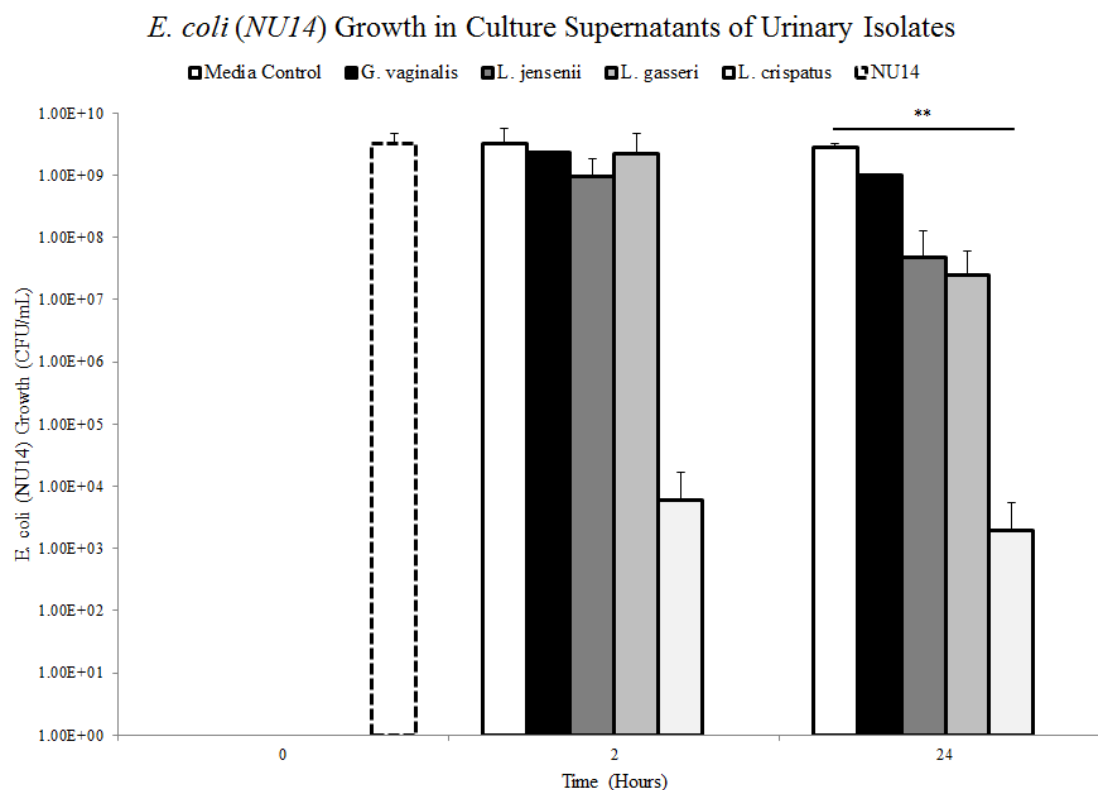


Figure 27. UPEC Growth Inhibition by Urinary Isolates in Liquid Culture. The spent culture supernatants of 3 different *Lactobacilli* and *Gardnerella vaginalis* (a non-lactic acid producer) were added to an overnight culture of UPEC (NU14). The supernatants were added in a 1:1 ratio. Cultures were incubated in aerobic, shaking environment. Timepoints were taken at 0hrs (before supernatants added), 2hrs post-addition, and 24hrs post-addition. The Media Control was MRS for the *Lactobacilli*, or TSB + 10% FBS for *G. vaginalis*. *E. coli* growth was measured as CFU/mL by serially dilutions and plating on TSB and incubating overnight in aerobic conditions. All samples have been repeated 3 times (3 separate isolates each tested once), with the exception of *L. gasseri* (twice) and *G. vaginalis* (once).

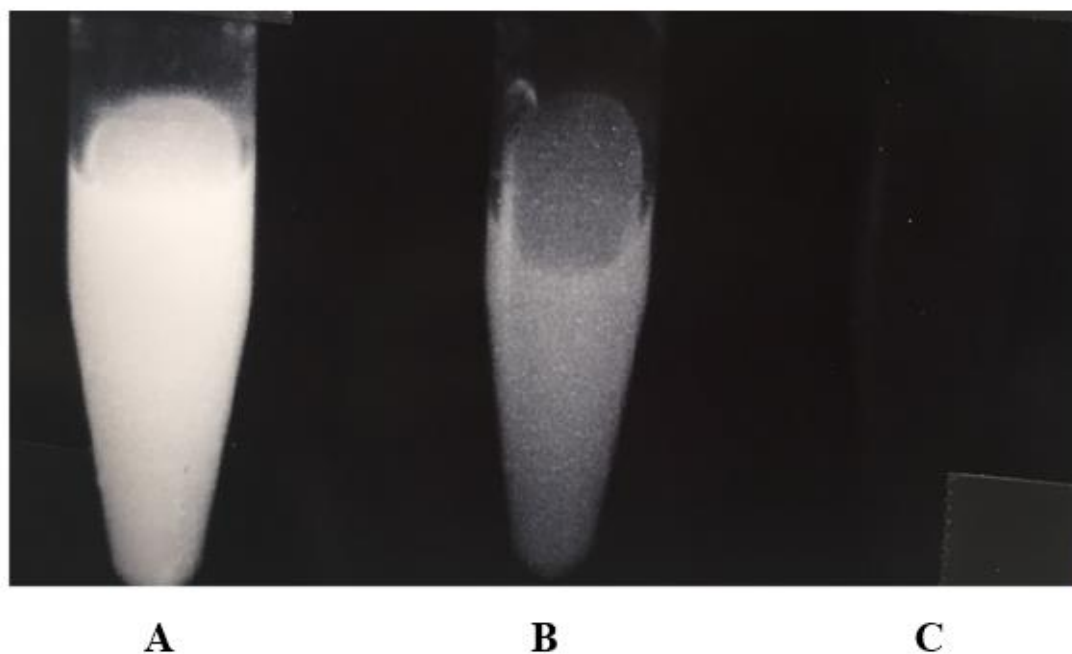


Figure 28. UPEC Susceptibility to EtBr after *L. crispatus* SCS treatment. (A). Positive control; 100°C heat-lysed UPEC. (B). 1:1 addition of *L. crispatus* SCS. (C). Negative control; media-treated UPEC. Image depicts EtBr DNA-binding visualized under UV light after 20 minutes.

***Lactobacillus crispatus*: Conclusions**

Further experiments are needed to provide the necessary information to understand how *L. crispatus* acts to kill UPEC. This refers to both the nature of the killing compound as well as the specific mechanism of action of this compound towards UPEC. With this knowledge, we can recommend the use of urinary isolates of *L. crispatus* as biotherapeutics for *E. coli* UTIs. We can also use the purified compound as a targeted antimicrobial agent against *E. coli* to treat UTIs. Nonetheless, these data show that the FUM does have a direct impact on urinary health.

CHAPTER VI

DISCUSSION & CONCLUSIONS

It is clear from the presented data that the FUM likely plays a role in LUTS. This knowledge is vital to the proper understanding of etiologies and treatments for these various urinary symptoms and disorders.

In *Chapter III*, the data show that the FUM is distinct in diversity, genus-level composition, and species-level composition between women with and without urinary incontinence. Biodiversity assessed by the Shannon-Wiener Index show a markedly lower diversity in the microorganism communities associated with continent women compared to those of women with urinary incontinence. In terms of composition, *A. schaalii*, *A. turicensis*, *A. sanguinicola*, *A. urinae*, and *Corynebacterium lipophile* group are all statistically associated with the Incontinence cohort. Many of these microorganisms are known or emerging uropathogens; therefore the idea that they may be a contributing factor to the development of urinary incontinence is not unlikely. *M. luteus* was the only microorganism associated with the Continence cohort suggesting that it may have a beneficial role in urinary health and the prevention of incontinence. What that role might be is unclear. The comparing of microbiota profiles highlights the dramatic difference in diversity of the FUM between the two groups. This assessment also shows a large difference in the number of EQUIC-negative samples. Over a third of the samples from the

Continence cohort were negative, whereas only about 10% of the samples from the Incontinence cohort were negative. This highlights a major confounding factor to the overall hypothesis that these microorganisms contribute to urinary symptoms. How could this be true if there are patients with symptoms who potentially have no FUM? This problem extends into **Chapter IV** as well, where there are some patients in the YES cohort who are EQUUC-negative despite their claim of severe urinary symptoms. There are a couple possible explanations for this. First, there are large limitations using EQUUC. The EQUUC conditions were developed to detect clinically relevant and commonly isolated microorganisms. But even if these conditions were expanded, there are estimates as high as 99% of naturally found microorganisms not being cultivatable using any known techniques. It is very likely that the EQUUC-negative patients are not devoid of a FUM. 16S rRNA sequencing of urine samples shows that this is in part a valid explanation, as about half of the EQUUC-negative samples in a particular study were found to be sequence-positive (i.e. bacterial genomic DNA was detected) (Pearce *et. al.*, 2014). Of the EQUUC and sequence-negative samples, there still may be a FUM present. As discussed earlier, many of the urinary microorganisms are able to associate with urothelial cells (Khasriya *et al.*, 2013). Therefore, it is valid to assume that some of these physical associations will be strong enough to prevent detachment during the urine voiding process, which would mean that we cannot detect their presence by EQUUC or sequencing in the TUC samples. Unpublished data assessing the microorganism content of bladder urothelial cell biopsies shows that there are microorganisms that are found solely attached to the tissue and are not detected in TUC or SPA samples from the same individual. Whether these microorganisms

are attached superficially to these urothelial cells or have been internalized is unclear. Nonetheless, it can potentially account for the EQUUC and/or sequence-negative samples.

Figure 9 of *Chapter III* details another important finding. This figure compares the microbiota profiles of the Continence cohort from the data detailed in *Chapter III* with that of data from Pearce *et al.*, 2014. The resulting figure demonstrates a strong reproducibility of data on the FUM. The two microbiota profiles are markedly similar in terms of their genus-level composition. Additionally, the diversity and species-level composition (*data not shown*) are also quite similar. This adds a degree of power to the rest of the conclusions made throughout, because it shows that there is a high level of stability to the data and little variation.

Collectively, the data comparing the FUM of women with and without urinary incontinence suggests that the FUM may act to induce or prevent symptoms of urinary incontinence through interactions with the host tissue. However, the data also show that the FUM is not substantially different between subtypes of urinary incontinence (UI and SUI). This shows that the FUM may also be, in part, a result of the physical environment of the urogenital tract. Women with either form of urinary incontinence have a wetter urethra and vaginal tract due to the involuntary leakage. This could promote ease of mobility for microorganisms to move between anatomical sites; it could also support the growth of a unique population of microorganisms. Whether this in turn would exacerbate symptoms is possible, yet unclear.

As a follow up to the data from *Chapter III*, *Chapter IV* looks at FUM associations with individual urinary symptoms, as opposed to an entire disorder, which may involve numerous symptoms. FUM diversity and composition in a patient population with UTI-like symptoms (which are broad and diverse) (YES cohort) differed dramatically from those in women with/without less severe UTI-like symptoms (NO cohort). These differences included diversity and composition. The YES cohort was comprised of communities of microorganisms with much lower biodiversity than that of the NO cohort. In terms of composition, *G. vaginalis*, *S. parasanguinis*, and *S. mitis/oralis/pneumoniae* were all found to be statistically associated with the NO cohort, while *E. coli* was strongly associated with the YES cohort. The microbiota profiles highlight the strong differences in diversity and composition.

The validated UTISA questionnaire was used to assess the associations between individual urinary symptoms with species of the FUM. Use of this questionnaire and the data from the original validation study (Clayson *et al.*, 2005) also was used to show that the NO cohort overall has statistically lower scores for severity and bother of the various urinary symptoms. Therefore, the dramatic differences in diversity and composition of the FUM between the cohorts can be directly connected to the degree of severity and bother of the urinary symptom.

Each UTI symptom was found to be associated with a unique bacterial profile at the species-level, in terms of degree of association, linear correlation, and covariance. These associations were not as apparent at the genus-level composition or with respect to

diversity of the FUM. These distinctions support the need to obtain species level identification for deeper understanding of the relationship between the FUM and lower urinary tract disorders.

In terms of associations between the FUM and the urinary symptoms, **Figures 19 and 20** tell somewhat similar stories. **Figure 19** looks at the associations in terms of how often a given microorganism was cultured from a patient with a particular symptom, whereas **Figure 20** looks at the associations in terms of the correlation between having a particular symptom and culturing a given microorganism. For some of the microorganisms, the two figures tell the same story. For example, this could mean that a microorganism associates *and* correlates highly with some of the seven symptoms. Therefore, not only is that microorganism found often in samples where the patients have those symptoms, but also out of *all* the patients with those symptoms, that microorganism is commonly found. An example of this is the uropathogen, *Candida parapsilosis*. *C. parapsilosis* is highly associated and correlated with frequency and urinary retention. These data can be inferred to mean that *C. parapsilosis* is likely directly contributing to the etiologies of those symptoms. Conversely, there are microorganisms that associate highly with particular symptoms, but do not correlate highly with them (or vice versa). An example of this is *Streptococcus anginosus*. *S. anginosus* is highly associated with frequency and urgency, and moderately associated with pelvic pain, and urinary retention (**Figure 19**), but it is strongly negatively correlated with all of the urinary symptoms (**Figure 20**). Therefore, of the patients with *S. anginosus* in their urine samples, quite a few of them have the four

symptoms, but of all the patients with each of the four symptoms, less than half of them had *S. anginosus* present. While this is a bit perplexing at first, it can actually be inferred to mean that *S. anginosus*, while typically considered a uropathogen, is not directly causing any of the symptoms, but rather it is commonly found with microorganisms that are contributing to the symptoms. This is vital to understanding the pathophysiology of the various urinary symptoms. *S. anginosus* may facilitate a microenvironment that is conducive to the colonization of an organism that causes the symptoms. Therefore, in determining proper treatment, the effects of *S. anginosus* must be considered. Furthermore, this has strong implications for women with recurrent UTIs. It's likely that organisms like *S. anginosus* are facilitating repeated colonization of other uropathogens leading to frequent infection. Proper treatment of recurrent UTIs likely will need to extend beyond the primary pathogen involved in the infection.

Additionally, urinary symptoms seem to cluster into two distinct groups as assessed by PCA. Frequency of urination, urgency of urination, and urinary retention vary less in their associations with the FUM than the other four symptoms (pelvic pressure, pain and burning during urination, lower back pain, and blood in the urine). This is an important finding because it shows that potential bacterial etiologies of the clustered symptoms are likely similar. This makes sense in the context of incontinence for example. Women with UII usually have symptoms of both urgency and frequency (OAB). Therefore, it is not surprising that the FUM of women who have either urgency or frequency are similar.

Unfortunately, many of these analyses did not consider CFU/mL of the cultured microorganisms. **Figure 21** shows that the CFU/mL of one microorganism (*Enterococcus faecalis*) effects the degree of association with the various urinary symptoms. Generally, the higher the CFU/mL, the higher the degree of association is with most of the symptoms. Therefore, the size of a bacterium's urinary population likely plays an integral role in symptoms. One could imagine that higher CFU would allow for greater dissemination of the bacteria throughout the bladder, allowing for greater potential impact on the host tissue as well as the rest of the microflora. Higher CFUs could also allow for synergistic effects of bacterial metabolites. Thus, CFU represents a substantial limitation to these findings and should be included in future analyses.

It is important to consider that the NO cohort is not actually devoid of urinary symptoms. In fact, much of this patient population has urinary incontinence. 47 patients of the 75 were clinically diagnosed with incontinence. By looking at these select individuals and comparing them to the Incontinence cohort (N=100) from **Chapter III**, one can assess the reproducibility of the data. The microbiota profiles are quite similar to one another (**Figure S10**) which shows consistency in the data. This is also important because it shows again that there are distinct communities of microorganisms associated with the symptoms of frequency and urgency that are different from other urinary symptoms.

Chapter V shows that one particular species of the FUM, *L. crispatus*, can impact symptoms. This organism is able to directly exclude and inhibit UPEC, which is known to cause the majority of UTIs and is clinically manifested as several urinary symptoms. This

inhibition occurs on urothelial cells *in vitro*, on solid media, and in liquid culture. The nature of the compound as well as the mechanism of action are unclear at this point. However, the data show that the compound is likely acting on the cell membrane of UPEC in a manner that is similar to antimicrobial peptides. Nonetheless, it is critical to follow up on these data and identify and characterize this compound. By introducing *L. crispatus* into the bladder environment as a probiotic or by introducing the compound as an antimicrobial, clinicians could potentially limit the growth and colonization of UPEC. This could act to cure several urinary symptoms brought on by UPEC, as well as to prevent recurrent infections.

Interestingly, the urinary isolates of *L. crispatus* that were sequenced were more closely related to a gut strain of *L. crispatus* (ST1) than to any known vaginal strains. This is rather striking considering the close proximity of the bladder and vagina. One might expect frequent microbial sharing between environments during voiding. Therefore, this finding shows that the urinary isolates are evolutionarily distinct from vaginal isolates (at least for *L. crispatus*). This immensely supports the notion that the microbiota of the bladder and vagina are distinct, not only in composition but also in their roles in urinary health and symptoms. This also shows that urinary microorganisms can potentially have vastly distinct and unique characteristics.

To conclude, with the knowledge presented in this discussion, we can now start to make directed hypotheses towards understanding whether some of the associations between the FUM and the symptoms are physiologically relevant, in terms of

understanding the process and mechanisms behind those associations as well as how to intervene to prevent and treat them more effectively. The possibilities are limitless for how the microbiota could influence various symptoms, but it is clear at this point that they are able to, and these interactions may be vital to fully understanding lower urinary tract symptoms.

APPENDIX A:

DEVELOPING AN OPTIMAL

URINE CULTURE PROTOCOL

Introduction & Rationale

Since the 1950's, the clinical practice for detecting infection in the bladder, including cystitis (bladder infection) and pyelonephritis (kidney infection), has been based on the detection of $\geq 10^5$ CFU/mL of a known uropathogen, using the standard urine culture protocol. This method was actually established to detect the patients that were likely susceptible to pyelonephritis (Kass, 1956). The method has since been adopted to include lower urinary tract infections as well, despite any empirical evidence that this was valid. Thus, many studies have provided evidence that this threshold of $\geq 10^5$ is insufficient to detect significant bladder infection (Stamm *et al.*, 1982, Stark *et al.*, 1984, Lipsky *et al.*, 1987, Thomas *et al.*, 2013). For example, many women present with some or all of the symptoms of a urinary tract infection, but their standard urine culture is deemed “NO GROWTH”. At this juncture, the patient is informed that they do not have a UTI. These patients are not given antibiotics and thus in many cases their symptoms persist. In rare cases, the urinary infection can lead to systemic life-threatening infections.

Using the EQUC protocol, bacteria can be detected in 70-90% of the urines that were deemed “NO GROWTH” by standard culture (85.71% - **Chapter III**, 70.37% - **Chapter IV**). Even in women who feel they have a UTI (**Chapter IV**), the false negative rate for standard culture was 35.00%. As assessed by EQUC, the standard protocol misses known or emerging uropathogens present in the urine of many individuals. These bacteria may be clinically relevant to the patients' symptoms. To develop an optimal urine culture protocol for detection of clinically relevant microorganisms that we could recommend to

the clinical microbiology community, we analyzed the data obtained from the patient population described in *Chapter IV* using the study outline from **Figure 11**.

UTI Study: A New Urine Culture Protocol

Overview of Study.

This study uses the same patient population and design as the study discussed in *Chapter IV*. **Figure 11** details the study outline. The TUC urine specimens that were collected from the 118 women were assessed for bacterial growth using the standard urine culture protocol as well as the expanded EQUC protocol (**Table 1**). The expanded EQUC protocol adds MacConkey agar and Campy gas mixture to the original EQUC protocol (Hilt *et al.*, 2014). MacConkey agar is ideal for the growth of typical uropathogens and is already used as part of the standard urine culture protocol. MacConkey agar also allows for quick phenotypic analysis of the bacterial growth based on the ability to ferment lactose. The Campy gas mixture provides an optimal environment for the isolation of fecal contaminant microorganisms. Additionally, three volumes of urine (1μL, 10μL, and 100μL) were used for each expanded EQUC, for a total of 21 plates (**Table 1**). This allows for the determination of an optimal protocol.

The standard urine culture was performed by the Clinical Microbiology Laboratory staff at Loyola University Medical Center. All patient care was a consequence of the results of the standard culture alone. The expanded EQUC results were not reported and thus did not impact patient care. In December 2014, the Clinical Microbiology Laboratory began using the modified urine culture protocol (**Table 1**). This change affected patient study

ID's 108-118. All data obtained for this study, was from combining the data obtained from the use of all three urine volumes of the expanded EQUC protocol at the 48 hour timepoint unless stated otherwise.

After 48 hours of incubation in the respected environmental conditions, the various plates were documented for morphologies and sub-cultured. The pure cultures were identified using MALDI-TOF MS, and were stored at -80°C for future study.

Determining an Optimal Protocol.

As expected, the greater the volume of urine plated, the greater the number of unique microorganisms were detected. These data are depicted in **Figure A1**, which presents them in the form of rarefaction curves. After 118 urine samples assessed (both the YES and NO cohorts), standard/modified urine culture detected 8 unique species. The 1µL expanded EQUC detected 31 unique species. This is rather striking considering that both protocols involve plating the same volume of urine, yet the expanded EQUC detected over four times more unique species. This demonstrates the power of using other environmental conditions and holding the plates for longer periods of time. The 10µL expanded EQUC detected 48 unique species, and the 100µL protocol detected 82 unique species. (**Figure A1**). Statistical analyses support the dramatic differences in diversity. Using the Shannon Diversity Index, the 100µL protocol had a high measure of biodiversity (3.862) while the standard culture had a much lower measure (1.169). The other protocols fell in an intermediate range (1µL – 2.879, 10µL – 3.393).

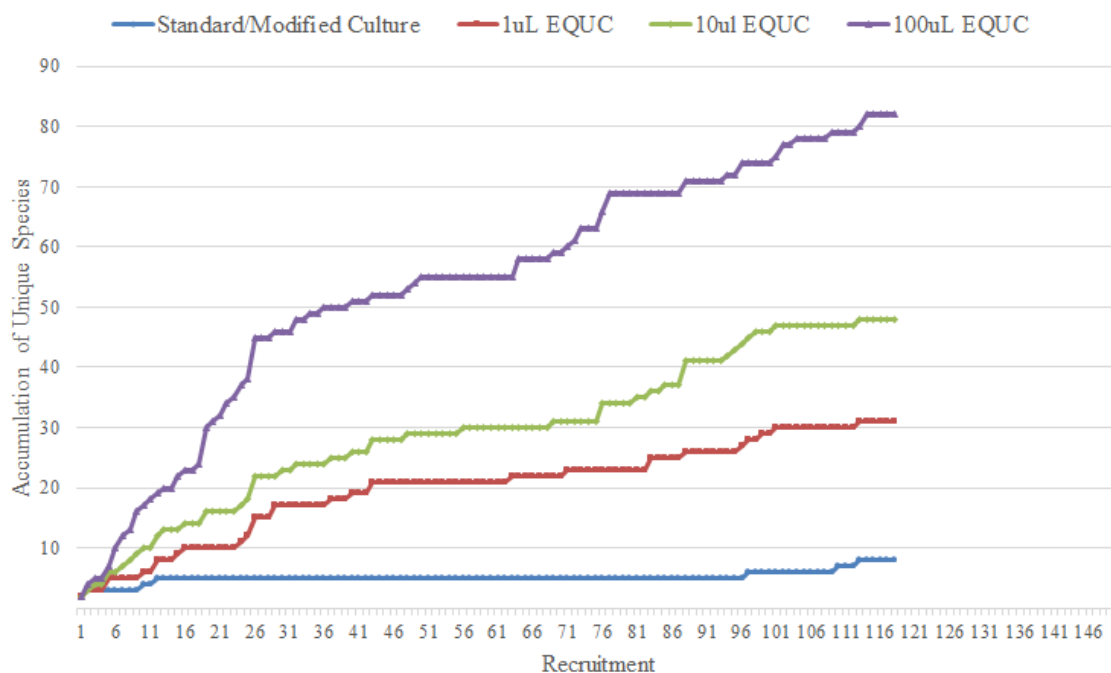


Figure A1. Rarefaction Analysis of Various Urine Culture Protocols. The Standard/Modified Urine Culture protocol (N=118) contains 8 unique species and is depicted by the blue circles. The 1 μ L Expanded EQUC protocol (N=118) contains 31 unique species and is depicted by the red squares. The 10 μ L Expanded EQUC protocol (N=118) contains 48 unique species and is depicted by the green diamonds. The 100 μ L Expanded EQUC protocol (N=118) contains 82 unique species and is depicted by the purple triangles.

Despite the dramatic difference in total number of unique species between protocols, not all of these microorganisms are currently understood to be clinically relevant (though they may be). **Table A1** looks at just the microorganisms known to be clinically relevant (known and emerging uropathogens) and the differences in their detection between the protocols. 18 known and emerging uropathogens were detected 126 times in this patient population: *Actinobaculum schaalii* (N=5), *Aerococcus urinae* (N=11), *Alloscardovia omnicolens* (N=6), *Candida albicans* (N=2), *Candida parapsilosis* (N=4), *Citrobacter koseri* (N=1), *Corynebacterium riegelii* (N=3), *Corynebacterium urealyticum* (N=1), *Enterobacter aerogenes* (N=3), *Enterococcus faecalis* (N=12), *Escherichia coli* (N=25), *Klebsiella pneumoniae* (N=8), *Proteus mirabilis* (N=1), *Pseudomonas aeruginosa* (N=1), *Serratia marcescens* (N=1), *Staphylococcus aureus* (N=6), *Streptococcus agalactiae* (N=10), and *Streptococcus anginosus* (N=26). The 100µL expanded EQUUC protocol detected 94% (119/126) of the total uropathogens. This value is not 100% because occasionally, a microorganism cultured at a high CFU/mL will overwhelm the plates on the 100µL protocol, and thus some uropathogens are actually missed. So a higher volume of urine is not always the best option. The 10µL expanded EQUUC protocol detected 58% (73/126) of the total uropathogens. The 1µL expanded EQUUC protocol detected 45% (57/126) of the total uropathogens. The standard/modified urine culture protocol detected only 26% (33/126) of the total uropathogens. **Table A1** summarizes these data.

Culturing Protocol	Uropathogens (N=126)	Urine Samples with Uropathogens (N=78)
100µl Expanded EQUC	94% (N=119)	100% (N=78)
10µl Expanded EQUC	58% (N=73)	73% (N=57)
1µl Expanded EQUC	45% (N=57)	59% (N=46)
Standard Culture	26% (N=33)	40% (N=31)

Uropathogens (Standard Culture/EQUC):

Actinobaculum schaalii (0/5), *Aerococcus urinae* (1/11), *Alloscardovia omnicolens* (0/6),
Candida albicans (0/2), *Candida parapsilosis* (0/4), *Citrobacter koseri* (0/1),
Corynebacterium riegelii (0/3), *Corynebacterium urealyticum* (0/1), *Enterobacter aerogenes* (1/3),
Enterococcus faecalis (1/12), *Escherichia coli* (23/25), *Klebsiella pneumoniae* (3/8),
Proteus mirabilis (0/1), *Pseudomonas aeruginosa* (1/1), *Serratia marcescens* (0/1),
Staphylococcus aureus (2/6), *Streptococcus agalactiae* (1/10), *Streptococcus anginosus* (0/26)

Table A1. Overview of Uropathogen Detection by Various Urine Culture Protocols.

(top) On the left are the various urine culture protocols. In the middle column are the percentage of uropathogens detected by the corresponding protocol. Out of the 118 patients, 126 uropathogens were detected. In the right column are the percentage of urine samples or patients where at least one uropathogen was detected by the corresponding protocol. 78 out of the 118 patients had uropathogens detected. **(bottom)** Listed are all of the detected known and emerging uropathogens. In parentheses are the number of times that microorganism was detected by the Standard/Modified Urine Culture protocol over the number of times that microorganism was detected by any of the Expanded EQUC protocols.

From the YES cohort only, 54 uropathogens were detected in 43 patients. 96% (52/54) were detected with the 100 μ L protocol, 74% (40/54) were detected with the 10 μ L protocol, 69% (37/54) were detected with the 1 μ L protocol, and 52% (28/54) were detected by the standard/modified culture. These values, while much higher than those obtained for the entire patient population, are dramatically skewed by the detection of *E. coli*. For example, standard/modified culture detected *E. coli* in all 18 YES cohort samples that had *E. coli* detected by expanded EQUIC. This means that the standard/modified culture protocol only detected 10 of the remaining 36 uropathogens (28%) that were detected by expanded EQUIC in the YES cohort. In fact, when looking at the entire patient population, standard/modified culture detected 92% (23/25) of the *E. coli*, but only 10% (10/101) of the non-*E. coli* uropathogens.

As alluded to in **Chapter IV**, the CFU/mL with which a microorganism is cultured likely plays a clinical role (**Figure 21**). This is the basic idea behind using $\geq 10^5$ as a threshold for infection; however, this threshold appears to be ineffective as assessed by the data from this study. **Figure A2** depicts the average CFU/mL with which the various uropathogens were detected in either cohort (YES and NO). It appears that for the majority of the uropathogens, the average CFU/mL was higher in the YES cohort than the NO, suggesting that when an individual has UTI-like symptoms, the presence of a uropathogen was at a higher CFU than an individual who does not have symptoms (or less severe). When the $\geq 10^5$ CFU/mL threshold for infection is used, it does not do a good job capturing any uropathogens.

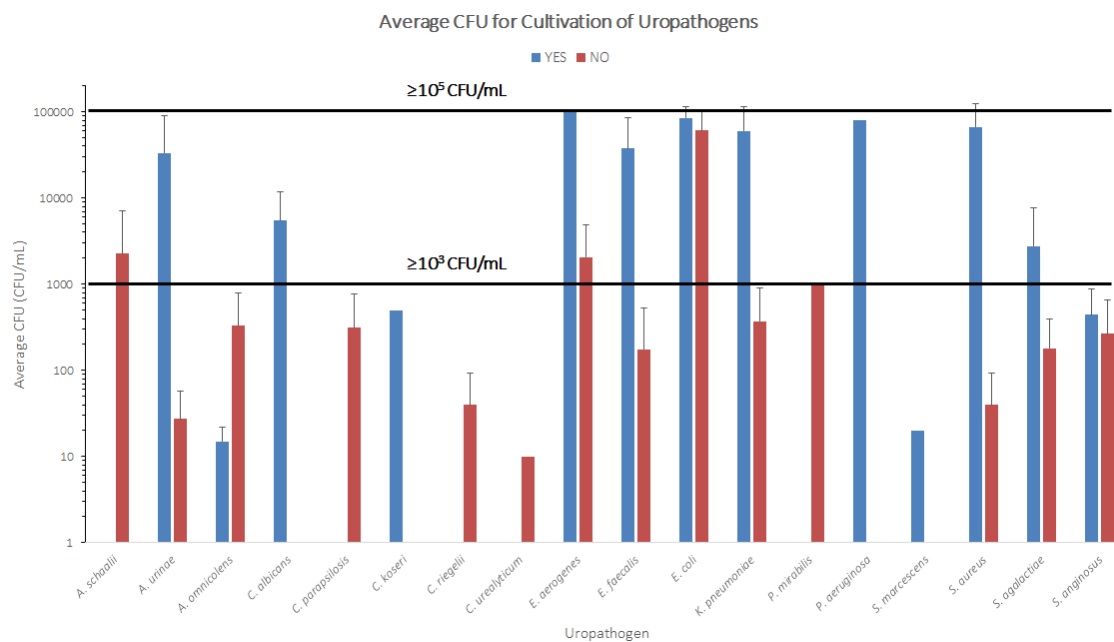


Figure A2. Average CFU/mL of Uropathogens between YES and NO Cohorts. The YES cohort (N=43) is depicted by the blue bars, and the NO cohort (N=75) is depicted by the red bars. Depicted are the average CFU/mL with which the various uropathogens were cultured for both cohorts. Two lines are drawn in to represent commonly used clinical thresholds for UTI diagnosis ($\geq 10^5$ CFU/mL and $\geq 10^3$ CFU/mL).

Even the lower threshold of $\geq 10^3$ CFU/mL (a common threshold for catheterized urine samples) is ineffective. This threshold captured uropathogens, but some of these are in the NO cohort, and some uropathogens in the YES cohort were still missed. Thus, the use of one threshold for all infections is not appropriate. Diagnosis of UTI must be on a person-by-person basis and thresholds should be established for each uropathogen. For example, these data suggest that >100 CFU/mL of *Aerococcus urinae* is indicative of clinical infection/symptoms, but <100 CFU/mL is not. However, the idea of a species-specific threshold would not work for *E. coli*, which appears to be at equivalent average CFU/mL between the two cohorts. In the case of *E. coli*, the problem rests with the inability to distinguish UPEC and asymptomatic bacteriuria (ABU) strains, which are close relatives. However, it is important to consider that the NO cohort is not necessarily devoid of urinary symptoms.

When looking at the seven different plating conditions, there also exists large differences in their abilities to detect microorganisms and specifically uropathogens. **Figure A3** represents a rarefaction analysis used to assess the differences in the number of unique species detected by each plating condition. It seems that BAP incubated in 5% CO₂ and the CDC anaerobe 5% sheep blood agar plate incubated anaerobically detected the highest number of unique species, 52. The Campy condition, CNA and Chocolate agar in 5% CO₂ all detected intermediate numbers of unique species (48, 45, 43 respectively). Interestingly, the two conditions that comprise the Standard Urine Culture protocol (BAP and MacConkey – aerobic incubation) detected the least number of unique species (36 and

6 respectively). These data come from a combination of the expanded EQUIC protocols and represent the 48 hour timepoint data.

Looking at cultivation of uropathogens between the various culturing conditions, there also exists large differences. **Table A2** lists the total number of each uropathogen detected in the various culturing conditions. BAP in 5% CO₂, CNA in 5% CO₂, and Anaerobic BAP incubated anaerobically, detected the greatest number of uropathogens. Together, they are able to detect each uropathogen individually. At 100µL of urine, held for 48 hours, these plates would have detected 87% (109/126) of the total uropathogens and 89% (48/54) of the uropathogens from the patients in the YES cohort. However, implementation of an anaerobic condition for urine cultures is not necessarily feasible for clinical microbiology laboratories. Thus, an optimal and feasible urine culture would be BAP, CNA, and MacConkey in 5% CO₂. Despite the low levels of detection of uropathogens by MacConkey agar (aerobically), this medium allows for phenotypic distinctions between morphologies, which is particularly useful in diagnosing *E. coli* and *Klebsiella pneumoniae* infections (the two most common uropathogens). Using 100µL of urine and holding for 48 hours in 5% CO₂ for these plates would have resulted in 79% (99/126) total uropathogen detection, and 87% (47/54) uropathogen detection in the YES cohort. These data stem from of holding the MacConkey agar in aerobic conditions. It is unclear how much of a difference incubating in 5% CO₂ will make.

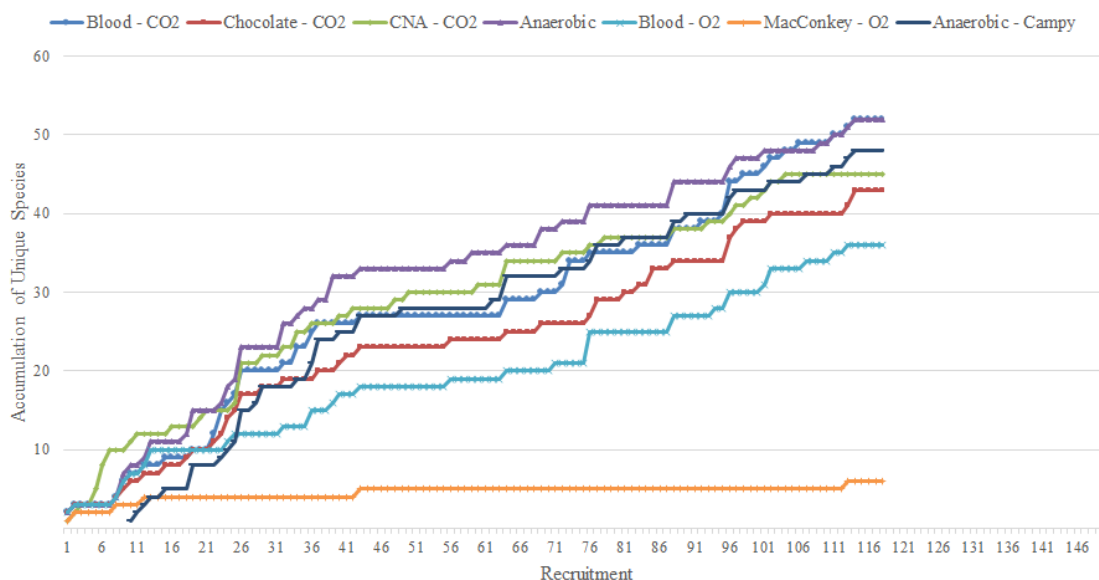


Figure A3. Rarefaction Analysis of Various Urine Culture Plating Conditions. The BAP in 5% CO₂ condition group contains 52 unique species and is depicted by the blue circles. The Chocolate agar in 5% CO₂ condition group contains 43 unique species and is depicted by the red squares. The CNA in 5% CO₂ condition group contains 45 unique species and is depicted by the green diamonds. The Anaerobic BAP incubated anaerobically group contains 52 unique species and is depicted by the purple triangles. The BAP incubated aerobically group contains 36 unique species and is depicted by the blue x's. The MacConkey agar incubated aerobically group contains 6 unique species and is depicted by the orange pluses. The Anaerobic BAP incubated in a Campy environment group contains 48 unique species and is depicted by the dark blue dashes.

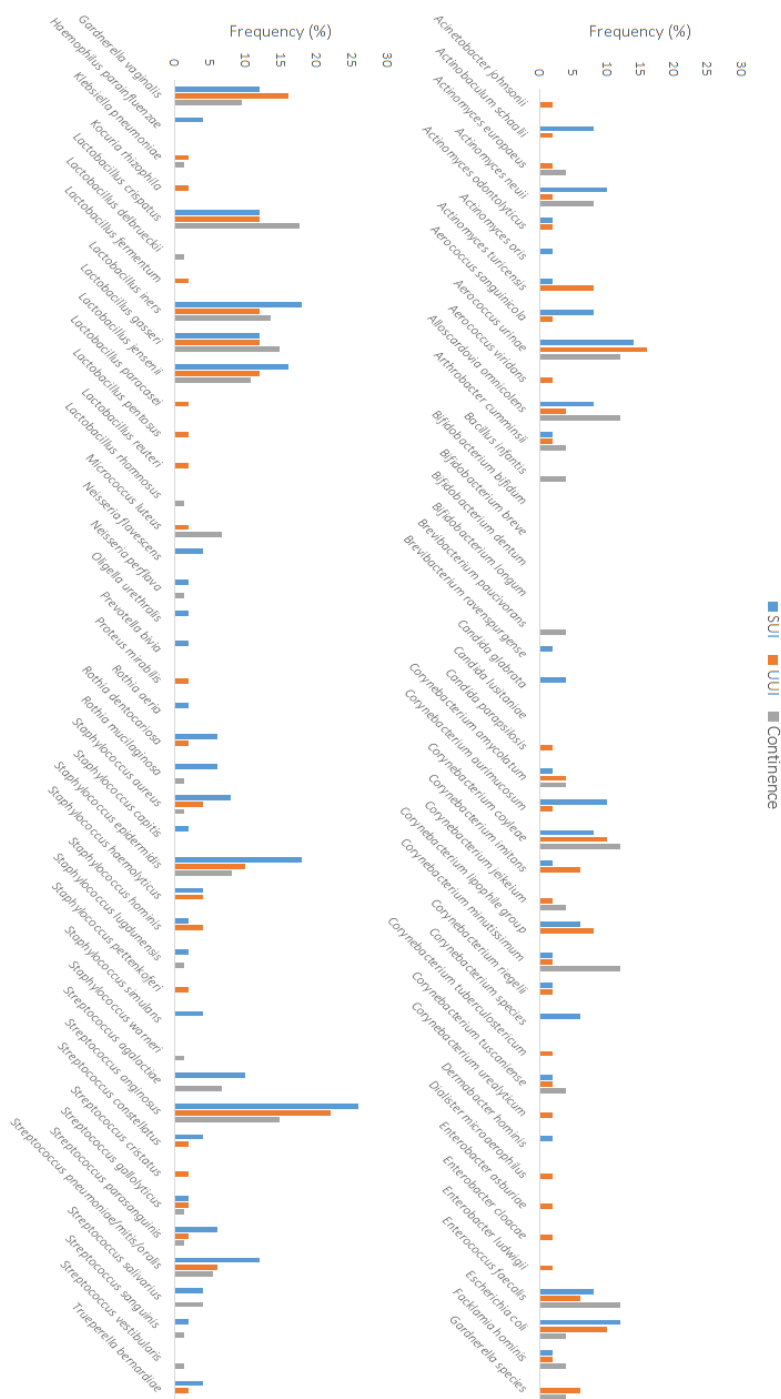
To conclude, it is clear from these data, that current methods and interpretation of data using the standard urine culture protocol are overwhelmingly inefficient. We recommend adopting the optimal urine culture protocol detailed above, as well as a reconsideration of the current thresholds for UTI diagnosis.

Uropathogen Detection		Blood CO ₂	Chocolate CO ₂	CNA CO ₂	Anaerobic	Blood O ₂	MacConkey O ₂	Anaerobic CAMPY
<i>Actinobaculum schaalii</i>	5	2	0	4	3	0	0	4
<i>Aerococcus urinae</i>	11	5	5	7	4	4	0	0
<i>Alloscardovia omnicolens</i>	6	3	1	4	4	0	0	1
<i>Candida albicans</i>	2	2	2	2	2	2	0	2
<i>Candida parapsilosis</i>	4	1	1	2	2	0	0	1
<i>Citrobacter koseri</i>	1	1	1	0	1	1	1	0
<i>Corynebacterium riegelii</i>	3	1	1	0	2	1	0	1
<i>Corynebacterium urealyticum</i>	1	1	0	0	0	1	0	0
<i>Enterobacter aerogenes</i>	3	2	2	0	1	1	2	2
<i>Enterococcus faecalis</i>	12	5	4	7	5	7	0	6
<i>Escherichia coli</i>	25	23	23	3	24	23	24	18
<i>Klebsiella pneumoniae</i>	8	5	4	0	7	6	5	5
<i>Proteus mirabilis</i>	1	1	1	0	1	1	1	1
<i>Pseudomonas aeruginosa</i>	1	1	1	0	1	1	1	1
<i>Serratia marcescens</i>	1	0	1	0	1	0	0	0
<i>Staphylococcus aureus</i>	6	3	3	3	2	3	0	3
<i>Streptococcus agalactiae</i>	10	8	6	5	6	6	0	5
<i>Streptococcus anginosus</i>	26	13	8	19	13	2	0	13
TOTAL	126	77	64	56	79	59	34	63

Table A2. Overview of Plating Conditions for Detection of Uropathogens. Depicted are a list of the uropathogens and the number of times each was cultured by the various culturing conditions.

APPENDIX B:

SUPPLEMENTAL FIGURES



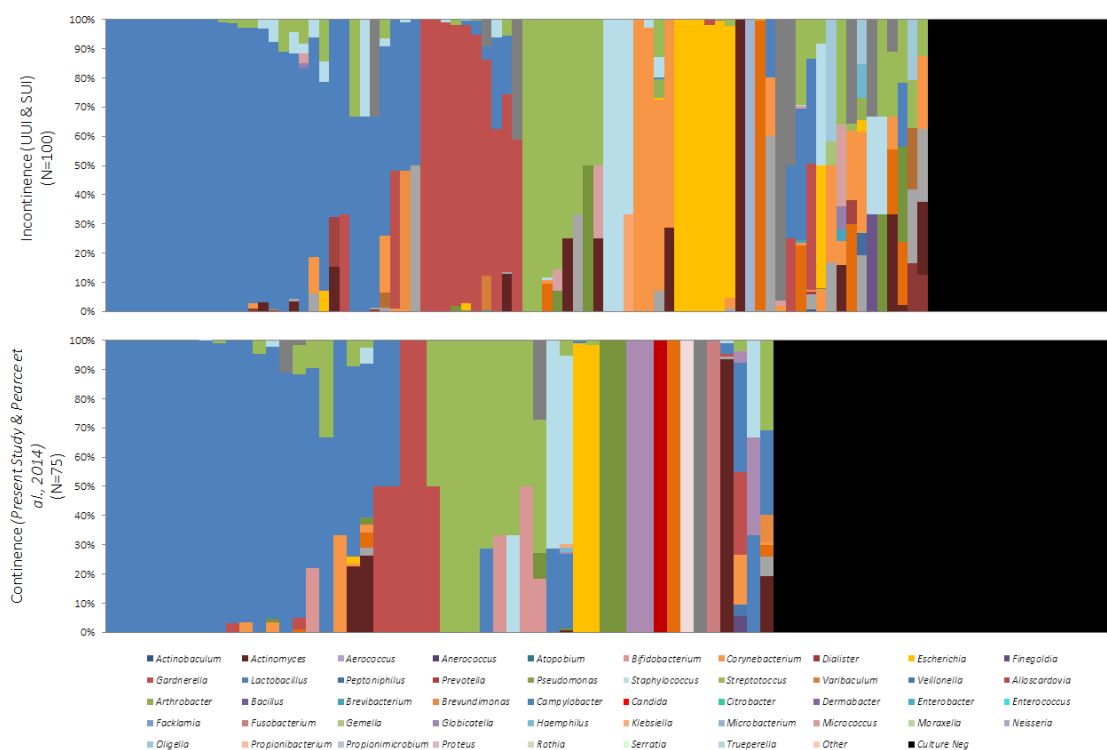


Figure S2. Microbiota Profiles of Incontinence & Continence Cohorts. Depicts the genus-level microbiota composition based on percent CFU/mL urine for the given patient urine sample. The Continence cohort combines data from *Chapter III* and from Pearce *et al.*, 2014 (*bottom*) and contains 75 urine samples. The Incontinence cohort combines the UII and SUI cohorts (*top*) and contains 100 urine samples. The figures have been stretched to be equivalent sizes for better qualitative comparisons.

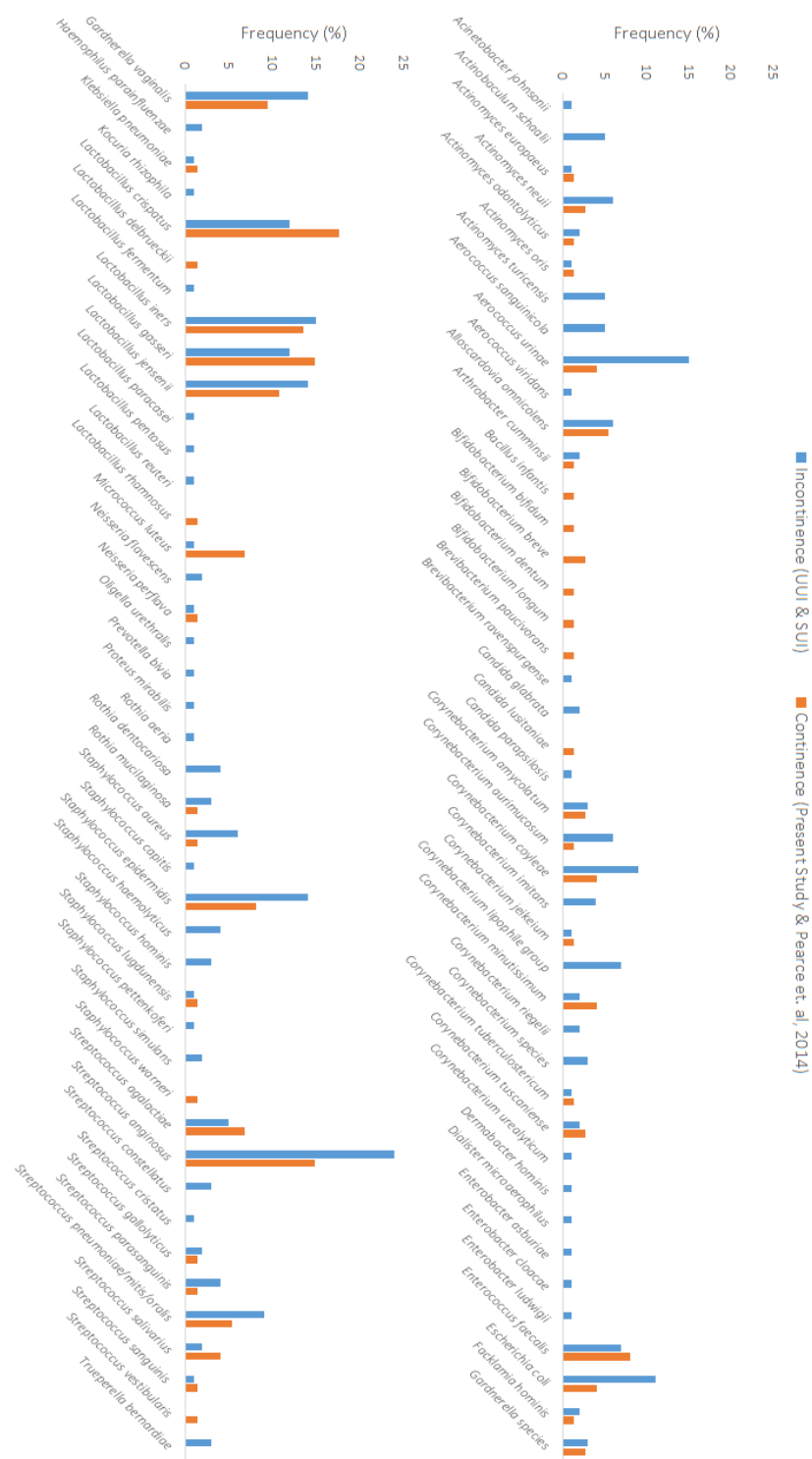


Figure S3. Frequency of Species in Incontinence & Continence Cohorts. The Incontinence cohort (N=100) is depicted by the blue bars and the Continence cohort (N=75) is depicted by the orange bars.

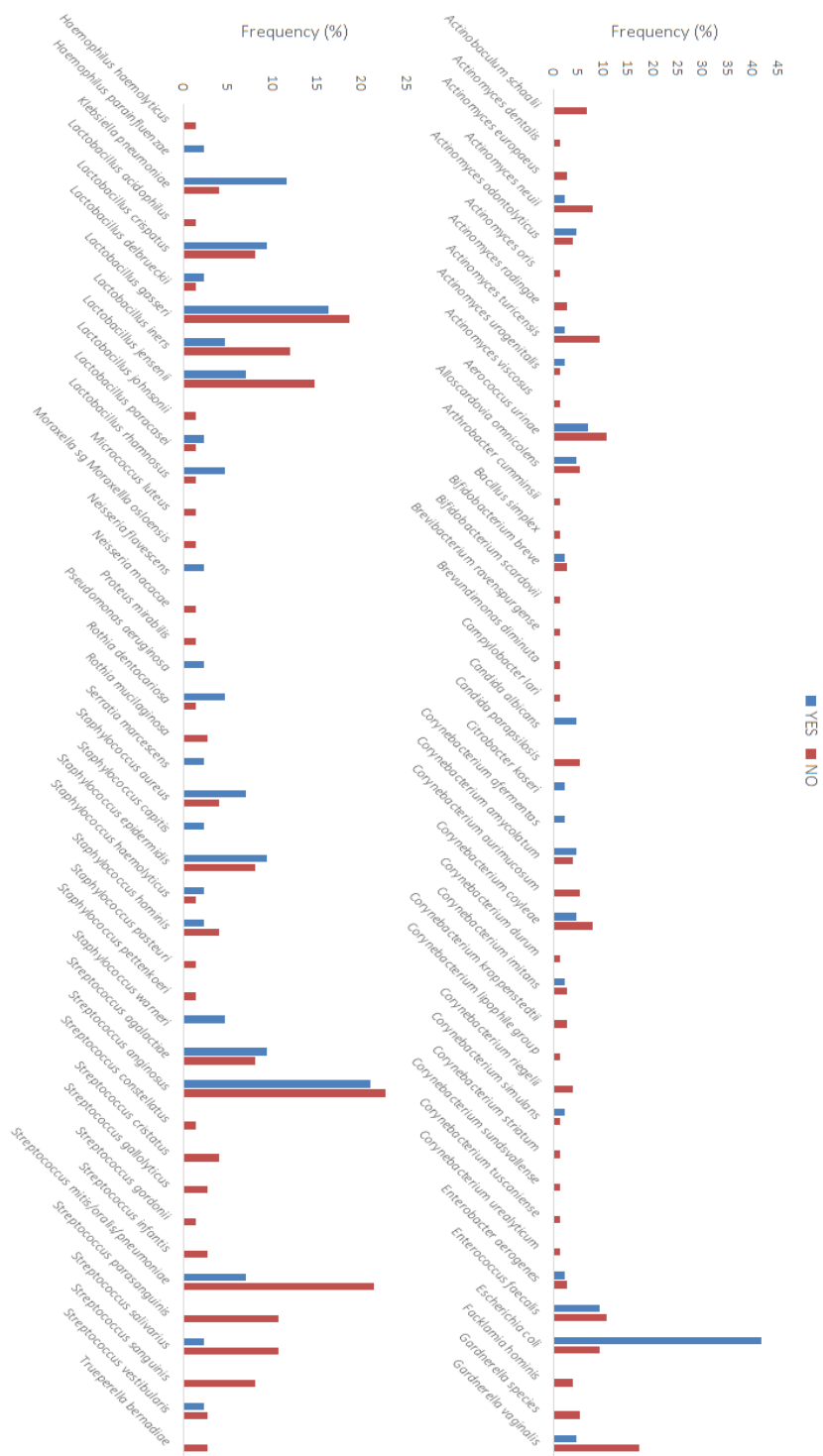


Figure S4. Frequency of Species in the YES & NO Cohorts. The YES cohort (N=43) is depicted by the blue bars and the NO cohort (N=75) is depicted by the red bars.

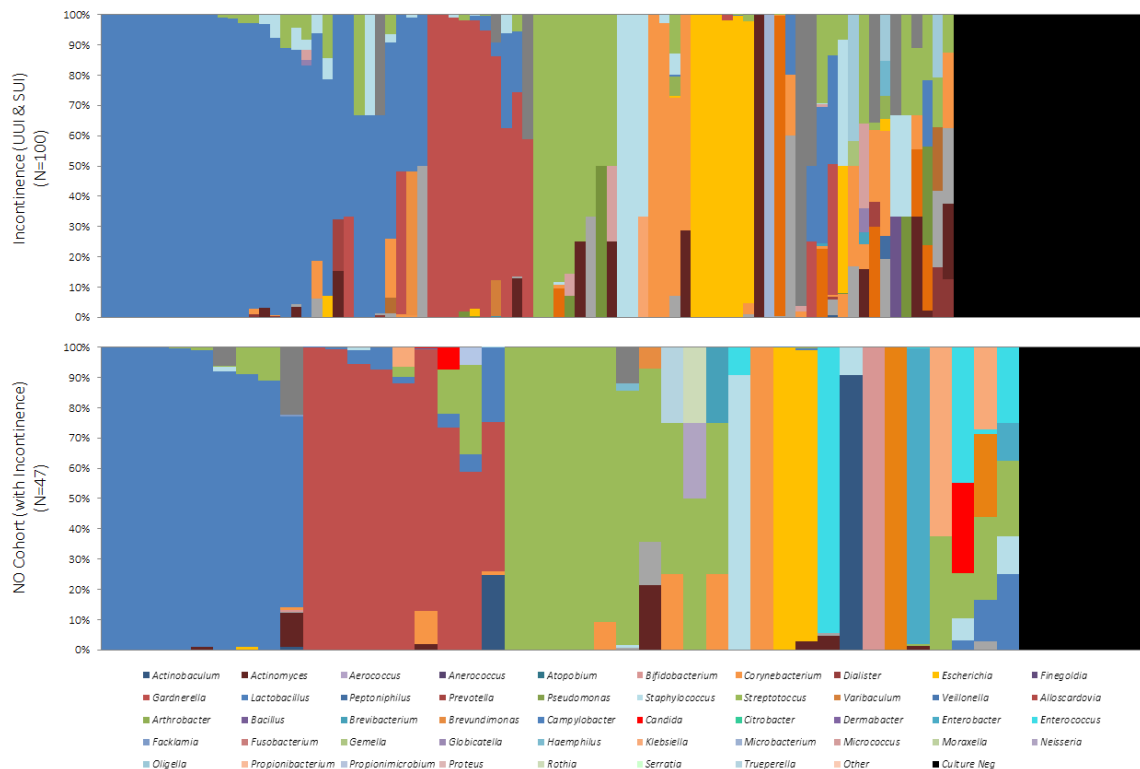


Figure S5. Microbiota Profiles of Incontinence Cohorts. Depicts the genus-level microbiota composition based on percent CFU/mL urine for the given patient urine sample. The Incontinence (UII & SUI) cohort from *Chapter III (top)* contains 100 urine samples. The NO Cohort from *Chapter IV (bottom)* contains 47 urine samples from individuals within the NO Cohort (N=75) who also had urinary incontinence. The figures have been stretched to be equivalent sizes for better qualitative comparisons.

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VITA

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