A Susceptibility Study of Urinary Isolates to Methenamine Hippurate and its Metabolites

Nancy Sloan

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<tr>
<td>BAP</td>
<td>Blood Agar Plates</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion broth</td>
</tr>
<tr>
<td>CFU/mL</td>
<td>Colony Forming Units per milliliter</td>
</tr>
<tr>
<td>EQUC</td>
<td>Expanded Quantitative Urine Culture</td>
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<tr>
<td>LUEREC</td>
<td>Loyola Urinary Research and Education Collaborative</td>
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<td>LUTS</td>
<td>Lower Urinary Tract Symptoms</td>
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<tr>
<td>MBC</td>
<td>Minimum Bactericidal Concentration</td>
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<tr>
<td>MH</td>
<td>Methenamine hippurate</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
</tr>
<tr>
<td>MRS</td>
<td>deMan, Rogosa, Sharpe medium</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>RUTI</td>
<td>Recurrent Urinary Tract Infection</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SPA</td>
<td>Supra-Pubic Aspirate</td>
</tr>
<tr>
<td>SUC</td>
<td>Standard Urine Culture</td>
</tr>
<tr>
<td>TUC</td>
<td>Transurethral Catheterization</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary Tract Infection</td>
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ABSTRACT

Urinary Tract Infection (UTI) is one of the most common bacterial infections in adult women, with an estimated 80% of adult women experiencing a UTI in their lifetime. A subset of those women (up to 44%) will experience a second episode within the next three months. This recurrence is known as recurrent UTI (RUTI), defined as at least three infections in a year or two in six months. As UTIs and RUTI are so prevalent, treatment for these conditions is one of the most common reasons for antibiotic prescription. The rise of antibiotic-resistant infections coupled with the shortage of new antibiotics creates an urgent need for alternative non-antibiotic treatments.

One alternative is the salt methenamine hippurate (MH), which is a non-antibiotic prophylaxis. Unlike conventional antibiotics, bacteria are not reported to acquire resistance to MH. It is also reported to be active against some common uropathogens including *Escherichia coli* and *Enterococcus* species, but its effects on the newly discovered bladder microbiota (urobiome) are undocumented. Given this knowledge gap, this study aimed to evaluate the drug’s spectrum of susceptibility on UTI-relevant bacteria. Thus, in Aim 1, I have characterized the effects of MH and its metabolites on common uropathogens and putative commensal species. We hypothesized that because of MH’s unique mechanism of action, some bacteria are more strongly affected than others and, in most cases, these sensitivities cannot be predicted *a priori*. In Aim 2, a study that is ongoing, we are determining the longitudinal effect of MH...
treatment on the urobiomes of women with RUTI and testing whether exposure to MH or its metabolites can decrease sensitivities.
CHAPTER I

INTRODUCTION

It is now known that the bladder is not sterile, new knowledge that has implications for many urinary disorders. The recent discovery of the urinary microbiome (urobiome) requires a re-evaluation of, among many other urinary disorders, urinary tract infection (UTI), which may not be strictly due to an invasion of a sterile environment by a pathogenic bacterium, as generally thought (Price et al. 2018). Furthermore, there are bacteria, such as *Lactobacillus* sp. and *Streptococcus* sp., present in the bladders of many asymptomatic women that may have a commensal relationship and may provide a protective effect against uropathogens (Hilt et al. 2014).

Antibiotic treatment for UTI is generally empirical and focused on the old dogma that UTIs are caused by suspected uropathogens invading the “sterile” bladder. In some but not all cases, the patient’s urine is tested for the presence of a single uropathogen by the typical or standard urine culture (SUC) protocol. However, SUC was designed to detect fast growing aerobic bacteria with no atypical nutrient requirements, biasing a positive urine culture toward members of the family Enterobactericeae (especially *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*) and a few related Proteobacteria (such as *Pseudomonas aeruginosa*) (D’Souza et al. 2004). As such, these species are ascribed to be the causes of UTI.
Women who experience recurrent UTI (RUTI) are vulnerable to the burden of increased antibiotic usage and bacterial antibiotic resistance. Antibiotic treatment is costly, inconvenient and disregards the presence of the urobiome. Methenamine hippurate is one alternative to the prophylactic antibiotics that are often prescribed to women who suffer from RUTI. Its antibacterial activity is reported to be effective on *E. coli* and some of its relatives; however, the spectrum of susceptibility beyond these uropathogens is unreported.

**Literature Review**

**History Behind the Dogma that “Urine is Sterile”**

The paradigm of “sterile urine” dates back almost two centuries, to the mid 19th century, when bacteriologists observed that a sealed vial of urine incubated in ambient conditions would not become cloudy. If the vial was left open, however, the urine became cloudy, evidence of growth of microorganisms (Duclaux 1920). These observations led the bacteriologists to falsely conclude that “fresh and healthy” urine is sterile (Roberts 1881, Bloom et al. 1994). At this time, “germ theory,” which states that pathogenic microbes cause disease, was becoming established. In keeping with this theory, bacteria detected in urine were considered pathogens.

The dogma that urine is sterile and its correlation with asymptomatic individuals was further propagated in the 1950s with the advent of culturing urine to diagnose patients’ kidney infections. To detect pathogens for diagnosis of kidney infection (pyelonephritis), infectious disease physician Dr. Edward Kass developed a non-invasive urine culture technique using midstream voided urine (Kass 1956, Kass 1957). He hypothesized that patients with pyelonephritis had urine that would grow large numbers of uropathogenic bacteria, as
compared to asymptomatic individuals who had urine that did not grow bacteria or grew smaller numbers. Kass distinguished these “contaminant” bacteria from infectious bacteria by setting a threshold of greater than $10^5$ colony forming units per mL (CFU/mL) of any species that contained known uropathogens.

SUC is considered to be the “gold standard” for determining bacteriuria for UTI diagnosis (Procop et al. 2017). Clinical microbiologists will report a SUC-negative culture as “no growth.” But “no growth” is more accurately “no E. coli” or other uropathogen capable of growing under SUC conditions. Although biased towards the specific culture conditions, this “no growth” has been falsely equated with “sterile” urine. This message that “healthy” urine is sterile has propagated throughout the education of medical students, ultimately affecting their practice of patient care as physicians.

The dogma that “fresh and healthy” urine is sterile was not seriously challenged until 1979 when Dr. Rosalind Maskell, an English clinical microbiology lab director, and her colleagues, Drs. Pead and Allen, observed that patients with UTI symptoms who had a negative result by SUC, often responded well to antibiotic treatment (Maskell et al. 1979). This team succeeded in culturing microbes from urine obtained by suprapubic aspiration (SPA) or by transurethral catheterization (TUC). Microbes that did not grow under ambient aerobic conditions could be cultured by incubating the urine samples in 5% carbon dioxide for more than 24 hours. From these observations, they concluded that SUC is inadequate for diagnosis of UTIs and other urinary disorders, including dysuria (defined as pain or discomfort when urinating). They further concluded that SUC is insufficient for culturing fastidious and/or slow-growing bacteria (Maskell 2010). They reported their findings and recommended a concerted
effort by clinicians and microbiologists to better understand urinary disorders. Unfortunately, their findings were deemed contamination or rejected (Maskell 1988) and the “sterile urine” dogma remained accepted until recently when the advent of culture-independent high throughput DNA-based methods permitted the sensitive and detailed study of the microbes that colonize humans.

**The Human Microbiome Project (HMP)**

It wasn’t until relatively recently in the timeline of the study of human-microbe interactions that it has been widely accepted that majority of human body sites are colonized by largely non-pathogenic bacteria. In 2007, the National Institutes of Health (NIH) funded the first large-scale study to characterize the human microbiome, a massive effort to understand the microbes involved in human health and illness (Peterson et al. 2009). Originally, the term “microbiome” was defined as "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (Lederberg and McCray 2001). Later, with the advent of culture-independent methods, it was defined as the collection of these microbes and their genetic content (Ursell et al. 2012). More recently, the definition has become more restricted to the genomes of the microbiota, which are the microbes within the host (Marchesi and Ravel 2015). Nonetheless, this more recent definition of microbiome is alike to another microbiome term, “metagenome,” defined as the collection of genes and genomes from the microbiota species (Handelsman 1998). The terms microbiome and microbiota are still often used interchangeably.

For the initial HMP Project, emphasis was placed on 5 body sites (niches): gastrointestinal tract, oral cavity, skin, vagina, and respiratory tract. The collaboration of
institutes collecting specimens from these niches used culture-independent identification methods, relying mainly on 16S ribosomal RNA (rRNA) gene amplicon sequencing (Peterson et al. 2009). The HMP discovered, among many important findings, that more than 10,000 species live within the human ecosystem and that large numbers of diverse non-pathogenic microorganisms inhabit humans. Little was known about most of these native residents of the human body. The HMP also found that there is a vastly greater number of microbial genes than human genes within the human body. The bladder was not included as one of the sites of focus in the HMP, mainly because it was regarded as a sterile niche.

**Evidence Confirms Live Bacteria in the Bladder**

In 2012, researchers from the Loyola Urinary Education and Research Collaborative (LUEREC) used the most common sequencing technique for classifying bacteria in a population, 16S rRNA gene amplicon sequencing, to provide evidence of bacterial DNA in urine taken directly from the bladders of adult women without active UTI and irrespective of other Lower Urinary Tract Symptoms (LUTS), who were undergoing urogynecological surgery (Wolfe et al. 2012). LUREC is an interdisciplinary team that includes basic scientists, clinicians, urogynecological fellows, clinical microbiologists, bioinformaticians and biostatisticians. To sample urine from the bladder, like Rosalind Maskell and her co-authors, they obtained urine by SPA and TUC. With one exception, these urine samples were culture-negative by SUC. In contrast, 16S rRNA gene sequencing detected bacteria DNA in many of the samples. Since sampling by SPA bypasses the vulvovaginal region, this was evidence of bacteria in the bladder. Furthermore, as the SPA and TUC results resembled each other and both were distinct from paired voided urine, TUC (the less invasive of the two procedures) was determined to be an
acceptable method for sampling urine from the bladder. Because the microbiome of voided
urine often resembled that of vaginal swabs, the authors concluded that data obtained using
voided urine cannot be interpreted as bladder microbes alone and instead must be interpreted
as a mixture of bladder microbes and everything downstream, including urethral and vulvo-
vaginal microbes.

LUEREC then developed the expanded quantitative urine culture (EQUC) protocol to
determine whether the detected DNA belonged to live bacteria, as opposed to non-living
bacteria or naked DNA. Relative to SUC, EQUC plates a larger volume of urine on a variety of
media under diverse atmospheric incubation conditions with double the incubation time.
Indeed, bacteria could be isolated from 90% of the SUC-negative cultures. Furthermore, most of
the bacterial taxa identified by sequencing were found to grow under EQUC conditions (Hilt et
al. 2014). Others have performed urinary microbiome studies with the conclusions that the
adult female bladder is not sterile (Fouts et al. 2012; Khasriya et al. 2013; Pearce et al. 2014;
Karstens et al. 2016; Coorevits et al. 2017; Ackerman and Underhill 2017). Thus, work over the
past decade has disproven the long-standing dogma that the adult asymptomatic female
bladder is sterile. Instead, the female bladder possesses its own indigenous community of
microbes called the female urinary microbiome/microbiota (FUM) that is disparate from
vulvovaginal contamination.

SUC has repeatedly had a false-negative rate of 90% (Hilt et al. 2014; Pearce et al. 2014;
Thomas-White et al. 2015). Our group and others have shown that SUC reproducibly detects *E.
coli* and some quickly growing, aerobic relatives in the family Enterobacteriaceae (e.g., *K.
pneumoniae* and *P. mirabilis*). Although it theoretically can detect other “clinically relevant
microorganisms,” SUC often misses them (Price et al. 2016). This category includes

*Enterococcus faecalis, Streptococcus anginosus, Streptococcus agalactiae* and *Aerococcus urinae*. These species either grow more slowly, require a selective medium to be detected (some Gram-positive genera), need atypical nutrients or elements, and/or require non-ambient atmospheric conditions. SUC misses these species because Kass designed it to detect the most common cause of pyelonephritis, *E. coli*, which grows rapidly within 24 hours under aerobic conditions.

SUC was effective at improving the outcome of patients with pyelonephritis as Kass intended it. His culture protocol, however, was not intended for diagnosis of bladder infection (cystitis) but today it is used for this purpose without sound evidence. Many studies since the 1950s have reported on the inadequacy of the threshold, all reporting that less than \(10^5\) CFU/mL for any uropathogen (even as low as \(10^2\) CFU/mL) can be indicative of cystitis (Gallagher et al. 1965; Stamey et al. 1971; Stamm et al. 1982; Stark and Maki 1984; Platt 1983). Furthermore, the threshold was designed for examining voided urine, which will naturally have higher mass than urine obtained from the bladder. Finally, this threshold was designed for adults, whereas there is no consensus on a threshold for pediatrics (Doern and Richardson 2016). In clinical microbiology labs, however, the SUC protocol continues to be implemented for cystitis diagnosis.

**The Microbiota/Microbiome of the Asymptomatic Adult Female Bladder**

It is critical to acknowledge and characterize the FUM of women with no lower urinary tract symptoms. To study the asymptomatic FUM, two independent but complementary approaches are used: culture-dependent (EQUC) and culture-independent (16S rRNA gene
sequencing). In a FUM study using both EQUC and 16S rRNA gene sequencing, the majority (58%) of urine samples had detectable bacteria by both methods, with a total of 90% having bacteria detected by either method or both (Pearce et al. 2014). Bacteria were cultured from 27% of sequence-negative samples, whereas bacterial DNA was sequenced from 6% of EQUC-negative samples. In some samples, EQUC detected genera that were not detected by sequencing. This was not due to the inability of the universal primers to amplify these genera, because they were detected by sequencing in other samples. Only one genus, *Trueperella*, was cultured but not sequence-detectable in any of the samples. Several genera, including *Atopobium* (a strict anaerobe), were sequence-positive but not EQUC-positive. Together these data demonstrate the limitations of each analysis method, but show that results are strengthened when both methods are used. Strong evidence that EQUC provides comparable results to sequencing (aside from difficult-to-culture strict anaerobic genera) has been further demonstrated (Price et al. 2020).

These complementary approaches determined the urotypes of the asymptomatic FUM. Urotype is a term used for the phylotype of any individual urinary microbiome. Phylotype is defined as a communities of sequences that share similarity likely based on evolutionary relationships; it is akin to the term “enterotype” used to describe gut microbiomes (Arumagam et al. 2011; Pearce et al. 2014). Urotypes are generated by taxonomical clustering of the urinary microbiota; often they are named by the predominant family, genus or species in a given sample. The *Lactobacillus* urotype has repeatedly been found to be the most common amongst asymptomatic women (Pearce et al 2014; Price et al. 2020; Price et al. in review). Other common urotypes are *Gardnerella*, *Streptococcus* and *Lactobacillus/Streptococcus* (Hilt et al.
2014; Brubaker and Wolfe 2016). When there is no predominant taxon, the urouptype is called “diverse.”

As the *Lactobacillus* urouptype is common across FUM profiles, it is important to discuss in further detail. In other niches, particularly the vagina and gut, a high abundance of *Lactobacillus* is considered beneficial; it is not a traditional pathogen. In fact, it has been revealed that *L. crispatus* associates with asymptomatic women, but rarely with women with LUTS (Pearce et al. 2014). Another species, *L. iners*, correlates with protection against post-instrumentation and post-operative UTI (Pearce et al. 2015; Thomas-White et al. 2015). Therefore, the microbiota of bladder, similar to the vagina, may have protective properties. A study to describe the relatedness of bladder versus vaginal microbiota isolated from the same individual revealed that similar species isolated from the bladder and vagina of an individual were highly related (Thomas-White et al. 2018). Interestingly this also demonstrates that microbiota migration is not limited to uropathogens but may also apply to commensal bacteria.

The discovery that a lack of symptoms correlates with certain bacterial genera is pivotal in the paradigm shift of LUTS disorders, particularly in UTI. As EQUC and sequencing have repeatedly shown that majority of adult female bladders are not sterile (“bacteriuric”), that appeals for a redefinition of the term urinary tract “infection.” For example, in a study by our group, detection of urinary bacteria correlated with greater symptom resolution and a decreased incidence of post-instrumentation UTIs (Pearce et al. 2015). Previous dogma proposed that commensal vaginal microbiota protects the sterile bladder from invading uropathogens. Similar to the bladder, the vaginal microbiota of asymptomatic women is frequently dominated by *Lactobacillus* species (Redondo-Lopez et al. 1990; Lloyd-Price et al.
2016). However, the bladder microbiota may provide its own protection from invading uropathogens.

While the FUM is detected in most asymptomatic women, it is not detected in all. Nonetheless, FUM “below the limit of detection” does not necessarily equate with “sterile;” these bladders are not necessarily without microbes (Brubaker and Wolfe 2017). For example, 16S rRNA gene sequencing does not detect fungi and EQUC can only detect members of the yeast genus Candida. One study showed that fungal species are present in the LUT; this “mycobiome” is in low biomass and discovery is challenging (Ackerman and Underhill 2017). These findings show that other microbes can be present in the LUT but are below the detection sensitivity.

**FUM and Associations with Lower Urinary Tract Conditions and Diseases**

We now know that the female lower urinary tract (the bladder and/or the urethra) contains resident bacterial communities (FUM). LUEREC, along with other groups, are committed to understand the FUM and its impact on pathophysiology. In contrast to UTI, which is universally considered to be an “infection” and is equated with a positive standard urine culture, non-UTI LUTS conditions are “diagnoses of exclusion,” meaning a diagnosis is made after ruling out others (Scott et al. 2015). These diagnoses might mean symptoms not falling into one category (e.g. no test to prove the illness exists, or ultimately course of treatment might be difficult) (Fred 2013). Many of these non-UTI LUTS conditions and diseases with previously no known bacterial etiology have now been associated with the urinary microbiota.

A pivotal study demonstrated that the microbiota of women diagnosed with overactive bladder (OAB) are disparate from continent control bladders (Pearce et al. 2014). More
precisely this study is of women with urge urinary incontinence (UUI), which is the incontinent ("wet") form of OAB. There have been no studies to date on “dry” OAB. It was discovered that, whereas a comparable number of women with and without OAB had detectable bacterial-positive urine samples, women with OAB were less frequently colonized by *Lactobacillus* species and more frequently colonized by *Gardnerella*. Thomas-White et al. provided evidence that there is a correlation between bacterial diversity and OAB treatment response (Thomas-White et al. 2016). These results show that the FUM can be correlated with disease. A study of voided urines from women with OAB also found that the average number of bacterial genera sequenced in both controls and OAB patients were equivalent, but *Lactobacillus* was less prevalent and *Proteus* more prevalent in the OAB cohort as compared to controls (Curtiss et al. 2017). Although voided urines were used, the finding that *Lactobacillus* is less frequent in OAB is in accordance with both studies using catheterized urine. Using 16S rRNA gene sequencing, Brubaker et al. found that in a cohort of 155 women with UUI, 39% had a detectable, “sequence-positive” microbiome. This subset of women had considerably higher Urgency Urinary Incontinence Episodes (UUIE) per day than the women who were “sequence-negative” (Brubaker et al. 2014). Pearce et al. also found that “sequence-positive” catheterized urines correlated with higher UUIE per day and, furthermore, that women with UUI have a higher prevalence of ten bacterial species. For example, *Aerococcus urinae*, an emerging uropathogen, was found to be concurrent with a decrease in *Lactobacillus* in UUI catheterized urines (Pearce et al. 2014; Pearce et al. 2015). On the species level, *L. gasseri* was more frequently detected in UUI urines, while *L. crispatus* was more frequent in asymptomatic controls. Karstens et al., however, found an opposite association in their study using catheterized urines; women with
lower alpha diversity scores had significantly increased daily episodes (Karstens et al. 2016). That an increase in uropathogens, both classical and emerging (which are associated with UTIs), and a decrease in certain *Lactobacillus* specie associate with women with UUI, signifies that perhaps the distinctions between LUTS disorders are less defined than has previously been thought.

In one study of women receiving surgery for uncomplicated stress urinary incontinence (SUI), urine specimens were collected and analyzed by sequencing. Of the 197 urine specimens, only 23 were collected by catheterization and the rest were voided. Of this large sample size, 86% of samples were “sequence-positive,” but no association was found between SUI symptoms and FUM bacterial diversity. Instead, UUI symptoms correlated with a loss of *Lactobacillus* predominance (Thomas-White et al. 2017). In a more recent study of 126 urogynecological SUI patients, SUI symptoms were found to associated with “sequence-positive” catheterized urines (Fok et al. 2018). More symptomatic patients’ urines correlated with a greater abundance of two bacterial species: *Atopobium vaginae* and *Finegoldia magna*. *Atopobium vaginae*, which is considered to associate with bacterial vaginosis (BV) (Onderdonk et al. 2016), was also found in paired vaginal and perineal swabs. In a study of mixed urinary incontinence (MUI), using only catheterized urine from 207 participants, *Lactobacillus* predominance did not correlate with whether the urine was from MUI or an asymptomatic control (Komesu et al. 2018). Due to the conflicting results, it cannot be determined whether a distinct MUI microbiota exists that is separate from an asymptomatic FUM.
On the contrary, some urinary conditions may not involve bacteria or other microbes. Interstitial cystitis/painful bladder syndrome (IC/PBS) is one disorder that has conflicting evidence as to whether the urinary microbiome has a symptomatic role. Via sequencing, voided urine from women with IC was found to have both reduced species diversity and richness compared to asymptomatic women (Siddiqui et al. 2012). In fact, it was observed that “more than 90% of the IC sequence reads were identified as belonging to the bacterial genus *Lactobacillus*” as compared to 60% in asymptomatic urines. Using sequencing and catheterized urines, the finding of less bacterial diversity in women with IC was established by another group, but reported lower abundance of *Lactobacillus* in IC urines than in asymptomatic urines (Abernethy et al. 2017). A higher abundance of urinary fungal species was reported to be correlated with greater symptom severity in women with IC/PBS (Nickel et al. 2019). A study by the same group and another, however, have shown that there are no differences in the urinary bacterial microbiota profiles of IC/PBS female patients and asymptomatic controls (Nickel et al. 2019; Bresler et al. 2019). A study comparing both the vaginal and voided urine microbiota of women with IC/PBS compared to asymptomatic controls found no variations between cohorts (Meriwether et al. 2019). In a 2020 study by Jacobs et al., 49 IC/PBS women patients and 40 women controls provided catheterized urine samples (Jacobs et al. 2020). By EQUC and 16S rRNA gene sequencing of some samples, *Lactobacillus* was found to be a urotype in both groups (about 50% in each) and was not correlated with symptom severity. Since EQUC can detect *Candida*, this study provided evidence against the hypothesis that this particular yeast is related to IC/PBS symptoms. Therefore, IC/PBS symptoms may not be due to urinary microbiota differences from those who are asymptomatic.
There are several conditions that are not traditionally associated with a bacterial etiology. Once attributed to particularly urease-producing bacteria, kidney stones have been found by our group and others to harbor diverse, often uropathogenic, bacteria (Barr-Beare et al. 2015; Mehta et al. 2016; Bajic et al. 2018; Dornbier et al. 2019; Xie et al. 2020). These results signify that bacteria might contribute to formation of some kidney stones. Although more studied in gut microbiota, Type 2 diabetes mellitus (of which, a major complication is LUTS) has been found by a few groups to associate with lower urinary microbiota diversity in female midstream voided urine (Cameron et al. 2006; Liu et al. 2017; Chen et al. 2019; Penckofer et al. 2020). One group attributes the alteration in diabetic patients’ microbiota to an increase in the proinflammatory chemokine interleukin, IL-8 (Ling et al. 2017). In patients with non-dialysis-dependent chronic kidney disease (NDD-CKD), Kramer et al. found higher bacterial diversity in midstream voided urine that associates with higher estimated glomerular filtration rate, which is a measure of kidney function (Kramer et al. 2018).

UTIs disproportionately affect adult women, which is likely due in part to a shorter female urethra (typically about three centimeters in length whereas the male urethra can nearly five times longer) (Abelson et al. 2018). The short length of the female urethra is thought to facilitate ascension of uropathogens into the bladder (Haddock 2015). Besides being female, other risk factors include sexual intercourse, use of certain body products (e.g. douches), diet (e.g. alcohol and other inflammatory foods), having an indwelling catheter and overuse of antibiotics (Hooton et al. 1996; Flores-Mireles et al. 2015; Storme et al. 2019). Although there are a multitude of risk factors, a lack of beneficial bacterial species providing protection against UTI has not been examined until recently.
The predominance of uropathogens, and a lower abundance of certain *Lactobacillus* species in the bladders of women with UUI, may represent a higher risk for developing a UTI. In another recent study of UI patients undergoing pelvic organ prolapse and urinary incontinence (POP-UI) surgery, their catheterized urines were assessed by SUC on the day of surgery (Thomas-White et al 2018). Less than 10% of samples had urinary microbes detected by SUC (SUC-positive) and about half had SUC-negative cultures, but the microbiome was detected by sequencing. The 10% SUC-positive samples tended to be predominated by Enterobacteriaceae and 32% of these patients went on to develop a post-operative UTI. The SUC-negative group had a mere 3.7% post-operative UTI rate. Of that group, those with microbiomes detectable by sequencing tended to be dominated by *Lactobacillus* instead of Enterobacteriaceae. In addition to the correlation of post-operative UTI risk and pre-operative microbiome profile, the abovementioned study provides evidence that utilizing SUC may not be in a patient’s best interest; EQUC and sequencing are more sensitive techniques to determine what microbes are present and their judicious use may improve patient outcomes.

Consortia of viruses (virome) have now been associated with UTI and non-UTI voided urine samples (Santiago-Rodriguez et al. 2015). In this study, bacteriophage (viruses that infect bacteria) vastly outnumbered eukaryotic viruses (those that infect human cells), most often human papillomaviruses (HPV), which were found in 95% of the samples. This study had a small sample size of 20 participants and did not find an association between the virome profile and UTI status. In another study using catheterized urines from a previously published study investigating the OAB microbiota profile (Pearce et al. 2014), 20 urines from women with OAB and 10 urines from women without LUTS were analyzed using metagenomic sequencing,
specifically for viral sequences (Garretto et al. 2018). After overcoming significant challenges in isolating viral species, 90% of bladder urines had detectable sequences predicted to be viral, which included bacteriophages and eukaryotic viruses. The frequency of viral sequences represents another plausible association between the urinary virome, LUTS and urinary tract health.

In addition to bacteria and viruses, diverse fungal species have also been detected in the bladder. A community of fungal species in a host environment is termed the “mycobiome.” EQUC coupled with MALDI-TOF is able to detect Candida spp. isolated from the bladder, but beyond Candida the urinary mycobiome likely requires culture-independent methods. A pivotal study exploring the urinary mycobiome (after facing significant protocol challenges) revealed fungal species diversity beyond Candida spp. and highly variable mycobiomes between individuals sampled (Ackerman and Underhill 2017).

These studies provide evidence for correlation between the FUM and components of incontinence, infection and health that were once presumed to not have a bacterial component. Correlations exist; however, it has not been established whether the urotype is a biomarker or whether it is a cause of the symptoms and disease state. In order to answer these questions, the last couple points of Koch’s postulates must be performed (i.e. the specific disease is mimicked when a pure culture of the bacteria is inoculated into a healthy susceptible host and these bacteria must be isolatable from the experimentally infected host).

**Microbiomes of the Different Female LUT Niches Differ**

Studies of the urobiome generally use either catheterized or voided urine samples. By collecting voided urine, all LUT niches (bladder, urethral niche, periurethral niche plus the
vulvovaginal niche) are indiscernibly analyzed as the urinary microbiome (Southworth et al. 2019). A study to characterize the female urethral microbiota was recently performed (Hochstedler 2019; Chen et al. 2020). A voided urine, periurethral swab, transurethral brush and catheterized urine were collected from 49 women with pelvic floor symptoms and cultured by EQUC. Collecting paired voided and catheterized samples, along with the swabs, presents the opportunity to ‘deconstruct’ voided urine as to what microbial contributions each of the niches are adding. This study found the urethra is a separate microbiological niche from the bladder and is more similar to the periurethra. Through analysis of the abundances in each niche, they found that some bacterial genera preferentially colonize the urethra or the bladder, while others are generalists and reside throughout the LUT. For example, *E. coli* is considered to be a bladder-colonizing species and this study found that, whereas the frequencies of *E. coli* detection in the periurethra, urethra and bladder were similar, *E. coli* abundance was orders of magnitude greater in the bladder. The opposite was true of *Corynebacterium*. In contrast to these ‘niche specialists,’ *Lactobacillus* was found in all three niches in similar frequency and abundance. Given that the urethra possesses a unique microbiota and that voided urine often more closely resembles the urethral microbiota, the fact that *E. faecalis* was more frequently isolated from voided urine than catheterized urine suggests that *E. faecalis* may cause urethritis in women who have this species. Further studies are required to determine the clinical significance of the urethral microbiome, but its existence can no longer be disregarded.

**Urobiome Compositions of Women Are Dynamic but Resilient**

The urinary tract consists of distinct niches that harbor somewhat different microbial communities. All the cross-sectional studies that illuminate the compositions of these niches...
communities are likely glimpses of dynamic microbiota, altered by intrinsic and extrinsic factors. A recent study by Price et al. showed that the lower urinary tract urobiome can be dynamic and that both menstruation and vaginal sex influence those dynamics (Price et al. 2020). In this study, eight young and fit women without LUTS provided a midstream voided urine sample each day for 3 months. As a control for post-urethral contamination, they also provided a daily periurethral swab; an exclusion criterion was the inability to provide urine samples microbiologically distinct from their peri-urethral swab. To detect and identify bacterial taxa, both EQUC and 16S rRNA sequencing were used.

In this study, three distinct patterns were observed and changes in the patterns were on days when women reported menstruation or after vaginal sex. The patterns observed were: (1) *Lactobacillus* predominance, (2) alternating *Lactobacillus* and *Gardnerella* predominance and (3) changing ratios of *Streptococcus*, *Staphylococcus*, and *Corynebacterium*. During menstruation, the diversity and composition of the microbiome changed. For example, one woman had blooms predominated by *Actinomyces* every time she menstruated. Following almost every report of vaginal sex, *Streptococcus* species bloomed with a concomitant loss of the previously predominant species (most often of the genus *Lactobacillus*); this bloom promptly dropped off in the following days. Although most women’s LUT microbiota fluctuated, it remained relatively stable over time as the bacterial species pattern was resilient, bouncing back even after agitation by menstruation or sex. Thus, the urobiome is a dynamic but resilient community.

On the basis of the observation that vaginal sex alters the urobiome, our group performed a pilot study to further explore how sexual activity affects the urobiome and
whether its constituents can be exchanged between heterosexual sexual partners (Price 2019; Mores et al. 2020; Mores et al. in prep). The study followed a single monogamous couple that had vaginal sex once a week for three weeks. The female partner provided oral, vaginal, periurethral and voided urine samples everyday, while her male partner provided oral, penile swabs and voided urine samples once weekly prior to and immediately following vaginal sex. They each filled out a lifestyle questionnaire, particularly taking note of when they had sexual intercourse. Multiple strains of *Streptococcus mitis* (considered to be an oral microbe) were isolated from the female and male and on multiple days from different sites. In particular, *S. mitis* was isolated in high abundance from the urine following vaginal intercourse, but was only isolated once from a vaginal swab. In order to determine whether the strains were identical and being transmitted between the partners, 39 isolates were sequenced by whole-genome sequencing. By genome assembly and comparison, all female isolates were found to be very closely related (“clonal population”). Only one of the male’s isolates from the oral cavity was related to the female isolates (“clonally related”); several others were not. The authors concluded that vaginal sex caused a bloom of *S. oralis* resident in the female’s lower urinary tract.

Vaginal sex is one of the largest risk factors for pre-menopausal women contracting UTI symptoms (post-coital UTI). In order to study the clinical relevance of a high abundance of *S. mitis* the day after sexual intercourse and how putatively relates to UTI, *in vitro* assays of inhibition towards uropathogenic *E. coli* (UPEC) were performed. Culture supernatants from obtained from strains of either *S. mitis* or *Lactobacillus jensenii* (the predominant species isolated from the female participant on most days after not having reported sexual
intercourse). These supernatants were separately incubated with UPEC cultures. In the presence of the *L. jensenii* supernatant, the UPEC culture exhibited no growth, suggesting the supernatant has bacteriostatic activity. This effect was found to be dose-dependent. In the presence of the *L. jensenii* supernatant, the UPEC culture exhibited no growth, suggesting the supernatant has bacteriostatic activity. This effect was found to be dose-dependent. In the presence of the *S. mitis* supernatant, however, the UPEC culture grew at a rate comparable to that of the sham control. By utilizing a UPEC strain lacking a gene for bacterial adhesion important for biofilm formation, it was shown that the inhibition of UPEC by *L. jensenii* supernatant is not due to prevention of biofilm formation, but rather more likely due to general growth inhibition. Furthermore, it was shown that UPEC growth is likely inhibited due to *L. jensenii* H$_2$O$_2$ production. From a clinical standpoint, vaginal sex disrupted the FUM causing a decrease in *L. jensenii* and a bloom of *S. mitis*, which is less adept at fending off UPEC. These data provide strong evidence for a potential mechanism of post-coital UTI. The temporary loss of a protective microbe, such as *L. jensenii*, could make a female susceptible to UTI if exposed to a uropathogen.

**Evidence of Male Urinary Microbiota**

The male urethral microbiome has been studied for at least a couple of decades due to its implications in nongonococcal inflammation of the urethra (urethritis) (Bowie et al. 1977; Ivanov 2007). Until recently, however, the male bladder microbiota has not been investigated. In 2010, Nelson et al. performed a study investigating the urinary microbiomes of men with and without STI (*Chlamydia trachomatis* or *Neisseria gonorrhoeae*) (Nelson et al. 2010). By sequencing voided urine, they found all urines to be sequence-positive. They discovered that
bacteria associated with the female vulvovaginal region were abundant in urines from STI-positive males when compared to STI-negative males. This was a significant finding, as these microbes had not previously been known to colonize the male urethra, let alone associate with *C. trachomatis* or *N. gonorrhoeae* infections. However, as this study was accomplished using voided urines, no conclusions about the male bladder alone were made.

A recent study investigated the lower urinary tract microbiota of males as it relates to LUTS in males (Bajic et al. 2020). In this study, 49 male participants were arranged in cohorts based on LUTS symptoms severity. Each participant provided paired voided/catheterized urines for EQUC and sequencing. The voided and catheterized urines, regardless of participant group, were found to be significantly different. Of the catheterized urines, 39% had detectable microbiota; of the voided urines, 98% had detectable microbiota. Of men with mild LUTS, microbiota was detected in catheterized urines of 22.2% of men, 30.0% with moderate LUTS, and 57.1% with severe LUTS. These data suggest that an abundance of bladder microbiota may associate with increased LUTS. In order to determine whether specific taxa are associated with LUTS or asymptomatic males, additional studies will be required.

More than two decades ago, Krieger et al. discovered that the prostate is colonized by microorganisms and that these may associate with chronic prostatitis (Krieger et al. 1996). Chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) has been associated with higher bacterial diversity in voided urine (Shoskes et al. 2016). No bacterial diversity difference was found in voided urine from patients with prostate cancer and those without prostate cancer (Shrestha et al. 2018). The urobiome of people with bladder cancer (which is more common in men), however, differs from those without bladder cancer (Bučević Popović et al. 2018; Wu et
al. 2018; Bajic et al. 2019). This discovery has implications in early-stage treatment using a very common, intravesical, immunotherapeutic Bacillus Calmette-Guerin (BCG).

**Lessons Learned About Urine Sample Collection**

An important consideration with regards to urobiome variability is that the urobiome reported for women without LUTS varies considerably across studies, likely due to use of different analytical and urine collection methods. Longitudinal urobiome studies and population studies have not been feasible in part because of the impracticality of catheterizing patients daily. Obtaining midstream voided urine is simpler because study participants can collect their own urine, whether in the clinic or at home. Voided urine, however, is most often not an accurate reflection of bladder urine as it is often not a true “clean-catch”. In fact, voided urine has been found to be more descriptive of periurethral skin than either the bladder or urethra (Hochstedler 2019; Chen et al. 2020). Nonetheless, as previously mentioned, certain genera (e.g. *Lactobacillus*) are present across all LUT niches, including vulvovaginal. Therefore, subtractive methods to deduce the bladder composition based on voided urine cannot be used. A solution to a cleaner-catch maybe a urinal device called Peezy (Forte Medical). By reducing post-bladder contamination, Peezy appears to be a cleaner-catch voided urine (Southworth et al. 2019). The widespread use of Peezy may make longitudinal urinary microbiome and community-based studies possible.

**Potential Microbiome Treatments**

The knowledge that microbiome profiles can protect from disease is being leveraged for treatment. Microbiome transplants known as fecal microbiome transplants (FMT) are established for gut disorders, particularly for treating stubborn and recurrent *Clostridioides*
*Clostridium difficile* infections. Recently, a group made attempts at transplanting a ‘healthy’ vaginal microbiome into 5 individuals with untreatable bacterial vaginosis (BV). The microbiome transplants were successful and the recipients had positive clinical outcomes (Lev-Sagie et al. 2019).

Microbiome treatment methods are arising for LUT disorders. The first successful in-human intravesicular *Lactobacillus rhamnosus* bladder instillation therapy for UTI in patients with any nervous system condition that causes LUT dysfunction (neurogenic lower urinary tract dysfunction) has recently been made (Groah et al. 2019). It has been deemed safe and well tolerated; furthermore patients could self-administer, which is beneficial for these patients who may not perceive symptoms of UTI, for example. With the evidence that *Lactobacillus* produces bacteriostatic and bactericidal molecules, this direct inoculation could be a powerful treatment for inhibition of uropathogens.

Phage therapy, the use of obligately lytic bacteriophages to treat bacterial infections, was implemented before the use of antibiotics in medicine. In fact, the first report of phage therapy was the attempt to lyse bacteria causing urinary infections (Caldwell 1928). Effective usage of this therapy will likely come with further characterization of the newly discovered bladder phage.

**RUTI**

The pursuit of both rigorous longitudinal urobiome studies and knowledge that disruption of the urinary microbial community can lead to development of infection are particularly important for recurrent UTI (RUTI) patients.
30-44% of adult women who have experienced one UTI episode will have another episode commonly within 3 months (Foxman 2014; Brubaker et al. 2018). This recurrence is known as RUTI, defined as at least three infections in a year or two in six months (Gupta and Trautner 2013).

It is generally unknown why some women experience RUTI. Risk factors for RUTI include having the first UTI before age fifteen, frequent sexual activity (particularly in pre-menopausal women), birth control methods such as nonoxynol-9 spermicide (which also kills commensal bacteria), lower neurogenic bladder dysfunction, and hypoestrogenic levels in postmenopausal women, which leads to vaginal atrophy and pH changes and bacterial overgrowth (Scholes et al. 2000). The aforementioned are just a handful of the many risk factors that have been associated with RUTI. With so many risk factors, however, the microbiological mechanisms of RUTI development have been understudied, although theories have been presented.

As patients commonly present with the same uropathogen at each episode (typically detected by SUC), a common hypothesis is the single uropathogen resides in reservoirs between infections as “persistor cells” (Kodner and Gupton 2010). In microbiology, a “persistor cell” is one that is highly antibiotic resistant, metabolically inactive (dormant) and is a variant from the parents without having undergone genetic change (Lewis 2010). Antibiotics cannot fully clear the uropathogenic persistor cells. Conceivably, these cells form biofilms, which increases the antibiotic resistance (Tapiainen et al 2014). Perhaps they remain in low abundance and potentially elsewhere in the body and then reemerge concurrently with UTI symptoms. These reservoirs of bacteria called quiescent intracellular reservoirs (QIRs) have been shown to reside in endosomes of the urinary bladder epithelium (urothelium) in a murine
model, whereby inducing epithelial shedding and the inhabiting QIRs results in decreased reoccurring infection (Mysorekar and Hultgren 2006). More commonly than forming QIRs, bacteria form expansive intracellular bacterial communities within urothelial cells, generating a niche that evades detection by the host (Jorgensen and Seed 2012). There is evidence that particularly *E. faecalis* also uses this mechanism in patients with chronic UTI (Horsley et al. 2013).

Another theory of bacterial etiology is that non-*E. coli* uropathogens go undetected. This limit of detection could be remedied by utilizing EQUC in place of SUC for analysis. As mentioned above, when a UTI patient’s urine is cultured, it is typically by the SUC method. It has been found that EQUC identifies bacteria in 90% of urine samples deemed ‘no growth’ by SUC and reproducibly detects more non-*E. coli* uropathogens than SUC (Price et al. 2016). More specifically it has been found that within an RUTI cohort, EQUC detects more potential uropathogens in both paired catheterized and voided urine samples (Hochstedler 2019, Hochstedler et al. in prep). Furthermore, a patient with UTI symptoms whose suspected uropathogen is not *E. coli* is more likely to have a better clinical outcome in terms of symptom resolution when their catheterized urine is cultured by EQUC rather than SUC (Barnes et al. in prep). Therefore, a couple common theories of RUTI mechanisms are unsatisfactory but more substantial theories have been proposed recently.

**Characterization of the Urobiomes of Women with RUTI and Longitudinal Analysis Reveals**

**Putative RUTI Microbial Mechanisms**

Based on SUC, *E. coli* is universally ascribed to contribute to up to 90% of all uncomplicated UTIs (i.e. UTIs in patients without structural or neurological urinary tract
abnormalities) (Hooton 2012). Using EQUC, a more accurate value is ~50% (Price et al. 2016). In this study of women who thought they had a UTI, 71% of the uropathogens detected by SUC were indeed *E. coli*. However, SUC missed 76% of the other uropathogens present.

While *E. coli* is present in about 50% of uncomplicated symptomatic UTI cases, RUTI microbiota profiles differ. In a cohort of 37 women with a history of RUTI, using paired catheterized and voided urine samples, Hochstedler et al. found *E. faecalis* to be the most common uropathogen detected and more likely to be associated with UTI symptoms versus *E. coli*. These data suggest that *E. faecalis*, rather than *E. coli*, might be the dominant species in RUTI etiology. When analyzing data from SUC versus EQUC abundance data, the authors recapitulated the evidence that SUC repeatedly fails to culture numerous uropathogens detected by EQUC, including *E. faecalis*, the most prevalent uropathogen in this cohort (Hochstedler et al. in prep).

Although with a small RUTI cohort, this study is nuanced and provides potential RUTI microbial mechanisms. Several participants had return clinic visits, which led to longitudinal analysis of the returning participants. By comparison of a baseline sample to a return sample for the participants, a few patterns were determined. The authors discuss the possibility that uropathogens correlated with RUTI may be capable of “(1) persisting/recurring despite treatment; (2) advancing up the lower urinary tract between episodes; or (3) persisting in a manner that allows the establishment of additional uropathogens at subsequent episodes.” These astute observations must be confirmed with a larger and longitudinal study. Akin to the methods of Mores et al. on *S. mitis* isolates, whole-genome sequencing analysis is necessary to determine if these ‘recurring/persisting’ uropathogens are indeed of the same strain.
RUTI Management

Due to the severity and frequency of their symptoms, women who suffer from RUTI receive frequent treatment for symptoms management. By national and international guidelines, the ‘gold standard’ of managing RUTI in adult women is long courses of low-dose antibiotics as a preventative treatment (Forbes et al. 2018). Treatment of patients with painful LUTS with a long-term antibiotic regimen has been shown to be effective (Swamy et al. 2019).

A chief component of the management of women with RUTI is prevention (Jung and Brubaker 2019). One alternative measure for prevention is a low dose antibiotic course intermittently, such as immediately after sexual intercourse (postcoital prophylaxis), or a low dose antibiotic for 3-12 months (continuous prophylaxis). Some women have the opportunity to recognize their own UTI symptoms and then start previously prescribed antibiotics (self-start therapy). A short course antibiotic treatment of 3-5 days is also recommended.

At the clinic, women with UTI symptoms are often treated empirically (i.e. that antibiotic treatment is prescribed based on symptoms without diagnostic testing) (Tomas et al. 2015; NICE 2015). Empiric treatment of UTI is likely contributing to antibiotic resistance, as UTI is one of the most common reasons for antibiotic prescription (Gupta et al. 2001). Alternatives to antibiotics exist, however, they should be cautiously considered as they are based on the now disputed E. coli-centric dogma.

Alternatives to Antibiotic Treatment for UTI

With numbers of microbiome studies and antimicrobial resistance on the rise, there is an increased interest in alternative treatment methods to antimicrobials. UTI treatment alternatives currently in use include cranberry products, D-mannose, oral and intravaginal
probiotics, vaginal estrogen, ascorbic acid (vitamin C) and two experimental treatments: a vaccine (Iromune) and an immunotherapeutic (OM-89 UroVaxom), and other biological therapies (Foxman and Buxton 2013).

Cranberry products are the most studied alternative, which along with other proposed benefits, are presumed to inhibit *E. coli* adhesion to urothelial cells (Guay 2009). There are mixed reports of whether it is an effective treatment or prophylaxis. A meta-analysis, which included recent and large clinical trials, suggests little or no effect of cranberry on treatment or prevention of UTI (Jepson et al. 2012). D-mannose is also proposed to prevent *E. coli* adhesion to urothelial cells and may be a treatment of infrequent UTI caused by *E. coli* (Domenici et al. 2016). One plausible rationale of using *Lactobacillus* oral and intravaginal probiotics is to restore an altered microbiome and prevent uropathogens from colonizing. The use of oral *Lactobacillus* probiotics may result in UTI prophylaxis, but the evidence is conflicting (Akgül and Karakan 2018; Wolff et al. 2019). Vaginal (unlike oral) estrogen supposedly lowers vaginal pH, increases *Lactobacillus* colonization while decreasing uropathogen colonizations (Raz 2011). Vaginal estrogen may reduce the number of UTI episodes in postmenopausal women (Raz and Stamm 1993). Ascorbic acid (vitamin C) is thought to improve immunity and decrease urine pH killing intolerant microbes, but has a lack of studies to support its usage (Hickling and Nitti 2013). A sublingual bacterial vaccine (Uromune) and a vaginal immunotherapeutic (OM-89 Uro-Vaxom *) are both based on lyophilized bacterial extract (UV, OM-89). Use of bacterial extracts as vaccines may have been implemented more than 40 years ago (Cruz et al. 2009). Meta-analysis has revealed that both Uromune and Uro-Vaxom may be moderately effective at reducing symptoms but require a ‘booster’ (Nickel 2018; Naber et al. 2009).
**RUTI Prophylaxis with Methenamine Hippurate**

Methenamine hippurate (MH) is a more than a century year-old RUTI prophylaxis. MH is an attractive treatment because it is considered an antiseptic or antibacterial rather than an antibiotic; it is bladder-targeted, has multiple antibacterial modalities, has little to no side effects, and bacteria are reported to not acquire resistance (Lee et al. 2012). The recommended dosage schedule is a 1-gram tablet twice daily. Larger doses of MH (8 g daily for 3 - 4 weeks) have caused side effects, including bladder irritation, blood in the urine and painful, and frequent urination (FDA 2006). The LD50 determined in mice was found to be 1500 mg/kg, which causes generally depressed behavior and seizures (NCBI). The average American woman weighs 77 kg and, therefore, the theoretical human LD50 is equal to 115.5 g, which is about 58 times the recommended daily dose.

In urine, MH disassociates into methenamine and hippuric acid. The hippuric acid acidifies the urine and is also reported to have mild bacteriostatic effects (Nahata et al. 1982). Hippuric acid is also a natural metabolite in urine (Toromanović et al. 2008). Under acidic conditions, methenamine salts hydrolyze to two metabolites: formaldehyde (the active ingredient) and ammonia. It is reported that, at pH 6 and below, formaldehyde is released within one hour of methenamine entering the bladder (Hamilton-Miller and Brumfitt 1977). One *in vitro* study showed that 108, 73, 40 and 37 ug/mL of formaldehyde was released from 0.5 mg/mL methenamine at pH 5.4, 6.1, 7.1 and 8.1, respectively (Greenfield et al. 1969). Formaldehyde activity was shown to be essentially unaffected by pH 5 – 8 (Hamilton-Miller and Brumfitt 1977). In air, formaldehyde slowly oxidizes to formic acid and most commercially
available stocks are reported to be contaminated with at least small amounts of formic acid (Gottschling et al. 1984).

Formaldehyde is a bacteriostat and bactericide, depending on the concentration (Mayrer and Andriole 1982). MH is reported to be active against some common uropathogens including *E. coli* and *Enterococcus* species (Gleckman et al. 1979), but its effects on urobioiome isolates or other species are undocumented. MH is commonly prescribed to RUTI patients, but there is a lack of rigorous clinical evidence and *in vitro* data to support its use.

There are numerous small studies that have compared the effectiveness of MH, but most have erroneous study parameters, and there is heterogeneity between studies. The evidence is not strong, but a few studies suggest that MH may be more effective at reducing RUTI after one year compared with placebo (Høvik et al. 1984; Gunderson et al. 1986; Cronberg et al. 1987). Most studies compare the effectiveness of MH to prophylactic antibiotics (Chwa et al. 2019). Ascorbic acid (Vitamin C) is sometimes paired with MH to further acidify the urine; vitamin C has been shown to not have an effect (Devenport et al. 1984; Strom and Jun 1993). A Cochrane review has deemed that MH may be safe and more effective for short duration treatment in individuals without renal tract abnormalities, where the outcome is symptomatic UTI (Lee et al. 2012). Another more recent review found that MH “appears to be a safe and effective option to prevent UTI in older adults with recurrent UTI, genitourinary surgical procedures, and potentially long-term catheterization” (Chwa et al. 2019). There remains a need for RCTs with long-term MH prophylaxis in patients without neuropathic bladders and more precise definitions of “symptomatic UTI” and patient outcomes. A large RCT study
comparing MH against the current standard of daily low-dose antibiotics in women with RUTI is currently being performed (Forbes et al. 2018).

There is currently little to no *in vitro* data providing bacterial susceptibilities to MH. The spectrum of susceptibility beyond the few reported uropathogens would provide insight on how the drug acts as a prophylactic. This is the purpose of my study.
CHAPTER II

MATERIALS & METHODS

*In Vitro* Susceptibility Study

Reagent Preparations

A stock solution of 400 mg/mL methenamine hippurate was made by pulverizing a 1 g (1000 mg active ingredient) enteric-coated tablet (Alvogen) using a mortar and pestle followed by weighing the powder with an analytical scale. The powder was dissolved in ~ 2.5 mL 1 mM KH$_2$PO$_4$, 155 mM NaCl, 3 mM Na$_2$HPO$_4$-7H$_2$O PBS, pH 7.4 (Thermo Fisher Scientific) by vortexing and then microwaving in 2 x 5” intervals for 10”. This solution was stored in 50 uL aliquots at -20°C until thawed at 4°C on the day of use. A stock solution of 1.09 mg/mL formaldehyde (Thermo Fisher Scientific) was vortexed in PBS. A stock solution of 400 mg/mL methenamine (Sigma-Aldrich) was made by weighing out powder on an analytical scale and vortexing in PBS to dissolve. Both formaldehyde and methenamine working stocks were made fresh on the day of use. A stock solution of 50 mg/mL hippuric acid was made by weighing hippuric acid powder (Fisher Scientific), vortexing in methanol (Millipore Sigma) and storing at -20°C. A stock solution of 120 mg/mL ammonia was made by weighing ammonium chloride (Sigma-Aldrich), vortexing in PBS and storing in 200 uL aliquots at -20°C until thawed at 4°C on the day of use.
Bacterial Isolates and Growth Conditions

Bacterial strains previously isolated from the bladders of women from 3 different health/disease states were used: (1) a history of RUTI (Hochstedler 2019; Hochstedler et al. in prep), (2) an active UTI (Price et al. 2016; Dune et al. 2017) and (3) asymptomatic controls (Pearce et al. 2014; Thomas-White et al. 2016; Price et al. 2020) were used. For each species, at least 3 urinary isolates from each health/disease state were selected. ATCC strains of *Escherichia coli* (ATCC 10798, 29425), *Enterococcus faecalis* (ATCC BAA-2128) and *Staphylococcus epidermidis* (ATCC 14490) were used. *Actinomyces neuii, Corynebacterium amycolatum, E. faecalis, E. coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, S. epidermidis* were statically grown in Brain Heart Infusion (BHI) medium (Sigma-Aldrich) in 5% CO\textsubscript{2} at 35°C for 24 hrs. *Lactobacillus jensenii* was statically grown in deMan, Rogosa and Sharpe (MRS) medium (Sigma-Aldrich) in 5% CO\textsubscript{2} at 37°C for 48 hrs. *Gardnerella vaginalis* was statically grown in NYCIII (REBLab formulation) medium plus 10% Newborn Calf Serum (NBCS) in a Coy Labs anaerobic chamber at 37°C for 24 hrs.

To standardize the bacterial CFU/mL to be treated, 5 x 10\textsuperscript{5} CFU/mL was used. Prior to treatment of each strain, the OD\textsubscript{600} value that corresponds to 5 x 10\textsuperscript{5} CFU/mL was determined. The selected strains were struck out from -80 °C freezer stocks (Brucella Broth cryovials, Hardy Diagnostics) and plated on their respective media and incubated in the species-specific conditions. Following incubation, a single colony was inoculated into 5 mL of medium and incubated in the same conditions. Biological triplicates were performed. The culture was then diluted to an OD\textsubscript{600} of 1.0 in fresh medium. A serial dilution (10\textsuperscript{-1} to 10\textsuperscript{-8}) was performed and 10
uL of each bacterial suspension dilution was plated. An uninoculated medium spot was included to control for contamination. Following incubation, spots with enumerable colonies (30-50) were counted. For each strain’s biological triplicates, the average CFU/mL corresponding to OD$_{600}$ was recorded.

**Determination of MICs and MBCs**

The Minimum Inhibitory Concentration (MIC) for each strain and drug were determined by broth microdilutions. Strains were struck out from -80 °C freezer stocks and plated on respective media and incubated. Following incubation, a single colony of each strain was inoculated into 5 mL of medium and incubated in the same conditions. The assay was performed following incubation. The OD$_{600}$ of the culture was measured in a BioTek plate reader and the culture was diluted to a bacterial suspension of $5 \times 10^5$ CFU/mL in fresh medium. When strains were treated in consortia (experimentally in parallel to their pure cultures), each strain was combined to $5 \times 10^5$ CFU/mL and this culture was treated as a single bacterial suspension. Microdilution was performed in biological triplicates for each strain. In a sterile, lidded 96-well flat-bottom microtiter plate, for each strain, the assay was composed of 3 vertical rows of medium sterility control (uninoculated medium), 3 vertical rows of growth control (diluent only), followed by a range of 10 concentrations of each drug. A row was left blank between each strain. To control for variance across wells and pipetting small volumes, master mixes of each drug were used (an equal volume was added to every well). To a sterile 96-well microtiter plate, using multichannel pipettes, equal volumes of each chemical master mix and culture were added for a final volume of 200 uL per well. After light mixing upon addition of the suspension, the lidded plate was incubated in species-specific conditions as
described above. A single read of the entire plate at OD\textsubscript{600} was performed in a BioTek plate reader and these reads were populated into Microsoft Excel. When growth curves were performed, the plate was incubated at 37°C in the BioTek plate reader contained in a 5% CO\textsubscript{2} chamber. During incubation, OD\textsubscript{600} of the plate was read every 15’, with a 5” orbital shake prior to each read, for 24 hrs as a kinetic cycle program. The MIC was manually determined to be the lowest concentration at which the corresponding OD\textsubscript{600} of the culture was equivalent to the uninoculated medium (unless contaminated, for which the experiment was repeated). The MIC for the strain was recorded as the average of the triplicate. Standard deviations (STD. DEV. P) were determined for reporting error.

Immediately following determination of the MICs, the Minimum Bactericidal Concentrations (MBC) were determined by plating directly from the microtiter plates onto appropriate medium supplemented with 1.5% agar plates using a multichannel pipet. From each well, including the 3 vertical rows of medium sterility control and 3 vertical rows of growth control, 10 uL was spotted onto the agar medium plate and allowed to dry before inverting the plate for incubation. After 24 or 48 hrs of incubation in the same conditions, the plate was examined. The MBC was determined to be the corresponding spot with no growth (equivalent to the sterility control unless contaminated). If the sterility control was contaminated, the experiment was repeated. If any spots growing colonies were contaminated, the experiment was repeated. The MBC for the strain was recorded as the average of the triplicate.

**Consortium Experiment with Commensal and Potentially Uropathogenic Species**

Three putative commensal species and three potential uropathogens were treated in a consortium alongside pure cultures. One RUTI strain of *G. vaginalis, L. jensenii, A. neuii, E. coli,*
E. faecalis and Staphylococcus aureus were each struck-out from -80 °C freezer stocks, plated and incubated in conditions described. S. aureus was plated on BHI medium supplemented with 1.5% agar and incubated at 35°C in 5% CO₂. Single colonies were picked and inoculated into 5 mL of fresh medium. Following incubation, the OD₆₀₀ of each culture was measured in a BioTek plate reader and diluted to an OD₆₀₀ of 0.001 in either 1 mL of fresh species-specific medium or 1 mL of particular medium for culturing the consortium (made from equal parts of each 3x concentrated MRS, BHI and NYCIII). The strains in isolation and the consortium were treated with 0, 5 and 15 mg/mL MH (final concentration) and allowed to incubate in glass culture tubes at 35°C in 5% CO₂ for 3 hours, which is one hour longer than the reported time for formaldehyde to act as a bacteriostat at pH 5.6 (Horton 2015). Untreated (0 mg/mL MH) cultures were immediately plated at t=0. Each species and the consortium were treated in biological duplicates. Following incubation of 3 hours, 1 uL of each culture was plated onto TSA supplemented with 5% Sheep Blood plates (BD BBL) and CFU/mL counts were enumerated after O/N incubation in 5% CO₂.

**pH-buffered Medium Assays**

To test for bacterial growth across a pH range, E. coli and E. faecalis were cultured in a range of pH-buffered BHI media. For each species, 2 strains from both RUTI and asymptomatic control bladders were selected. For the assay, pH 7.5 BHI buffered to pH 5.0- 7.0, 7.5- 8.5 and 9.0- 10.0 with autoclaved 200 mM sodium phosphate (Na₃PO₄), 50 mM Tris Base (C₄H₁₁NO₃) and 100 mM sodium bicarbonate (NaHCO₃), respectively, were used. The media were allowed to equilibrate in a 37°C water bath prior to pH’ing. An O/N culture from a single colony of each strain was inoculated in 5 mL BHI, pH 7.5. After O/N incubation, a 1:1000 subculture in fresh
pH-buffered BHI of each strain was made. The assay was performed in a sterile, lidded 96-well flat-bottom microtiter plate. The assay was composed of a well of each medium sterility control (uninoculated medium), 2 vertical rows of growth control (inoculated pH 7.5 BHI) followed by the range of pH 5.0 - 10.0 buffered-BHI inoculated cultures. Each strain was assayed in each pH in duplicate. The lidded plate was incubated at 37°C in a BioTek plate reader contained in a 5% CO₂ chamber. The OD₆₀₀ of the plate was read every 15’, with a 5” orbital shake prior to each read, for 24 hrs as a kinetic cycle program. The reads were populated into Microsoft Excel. The pH of each well was measured and recorded.

**Urinary Sampling and Treatment of Author with MH**

For 21 days, the author collected a periurethral swab (BD ESwab Collection and Transport System) and a voided urine sample (first voided urine of the morning) in a sterile cup from herself daily. Everyday a Daily Lifestyle Questionnaire (Price et al. 2010) was filled out. On Day 7, the author started taking 1 g MH (Alvogen) twice daily for one week. On days 7, 13 and 21, the author filled out the UTISA questionnaire (BJU International 2005), the Pelvic Floor Disability Index (PFDI) and the Overactive Bladder questionnaire (OAB-q). Each day, the pH of the urine was taken immediately and the paired samples were processed in the Wolfe Lab. 10 μL of voided urine and periurethral swab were plated onto 5% BAP and a CDC anaerobe 5% BAP plates. Plates were incubated at 35°C for 24 hours in 5% CO₂ or anaerobic conditions (CDC anaerobe 5% BAP). Each sample was frozen in 10% Assay Assure (Sierra Molecular) at -80 °C. Following incubation, each distinct morphology was counted and identified directly with Matrix-Assisted Laser Desorption/Ionization Time-of Flight (MALDI-TOF) mass spectroscopy.
Longitudinal Clinical Study

Participants

Following Loyola institutional review board (IRB) approval, 10 women with a clinical history of RUTI seeking treatment from the Urogynecology Clinic of Loyola University Outpatient Center will give verbal and written consent for the collection and analysis of their urine for research purposes. All participants will answer Pelvic Floor Disability Index (Barber et al. 2005) and Urinary Tract Infection Symptom Assessment (Clayson et al. 2005) questionnaires to assess the severity of any pelvic floor conditions and possibility of urinary tract infection, respectively. For each participant, the urogynecological clinicians will prescribe methenamine hippurate prophylactically.

Collection of Urine Samples

The clinicians will obtain urine by transurethral catheterization (TUC) at the participants’ initial visit, their 3-month visit and any unscheduled visits for acute symptoms of UTI. For obtaining urine by TUC, the urethral meatus is prepped with a routine betadine swab before a sterile Bard Clean-Cath Ultra 6" female catheter, 14Fr for intermittent catheterization, will be placed into the urethra and advanced until urine is returned. Urine specimens will be collected in a sterile BD Vacutainer. The participants will also collect samples daily during several specified windows: periurethral swab samples and midstream voided urines using a urinal device called the Peezy. Periurethral samples will be collected by swabbing (BD ESwab Collection and Transport System) the periurethral vagina 5mm from the urethral meatus. In order to use the Peezy, participants will be instructed to wash their hands with soap and water, unpack the Peezy device and attach the sterile collection tube containing a boric acid
preservative, use a sterilizing peri-urethral wipe (if appropriate), hold the Peezy device by the designated area, and void completely into the device. Participants will then allow the device to drain fully before unscrewing and capping the sterile midstream collection tube. All samples will be sent to the Wolfe Lab, where they will be divided into two aliquots: one immediately cultured using the Expanded Quantitative Urine Culture (EQUC) protocol and the other will be frozen for 16S ribosomal RNA (rRNA) gene sequencing.

**Sample Culture Methods**

The EQUC method involves inoculation of 100 μL of catheterized urine, or 10 μL of voided urine and peri-urethral samples, onto 5% BAP, chocolate agar, colistin and nalidixic acid (CNA) agar and CDC anaerobe 5% BAP plates. Plates will be incubated at 35°C for 48 hours in aerobic (BAP), 5% CO2 (BAP, chocolate agar, CNA), or anaerobic conditions (CDC anaerobe 5% BAP). Thus, the level of detection for EQUC is 10 CFU/mL for catheterized urine, or 100 CFU/mL for voided urine and peri-urethral samples, represented by 1 colony of growth on any of the plates.

**Identification of Bacterial Isolates**

Each morphologically distinct colony type will be counted and isolated on a different plate of the same medium to prepare a pure culture that will be used for identification with Matrix-Assisted Laser Desorption/Ionization Time-of Flight (MALDI-TOF) mass spectroscopy. MALDI Biotyper 3.0 software Realtime Classification will be used to analyze the samples. In the Realtime Classification program, log score identification criteria are used as follows: a score between 2.000 and 3.000 is species-level identification, a score between 1.700 and 1.999 is genus-level identification, and a score that is below 1.700 is an unreliable identification. A
Realtime Classification log score will be given for each bacterial isolate sample for every condition from which it is isolated.

Following identification, each unique isolate from the initiation of the study, intermediate time points and at the end of the study will be frozen at -80°C and treated using methods described above to determine MBCs using CFU/mL quantification as a readout for sensitivity.

**Statistical Analyses**

Culture data, consisting of species detected and CFU/mL, will be analyzed by various diversity measures. Alpha diversity measures will be used to compare species frequency, abundance, and evenness within populations/sample types. Beta diversity measures (Bray-Curtis Analysis and Principal Component Analysis) will be used to compare microbial community diversity between populations/sample types. In RStudio 1.1.423 (Boston, MA), non-parametric Wilcoxon signed-rank, Wilcoxon rank-sum, Kruskal-Wallis, and Chi-square or Fisher’s Exact tests will be used to test for significance.
CHAPTER III

DETERMINATION OF THE SENSITIVITIES OF URINARY ISOLATES TO

 METHENAMINE HIPPURATE AND ITS METABOLITES

Introduction & Rationale

I hypothesize that, within any given bacterial species, RUTI bladder isolates will be more resistant to MH and its active ingredient, formaldehyde, than isolates from women with an infrequent UTI or from asymptomatic controls. Furthermore, I hypothesize that when species are in a consortium with other species, sensitivities may be altered.

Determination of Sensitivities to MH Metabolites

I selectively chose E. coli and E. faecalis as representatives of common Gram-negative and Gram-positive uropathogens to determine sensitivities to MH and its metabolites (formaldehyde, methenamine, ammonia and hippuric acid).

I hypothesized that ammonia would not have bacteriostatic or bactericidal activities, whereas the other metabolites would. Based on a urine output of 1.0 – 1.5L output, it has been calculated that 15 mg/mL MH ideally catabolizes to 366 ug/mL formaldehyde (FDA 2006). Therefore, 0.5, 1.0 and 2.0 mg/mL MH should catabolize to 12.2, 24.4 and 48.8 ug/mL formaldehyde, respectively. Therefore, I treated the strains with these physiologically-relevant concentrations of formaldehyde, as well as higher concentrations. Similar calculations were performed to determine physiologically relevant concentrations of ammonia and hippuric acid.
For each species, I selected and cultured an RUTI strain and an asymptomatic control strain as described in the Materials and Methods. I monitored growth of the cultures in a BioTek plate reader for 24 hrs. Below, I show only the RUTI strains, as the asymptomatic strains exhibited similar behaviors.

Figure 1. *E. coli* RUTI Strain Treated with MH and Growth Monitored Over 24 hrs.

For *E. coli*, the RUTI strain was slightly inhibited by 0.5 mg/mL MH; it had a longer lag phase than the untreated control (Figure 1). The lag phase was even longer when the culture was treated with 1 mg/mL MH, but also the culture grew more slowly. Both treated cultures began to enter stationary phase at a lower biomass than the untreated culture, but exhibited faintly double sigmoidal curves, suggestive of adaptation that permitted growth to the same final OD$_{600}$ as the untreated culture. In contrast, growth was fully inhibited by 2 mg/mL MH, which was determined to be the minimal inhibitory concentration (MIC). As evident by a very
slight increase in \( \text{OD}_{600} \) at the end, however, perhaps had the kinetic cycle run longer, the culture would have overcome the inhibition.

**Figure 2.** *E. coli* RUTI Strain Treated with Formaldehyde and Growth Monitored Over 24 hrs.

Similar results were obtained from treatment with formaldehyde (Figure 2). Both 100 and 200\( \mu \text{g/mL} \) treatments resulted in longer lag phases; growth was completely inhibited by 500 \( \mu \text{g/mL} \) formaldehyde. Based on the calculations described above, the formaldehyde MIC equivalent to 2 mg/mL MH should in theory be 48.8 \( \mu \text{g/mL} \). However, growth was not inhibited by 48.8 \( \mu \text{g/mL} \) formaldehyde (Figure 3). However, there was an \( \sim \)3-fold increase in lag time compared to lower concentrations.

Methenamine and hippuric acid only slightly inhibited growth, as observed by the cultures reaching stationary phase at a lower \( \text{OD}_{600} \) (Figures 4 and 5). As suspected, ammonia had no obvious effect (Figure 6).
Overall, the *E. faecalis* exhibited similar end results to *E. coli*, although *E. faecalis* appeared to be slightly more sensitive; the curves varied more dramatically from the untreated control (Figures 7 – 12). For example, when treated with 0.5 and 1 mg/mL methenamine, the cultures’ growth rates were slower (Figure 10). When treated with 2 mg/mL, the culture had an even slower growth rate, a slightly longer lag phase and a lower final OD$_{600}$. The MIC for *E. faecalis* exposed to MH was 2 mg/mL based on the complete inhibition over 24 hrs.

![Figure 3.](image_url)

*Figure 3. E. coli* RUTI Strain Treated with Physiologically-relevant Concentrations of Formaldehyde and Growth Monitored Over 24 hrs.*
Figure 4. *E. coli* RUTI Strain Treated with Methenamine and Growth Monitored Over 24 hrs.

Figure 5. *E. coli* RUTI Strain Treated with Hippuric Acid and Growth Monitored Over 24 hrs.
Figure 6. *E. coli* RUTI Strain Treated with Ammonia and Growth Monitored Over 24 hrs.

Figure 7. *E. faecalis* RUTI Strain Treated with MH and Growth Monitored Over 24 hrs
**Figure 8.** *E. faecalis* RUTI Strain Treated with Formaldehyde and Growth Monitored Over 24 hrs.

**Figure 9.** *E. faecalis* RUTI Strain Treated with Physiologically-relevant Concentrations of Formaldehyde and Growth Monitored Over 24 hrs.
Figure 10. *E. faecalis* RUTI Strain Treated with Methenamine and Growth Monitored Over 24 hrs.

Figure 11. *E. faecalis* RUTI Strain Treated with Hippuric Acid and Growth Monitored Over 24 hrs.
Determination of Species’ Sensitivities to pH Over Time

As it is reported that acidic urine is required for metabolizing MH, I tested the viabilities of the RUTI and asymptomatic control strains of *E. coli* and *E. faecalis* in a range of pH-buffered media. I grew the strains in BHI buffered across the range of pH 5 – 10 and growth was monitored over 24 hrs at 37°C in 5% CO₂. I determined that “unbuffered” BHI had a pH of 7.3. Although I measured half pH values, for conciseness, I only present whole pH values in the graphs.

Control and RUTI strains exhibited similar growth behaviors; thus, I only present the RUTI strain data as representative. The *E. coli* strains grew similarly at all pHs, with the exception of pH 5, where growth was delayed and slow, and pH 9 - 10.0, where growth was inhibited (Figure 13). In contrast, the RUTI and control *E. faecalis* strains were considerably
more sensitive to pH and able to grow in all pH-buffered BHI tested (Figure 14). However, the curves varied substantially, including the final OD$_{600}$ measurements of these cultures after 24 hrs. For example, the culture in unbuffered BHI was the only culture to follow a sigmoidal curve. Although the starting pH of the unbuffered BHI was similar, growth in BHI buffered at pH 7.0 differed in terms of curve shape and final OD$_{600}$. BHI buffered at pH 5.0 supported a slower growth rate and growth to about half the final OD$_{600}$ as unbuffered BHI. BHI buffered at pH 9.0 supported growth similar to that supported by BHI buffered at pH 5.0, but with a slightly increased growth rate. Growth at pH 6.0 also looked very similar to pH 9.0. Based on the higher growth rate and final OD$_{600}$, *E. faecalis* seemed to prefer pH 8.0.

\[\text{Figure 13. E. coli RUTI Strain Cultured in a Range of pH-buffered Media and Growth Monitored Over 24 hrs.}\]
To determine if the buffering worked, final pH measurements of the *E. coli* and *E. faecalis* cultures were taken at the end of the time course. The values were generally unchanged compared to uninoculated medium of the same pH (*Table 1*).

Figure 14. *E. faecalis* RUTI Strain Cultured in a Range of pH-buffered Media and Growth Monitored Over 24 hrs.
Table 1. pH Values of Cultures Measured After 24-hour Time Course.

<table>
<thead>
<tr>
<th>Theoretical pH</th>
<th>Uninoculated</th>
<th>RUTI E. coli</th>
<th>Ctrl E. coli</th>
<th>RUTI E. faecalis</th>
<th>Ctrl E. faecalis</th>
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<td>Unbuffered</td>
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<td>9.9</td>
<td>9.9</td>
<td>9.8</td>
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</tr>
</tbody>
</table>

Determination of Formaldehyde and MH MICs and MBCs in Diverse Urinary Species

In addition to *E. coli* and *E. faecalis*, I treated other potential uropathogens with MH and formaldehyde to determine their MICs and MBCs. I selected *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* as they were frequently isolated from an RUTI patient cohort (Hochstedler et al. in prep). However, no RUTI strains of *P. aeruginosa* were available as frozen stocks.

I also treated species thought to be commensal and potentially beneficial species. I selected *Gardnerella vaginalis* as it is a common urotype of the asymptomatic FUM (Pearce et al. 2014; Price et al. 2020; Price et al. in review). I selected *Lactobacillus jensenii*, as it is also a common species of the asymptomatic FUM (Rivera et al. 2020; Miller-Ensminger et al. 2020). Furthermore, the genus has been shown to be protective against post-instrumentation and postoperative UTIs (Pearce et al. 2015; Thomas-White et al. 2015). I selected *Actinomyces neuii*, as it was the most frequently isolated non-uropathogenic species of the aforementioned RUTI
cohort (Hochstedler et al. in prep) and, although it has been associated with UUI symptoms (Pearce et al. 2014), it is a common species of the female urogenital tract (El Aila et al. 2009). I selected at least three strains of each species from each disease state for susceptibility testing.

If RUTI isolates were more resistant to MH and formaldehyde than isolates of the same species from different disease states, then they would have higher MICs and MBCs. However, RUTI isolates rarely had higher MICs and MBCs (Table 2). Generally, I observed no difference in values between isolates of the same species. Potential uropathogens (E. coli through P. aeruginosa) had generally higher MICs and MBCs than commensal bladder species (A. neuii through S. epidermidis). Of all the species tested, Gram-negative species (E. coli, K. pneumoniae, P. mirabilis and P. aeruginosa) had higher values than Gram-positive species (E. faecalis, A. neuii, C. amycolatum, L. jensenii and S. epidermidis), with E. faecalis as an exception. The Gram-variable species (G. vaginalis) also had lower values than the Gram-negative species.

Whereas MH and formaldehyde had bactericidal activities against E. coli and E. faecalis (see below), the other metabolites did not (data not shown). The metabolites, aside from formaldehyde, had little to no inhibitory and no bactericidal activities; thus, I did not determine their MBC values for the other species.
Table 2. Heat Map of Inhibitory (MIC) and Bactericidal (MBC) Concentrations of Formaldehyde and MH Against All Bacterial Species Tested. Green, yellow and red/orange concentrations represent least to most resistant species of those tested, respectively. Concentration values are averages of at least three strains, for each “disease state” that the bacterial isolates were cultured from (each strain in biological triplicate).

<table>
<thead>
<tr>
<th>Species</th>
<th>Disease state</th>
<th>Formaldehyde (ug/mL)</th>
<th>MH (mg/mL)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td>E. coli</td>
<td>RUTI</td>
<td>306 ± 24</td>
<td>711 ± 69</td>
</tr>
<tr>
<td></td>
<td>UTI</td>
<td>278 ± 24</td>
<td>722 ± 139</td>
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<tr>
<td></td>
<td>Ctrl</td>
<td>261 ± 16</td>
<td>667 ± 94</td>
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<td>ATCC</td>
<td>200</td>
<td>TBD</td>
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<tr>
<td>E. faecalis</td>
<td>RUTI</td>
<td>311 ± 31</td>
<td>989 ± 135</td>
</tr>
<tr>
<td></td>
<td>UTI</td>
<td>306 ± 8</td>
<td>900 ± 73</td>
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<tr>
<td></td>
<td>Ctrl</td>
<td>361 ± 24</td>
<td>1000 ± 102</td>
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<td></td>
<td>ATCC</td>
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<td>TBD</td>
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<td>K. pneumoniae</td>
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<td></td>
<td>Ctrl</td>
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<td>833</td>
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<td>P. aeruginosa</td>
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<tr>
<td></td>
<td>Ctrl</td>
<td>178 ± 16</td>
<td>467</td>
</tr>
<tr>
<td>A. neuii</td>
<td>RUTI</td>
<td>100</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td>UTI</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>C. amygolatum</td>
<td>RUTI</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>G. vaginalis</td>
<td>RUTI</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>UTI</td>
<td>94 ± 8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>94 ± 31</td>
<td>100</td>
</tr>
<tr>
<td>L. jensenii</td>
<td>RUTI</td>
<td>133</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>UTI</td>
<td>133</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>139 ± 8</td>
<td>150</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>RUTI</td>
<td>267</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>Ctrl</td>
<td>262 ± 31</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>ATCC</td>
<td>200</td>
<td>TBD</td>
</tr>
</tbody>
</table>
Determination of Sensitivities in Consortia

To test whether species’ sensitivities to MH were altered when in the presence of others, I treated species both in isolation and as a consortium. One consortium was comprised of 3 commensal species (G. vaginalis, L. jensenii and A. neuii) and 3 classically uropathogenic species (E. coli, E. faecalis and S. aureus). One RUTI strain of each species was selected. The 3 commensal species and the 3 potential uropathogens were cultured in species-appropriate medium O/N. Following incubation, each species was diluted to an OD$_{600}$ of 0.001 in either 1 mL of fresh species-specific medium for culturing in isolation or 1 mL of particular medium for culturing the consortium (as described in Methods & Methods). The strains in isolation and the consortium were treated with 0, 5 and 15 mg/mL MH (final concentration) and allowed to incubate for 3 hours. Untreated (0 mg/mL MH) cultures were immediately plated at t=0. Following incubation of 3 hours, 1 uL of each culture was plated and CFU/mL counts were enumerated after O/N incubation.

As depicted in Figure 15, the leftmost set of CFU/mL measurements were taken from G. vaginalis in isolation and next set was taken from G. vaginalis in the consortium. Each colored bar within each set represents the CFU/mL enumerated corresponding to a treatment (0, 5 or 15 mg/mL MH). This pattern is repeated for all 6 species. Based on the comparison of CFU/mL values quantified at the initiation of the experiment, as compared to CFU/mL assessed after 3 hours (dark orange and gold, respectively), G. vaginalis maintained its CFU/mL over the course of 3 hours both when in isolation and when in a consortium. Although this species grew to about a log less when in the consortium, G. vaginalis was resistant to 5 mg/mL MH (green bar) when in the consortium whereas it was sensitive to this concentration when tested in isolation.
*L. jensenii* was about equally resistant to MH either in isolation or in the consortium. Shown by at least 2 log CFU/mL values less than those of the untreated cultured enumerated after 3 hours, 5 and 15 mg/mL MH (burgundy bar) seem to be bacteriostatic, but not bactericidal, to *A. neuii* when in a pure culture. As compared to the commensal species, *E. coli*, *E. faecalis* and *S. aureus* grew at least a log after 3 hours of culturing, likely because they grow more quickly because they are better suited to the media. Based on CFU/mL counts for each concentration, *E. coli*, *E. faecalis* and *S. aureus* were all slightly less resistant in the consortium. This is less notable, however, as *E. coli* and *S. aureus* grew to about a log less in the consortium. Based on similar CFU/mL counts in all treatments in both isolation and the consortium, *E. faecalis* was the most resistant to MH and least affected by being in a consortium of the 6 species.
Figure 15. Three Commensal Species and Three Potential Uropathogens Treated with MH in Isolation and in a Consortium. Three commensal species (G. vaginalis, L. jensenii and A. neuii) and three potential uropathogens (E. coli, E. faecalis and S. aureus) were treated in pure cultures and in a consortium (x-axis) with 0, 5 and 15 mg/mL (final concentration) of MH and allowed to incubate for 3 hours. Untreated (0 mg/mL MH) cultures were immediately plated at t=0 (dark orange bars). All cultures were plated after 3 hours. Each species and the consortium were treated in biological duplicates.

In a more recent experiment, a consortium of frequently isolated species from a cohort of women with UTI were cultured and treated with formaldehyde and MH. This ‘UTI consortium’ consists of E. coli, Staphylococcus epidermidis and Corynebacterium amycolatum. As described in Materials & Methods, 3 RUTI strains of each species were cultured in BHI medium in isolation and as combinatorial consortia. Whereas the previous experiment used OD$_{600}$ of 0.001 for each of the cultures, this consortium treated the more accurate standard of CFU/mL ($5 \times 10^5$ CFU/mL). MICs of formaldehyde and MH against the individual species and the
consortium were determined (Table 3). Each strain and consortium were tested in biological triplicate. *E. coli* had the highest MICs of formaldehyde and MH when in isolation.

**Table 3. Determination of Formaldehyde and MH MICs of the ‘UTI Consortium’ in Pure Cultures and in a Consortium.** For each species, three RUTI strains were treated in biological triplicates in isolation or in consortia. Values represent averages of strains and biological triplicates.

<table>
<thead>
<tr>
<th>Species (s)</th>
<th>Formaldehyde MIC (ug/mL)</th>
<th>MH MIC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>200</td>
<td>2.0</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td><em>C. amycolatum</em></td>
<td>100</td>
<td>0.5</td>
</tr>
<tr>
<td><em>S. epi + C. amy</em></td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td><em>E. coli + S. epi</em></td>
<td>133 ± 47</td>
<td>2.0</td>
</tr>
<tr>
<td><em>E. coli + C. amy</em></td>
<td>167 ± 47</td>
<td>2.0</td>
</tr>
<tr>
<td><em>S. epi + C. amy + E. coli</em></td>
<td>100</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Interestingly, when *E. coli* was in a consortium with either *S. epidermidis* or *C. amycolatum*, the formaldehyde MIC of the culture was lower, and lowest still when in the presence of both other species. Although, the MH MICs were consistently 2.0 mg/mL whenever *E. coli* was present.

**Urinary Sampling and Treatment of Author with MH**

As the clinical study of the longitudinal effects of MH on the urobiome of women with RUTI had not commenced, I was curious as to how MH affects the urobiome *in vivo* and in someone both without RUTI and without recent prior antibiotic treatment. Daily for 21 days, I collected a periurethral swab and a voided urine sample (first voided urine of the morning) in a sterile cup. Each day the paired samples were processed in the Wolfe Lab as described. The
data are presented as 3 histograms of urinary microbiota profiles over 7 days. On Day 7 (represented in Day 8’s samples), the author started taking 1 g MH (Alvogen) twice daily for one week. On day 10 (represented in Day 11’s samples) menstruation started. Prior to taking MH and menstruation, the urotype of the samples was generally *Lactobacillus*-predominant *(Figures 16 & 17)*. Based on relative abundances of species present, the paired voided urine and peri-urethral swab samples were similar. During treatment and partially coinciding menstruation (represented as a red line across Days 11-13), the urotype of the samples was *Streptococcus*-predominant *(Figure 17)*. Again, based on relative abundances of species present, the paired samples were similar. Post-treatment and menstruation, the urotypes of the samples were mixed genera *(Figure 18)*. Genera that had not been isolated in the two weeks prior, such as *Aerococcus* were isolated. Furthermore, only on a couple days did the periurethral swab resemble the voided urine, whereas for the first couple weeks they were generally similar.
Figure 16. Pre-treatment Week of the 21-day Experiment of Urinary Sampling and MH Treatment Microbiota Profiles. Profiles of the paired periurethral swabs and voided urine samples are presented over time (x-axis) as CFU/mL relative abundance (y-axis). The legend at the bottom is for the most common species isolated. As presented as single black bars, total CFU/mL counts (secondary y-axis) of each sample were determined.
Figure 17. Treatment Week of the 21-day Experiment of Urinary Sampling and MH Treatment Microbiota Profiles. Profiles of the paired periurethral swabs and voided urine samples are presented over time (x-axis) as CFU/mL relative abundance (y-axis). The legend at the bottom is for the most common species isolated. As presented as single black bars, total CFU/mL counts (secondary y-axis) of each sample were determined. The red line represents menstruation.
Figure 18. Post-treatment Week of the 21-day Experiment of Urinary Sampling and MH Treatment Microbiota Profiles. Paired periurethral swabs and voided urine were collected daily and processed. As presented as single black bars, total CFU/mL counts of each sample were determined.
CHAPTER IV

DISCUSSION

We now know that the bladder is not sterile and that certain microbiota profiles may provide protective effects against LUTS. Thus, native species’ sensitivities to antibiotics and antibacterials can no longer be disregarded. If a native urobiome species is more sensitive to a particular drug than the target group of species, treatment by that drug could exacerbate symptoms by loss of protection and a bloom of potentially uropathogenic microbes. It is archaic for clinicians to consider all urinary microbes a human threat worthy of being killed by broad-spectrum antimicrobials. Ideally, a more contemporary approach would be to recognize that most adult women are bacteruric and, although common urotypes exist, FUMs are not identical. Given the multitude of risk factors for UTI, individualization must be involved. As such, there is no “gold standard” or single treatment for UTI, especially RUTI. A treatment strategy for RUTI should focus on effective relief and prevention of recurrence, while minimizing off-targets effects (e.g. disturbance to the native microbiome and multi-drug resistant infections).

Mine is a novel study determining the susceptibilities of the native urobiome and potentially uropathogenic species to the antibacterial MH and its metabolites. My results support the conclusion that formaldehyde is indeed the active ingredient of MH. In contrast, although we hypothesized otherwise, there were no differences in susceptibility of isolates from different disease states.
Growth curves used to determine the MICs of metabolites against species revealed nuances that endpoint readings of MICs did not provide. For example, the lag phases of the *E. coli* cultures treated with high concentrations of formaldehyde were extended with increasing concentrations until completely inhibited by 500 ug/mL (Figure 2). If an endpoint reading for the MIC had been taken at about 15 hrs (915 mins) instead of 24 hrs, it would be determined to be 200 ug/mL instead of 500 ug/mL. From a physiological standpoint, an extended lag phase for one species might allow for opportunistic growth of another. Growth curves also established differences in growth rates and/or maximum biomass that would go completely unnoticed in an endpoint reading (Figure 10). As I observed slight sensitivity to the concentrations of hippuric acid tested, it would be important to monitor the pH of the medium upon addition of this acid to gauge whether the inhibition is due to change in pH.

Determination of the sensitivities of *E. coli* and *E. faecalis* to a range of pH-buffered media revealed that both *E. coli* and *E. faecalis* can grow in both acidic and alkaline media. As acidification of urine is another mode of MH’s action against UTI, both of these uropathogenic species could theoretically survive harsh pH extremes that other species might not. This ability to grow in a wide range of pH is relevant, as the pH of urine from asymptomatic individuals can range from 4.5 to 8.5 (Bilobrov et al. 1990); my data show that both uropathogens could theoretically thrive under those extremes.

Of the species tested, those with uropathogenic potential were more resistant to MH and formaldehyde than species that are considered to be commensals or native (Table 2). I have reported these susceptibility values as either ug/mL or mg/mL for formaldehyde and MH, respectively. It is likely more accurate to report them as molarity (moles of solute/liter of
so that they can be directly compared between them. Based on molecular weights (grams/mole), formaldehyde to MH is about 1:10. I expect my experimental susceptibilities to theoretically correspond to this ratio. I determined the ATCC strain of *E. coli*, for example, to have MICs of 200 ug/mL and 2000 ug/mL for formaldehyde and MH, respectively. These values are exactly a 1:10 ratio and a couple of the other species have similar ratios, although most have lower ratios.

It has been reported that *P. aeruginosa* and *P. mirabilis* are resistant to MH (FDA 2006). I speculate that this resistance is, in part, because these genera are able to catabolize urea for nutrient acquisition (urease-positive), thus increasing the pH of the milieu and that they are biofilm-formers (Armbruster et al. 2017; Khatoon et al. 2018). Indeed, *P. aeruginosa* had MH MICs that were more than 2-fold higher than for the rest of the species tested which does suggest some resistance to MH. *P. mirabilis* also had some of the highest formaldehyde MICs and MBCs and the second highest MH MBCs, which also suggests some resistance. Thus, initiation of prophylaxis with MH may select for uropathogens and inhibit commensals, thereby promoting recurrence or prolongation of an individual’s UTI symptoms. Phenotyping a patient’s urobiome prior to prescription should be recommended. However, as the standard urine culture and the manner in which results are reported by clinical microbiology labs produce an incomplete description of the microbiota of the lower urinary tract, more advanced approaches should be considered.

A caveat to this study is that I used different media to determine MICs and MBCs. Testing methods in clinical microbiology labs are standardized to make the test results reproducible as parameters such as the medium, incubating temperature and duration can all
influence the results. However, this approach is problematic for interpreting MIC and MBC results in general, as these values are not exact representations of the therapeutic doses required to inhibit/kill microbes in vivo. With these caveats in mind, I could not use the same medium for all the species tested in my study. For example, L. jensenii is a fastidious species and requires specific conditions; I had to use MRS medium. Whereas E. coli can grow slightly in MRS medium, other species tested cannot grow in MRS. Thus, without the use of MRS medium, it would not have been possible to include this common urinary tract species. A compelling way to determine the effects of growth medium on the susceptibilities would be to determine the susceptibilities for two easily cultivatable species, E. coli and E. faecalis, in minimal medium and compare them to those determined in BHI by this study.

Another caveat to this study is that the susceptibilities were determined in static, rather than shaking, cultures. Most of these species including E. coli, E. faecalis, S. aureus, S. epidermidis, K. pneumoniae, P. mirabilis and P. aeruginosa form biofilms, which are more in keeping with a persistent infection model and are known to alter susceptibilities of antibiotics (Hall-Stoodley et al. 2004; Van Acker et al. 2014). By interfering with biofilm formation, much larger volumes (greater than 200uL) and continuous shaking of the cultures prior to determining the susceptibilities would be a way to avoid the complicating effects of the biofilms.

As a niche is likely not colonized by a single species but rather by a polymicrobial community (a consortium), it is important to determine the sensitivities of species in the background more in keeping with a native environment. Clear biases were introduced in the consortia experiment with three potential uropathogens and three commensal species,
specifically the standardization of inoculum based on OD$_{600}$ rather than CFU/mL; the medium for culturing the consortium used was not ideal and the commensal species did not grow within the three hour timeframe. The parameters and biases should be considered when interpreting the results and for performing consortia experiments in the future (see below). An ideal medium for urinary bacterial isolate consortia experiments would better mimic urine composition and bladder conditions. It would be pertinent to determine the growth rates of the individual species when in pure culture and when combined. Obtaining growth rates in vitro is simpler than in vivo (or “ex vivo”) where one can perform shotgun metagenomic sequencing, determine the ratio of sequences from the origin and termini of replication and compare the ratios of the commensal and pathogenic species. Although there are explained biases, in both this experiment and with the “UTI consortium,” it does seem that species’ sensitivities were altered when co-cultured with other species (Figure 15 and Table 3). Further consortia experiments are required to make sound conclusions.

In an effort to understand the longitudinal effects of MH and how it affects the asymptomatic, untreated FUM in vivo, I sampled myself daily over 21 days. I was expecting to see a drop in the pH of the urines during the treatment; however, the weekly averages of the pH of the urine samples were almost identical, at about pH 6.5. Voided urine was used and menstruation coincided with treatment and are both confounding variables in determining the direct effects of the drug. As mentioned in the Introduction, it has been found that menstruation alters FUM by increasing alpha diversity of the urine; however, the urobiome profile tends to return to pre-menstrual urotype (Price et al. 2020). Interestingly, after both menstruation and the treated ended, the urine profile of Lactobacillus-predominance was not
restored and the samples were more diverse, particularly with the number of uropathogens detected. Perhaps had I continued to sample myself, I would have seen that a one-week washout was insufficient and that the *Lactobacillus* urotype was restored after two weeks, for instance. Ideally, I would have sampled myself for another week or at a time circumventing menstruation. It should be noted that, while these potential uropathogens were present, I was without LUTS. This finding of a bloom of potential uropathogens has potential implications for administration of MH. The planned longitudinal study will provide a formal depiction of the effects of MH on the urobiomes of women with RUTI.

As benchwork was put to a halt due to the COVID-19 pandemic, there are many further experiments of interest. As proposed for Aim 2, there is an ongoing longitudinal clinical study to determine the effects on the urobiome of women with RUTI, for which Dr. Acevedo Alvarez will be recruiting. This study will be the first longitudinal MH clinical study to phenotype women prior to prescribing MH and to examine the effects of MH on the urobiome. It also will contribute longitudinal data focused on the urobiome of women with RUTI.

One set of experiments that can be done at the bench is additional consortia experiments. In a large study of patients with UTI (N=3,124), a company called Pathnostics discovered that urinary isolates’ resistances to 8 different common antibiotics increase when species are consortia (Vollstedt et al. in review). Their findings exemplify the importance of testing susceptibilities within the native environment confines. Particularly, additional consortium experiments mixing RUTI uropathogens and RUTI native or commensal species.

Other future experiments include (1) testing further RUTI-relevant and commensal species for their MICs and MBCs to formaldehyde and MH via growth curves, which would
provide more data for phenotyping women in the clinical study and (2) treating the species tested, particularly *E. coli* and *E. faecalis*, with formaldehyde and MH in different incubation conditions and with higher concentrations of the metabolites for which there was slight inhibition but neither MICs or MBCs could be verified. It would be pertinent to determine the MICs and MBCs of the facultative anaerobic species in anaerobic and/or microaerophilic conditions more closely resembling the bladder niche. These data could be used to assess whether the drug is more effective in the bladder.

As MH is used for suppression of growth rather than treatment, it would also be applicable to determine the MICs and MBCs of strains at lower CFU/mL rather than the higher $5 \times 10^5$ CFU/mL, which is more of a treatment model, and determine whether MH is more effective at these lower CFU/mL. It would also be relevant to determine and compare species’ MICs and MBCs of commonly prescribed UTI antibiotics to be used as references of effectiveness.

As it is reported that bacteria do not develop resistance to MH (FDA 2006), experiments to analyze this claim were attempted. In one design, working in parallel with my own sampling, urine was treated with increasing concentrations of formaldehyde similar to the Materials and Methods described for the three uropathogens and three commensal species consortium. The goal was to be able to isolate species that exhibited resistance and re-retreat them in culture to test whether resistance could be evolved. The undiluted treated and untreated urine samples were plated after three hours of incubation. After O/N incubation, the plates were examined, comparing surviving colonies to those of the plated untreated sample. On a few attempts at this design, I observed a recurring problem - the untreated sample did not contain *E. coli*;
however, upon treatment, E. coli would grow and completely overtake the sample plated. I do not believe that the E. coli is merely contamination of those treated samples, but I do hypothesize that it is still opportunistically overtaking the culture nonetheless. Perhaps E. coli was not visible to the naked eye upon examination of colonies but bloomed when other species were inhibited. To potentially inhibit this overwhelming growth of other species, urine could be spun down and the pellet could be resuspended in innocuous PBS or in urine composition medium. That bacterial suspension could be aliquoted, treated with formaldehyde and MH and plated immediately.

A way to gain insight on the mechanism of resistance to formaldehyde and MH would be to query the genomes of the species in question looking for resistance genes. In a preliminary literature review of genes associated with formaldehyde resistance, a few were found. These genes are a formaldehyde-sensing and detoxifying operon called frmRA(B) (Denby et al. 2016). By querying genomes on my behalf, one of my colleagues, Cesar Eber Montelongo Hernandez, found that the three E. coli RUTI isolates possess these genes.

As MH is well-tolerated with little to no reported side effects and it is reported that much higher concentrations of formaldehyde are required to be toxic to mammalian cells (FDA 2006), toxicity could be assayed using urothelium in tissue culture with a range of concentrations of formaldehyde in isolation and in culture with bacterial strains. The urothelial cells could be examined microscopically for viability.

Through the use of growth curves and static cultures to determine the formaldehyde and MH MICs and MBCs, assays of inhibition in different pH-buffered medium, treatment of consortia to determine how sensitivities change and treatment of myself with MH, I found that
MH might select for uropathogens and inhibit putative commensal species. Based on these data, initiation of prophylaxis with MH might promote recurrence or prolongation of an individual’s UTI symptoms. The ongoing clinical study on the effects of MH on the urobiome of women with RUTI will provide sound in vivo data for this hypothesis.


Caldwell JA. Bacteriophagy in urinary infections following the administration of the bacteriophage therapeutically. *Arch Intern Med* 41, 189 (1928).


Curtiss, N et al. A case-controlled study examining the bladder microbiome in women with Overactive Bladder (OAB) and healthy controls. *European journal of obstetrics, gynecology, and reproductive biology* 2017;214:31-5. PMID:28463826


FDA. (2006). *UREX (methenamine hippurate).*
https://www.accessdata.fda.gov/drugsatfda_docs/label/2008/016151s025lbl.pdf


Fok, CS et al. Urinary symptoms are associated with certain urinary microbes in urogynecologic surgical patients. *International urogynecology journal* 2018;29(12):1765-71. PMID:30116843


*Urology* 2019;16(7):422-32. PMID:31073244


Guay, DR. Cranberry and urinary tract infections. *Drugs* 2009;69(7):775-807. PMID:19441868


Gupta, K, and BW Trautner. Diagnosis and management of recurrent urinary tract infections in non-pregnant women. *BMJ (Clinical research ed.)* 2013;346:f3140. PMID:23719637


Hilt, EE et al. Urine is not sterile: use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. Journal of clinical microbiology 2014;52(3):871-6. PMID:24371246


Kaewsrichan, J et al. Selection and identification of anaerobic lactobacilli producing inhibitory compounds against vaginal pathogens. *FEMS immunology and medical microbiology* 2006;48(1):75-83. PMID:16965354


Kass, EH. Bacteriuria and the diagnosis of infections of the urinary tract; with observations on the use of methionine as a urinary antiseptic. *A.M.A. archives of internal medicine* 1957;100(5):709-14. PMID:13468815


Komesu, YM et al. The urinary microbiome in women with mixed urinary incontinence compared to similarly aged controls. *International urogynecology journal* 2018;29(12):1785-95. PMID:29909556


Lewis, K. Persister cells. *Annual review of microbiology* 2010;64:357-72. PMID:20528688

Ling Z, Liu F, Shao L, Cheng Y, Li L. Dysbiosis of the Urinary Microbiota Associated With Urine Levels of Proinflammatory Chemokine Interleukin-8 in Female Type 2 Diabetic Patients. *Front Immunol.* 2017;8:1032. PMID:28943876


Meriwether, KV et al. The Vaginal and Urinary Microbiomes in Premenopausal Women With Interstitial Cystitis/Bladder Pain Syndrome as Compared to Unaffected Controls: A Pilot Cross-Sectional Study. *Frontiers in cellular and infection microbiology* 2019;9:92. PMID:31024861


Platt, R. Quantitative definition of bacteriuria. The American journal of medicine 1983;75(1B):44-52. PMID:6349344


Shoskes, DA et al. The Urinary Microbiome Differs Significantly Between Patients With Chronic Prostatitis/Chronic Pelvic Pain Syndrome and Controls as Well as Between Patients With Different Clinical Phenotypes. *Urology* 2016;92:26-32. PMID:26970449


Ursell, LK et al. Defining the human microbiome. *Nutrition reviews* 2012;70 Suppl 1:S38-44. PMID:22861806


VITA

The author, Nancy Sloan, was born in Berkeley, CA on August 5, 1992 to Francie and John Sloan. She attended the University of California-Santa Cruz where she earned a Bachelor’s of Science in Molecular, Cell and Developmental Biology in December 2014. After graduation, Nancy worked as a research assistant for three years in two bioengineering labs in Berkeley. In 2018, she matriculated into the Loyola University Chicago Stritch School of Medicine Microbiology and Immunology Graduate Program and began her graduate education in the Microbiology and Immunology Program under the mentorship of Dr. Alan Wolfe.

Nancy’s thesis work on determining the susceptibilities of urinary microbiome bacteria to the UTI drug, methenamine hippurate, was supported by an RO1 grant. After completion of her graduate studies, Nancy will pursue a career as a microbiologist in industry.