URINARY AND VAGINAL MICROBIOME COMMUNITY STRUCTURE IN WOMEN WITH URGENCY URINARY INCONTINENCE

Identification of bacteria in the urinary and vaginal microbiomes associated with urgency urinary incontinence using microbial co-occurrence networks

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Abstract

Urgency urinary incontinence (UUI) is a chronic, burdensome condition with urges to urinate that are difficult to defer and results in urinary leakage. UUI impinges on an individual's quality of life, and affects about 15% of American women. Despite the heavy burden of UUI, we still have a poor understanding of the condition and how to most efficiently triage patients to the most effective treatment.

The microbiome is recognized to play a role in genitourinary health. The vaginal microbiome is long known to inhabit the vaginal tract and play an important role in maintaining genitourinary health and preventing disease. Conversely, the bladder was thought to be sterile in the absence of an acute clinical infection, but recent studies have overturned this paradigm and have identified microbes in the bladder of healthy individuals. This has led to emerging evidence to show that changes in the microbiome of the bladder may play an important role in the pathophysiology of UUI.

More specifically, decreased diversity in the urinary microbiome is associated with increased symptom severity for women with UUI. Moreover, the presence or absence of specific microbes is associated with UUI. In contrast, little work has explored the relationship of the known vaginal microbiome to either the urinary microbiome or UUI symptoms. Moreover, current computational methods used to study the urinary microbiome neglect the underlying ecology of bacteria.

Here, we leverage network-based methods to propose a framework to generate microbial co-occurrence network structures to identify bacteria in the urinary and vaginal microbiomes associated with UUI. Network-based methods allow for hypothesis generation of the underlying microbial community dynamics. Ultimately, these network structures facilitate exploration of novel, therapeutic microbial targets while also being ecologically aware of the therapeutic effects on the community structure.

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1 Introduction

Urgency urinary incontinence (UUI) is a common but chronic condition characterized by a sensation ("urge") to urinate that is difficult to defer and often results in involuntary loss ("leakage") of urine (Abrams et al. 2002). It affects women to a much greater extent than men, which negatively impacts their quality of life, mental health, and financial well-being (Powell et al. 2018; Dugan et al. 1998; Robinson and Pearce 1998).

There are three known etiologies for UUI: detrusor overactivity, poor detrusor compliance, and bladder hypersensitivity (Aoki et al. 2017). Each of these etiologies involve different anatomical structures of the bladder. Detrusor overactivity is the spontaneous contraction of the detrusor muscle that an individual cannot defer. Poor detrusor compliance occurs when the detrusor muscles are unable to properly stretch when filling with urine, resulting in increased pressure that leads to leakage. Last, emerging evidence suggests that the bladder urothelium, the epithelial cells that line the inside of the bladder, is not a passive barrier, but is involved in bladder sensory signaling. Furthermore, people with urinary urgency have an increased number of these sensory receptors (Li et al. 2011).

The approach to treatment is iterative and tiered, starting with behavioral changes, followed by medication management and then finally surgery if symptoms do not respond to these previous measures. However, all levels of treatment have high attrition rates (Wyman, Burgio, and Newman 2009; Komesu et al. 2011). Anticholinergic drugs are the most common class of medications and they work by relaxing the detrusor muscle, the smooth muscles that line the bladder. However, up to 50% of women have intolerable side-effects including dry mouth, dry eyes, and constipation (Hartmann et al. 2009). In elderly patients, these drugs can worsen symptoms of dementia, which can then be difficult to

prescribe to older patients (Gray et al. 2015; Coupland et al. 2019; American Urogynecologic Society (AUGS) Guidelines Committee with the assistance of Tonya N. Thomas and D. 2017). Moreover, these side-effects may worsen more in the elderly because of interactions with other drugs they may be taking and the changes in their body's ability to metabolize drugs (Thüroff et al. 1998).

More recently, with the recognition that the bladder urothelium is an active and responsive barrier, attention is being focused on how inflammation and infection can contribute to the etiology of urinary incontinence. Historically, the bladder was thought to be sterile in the absence of an acute infection. However, with more widespread use of high-throughput sequencing and use of transurethral catheters to avoid contamination, it has been realized that bacteria are normally present in the bladder (Wolfe et al. 2012). Additionally, the development of an alternative culturing method, called expanded quantitative urine culture (EQUC), has further demonstrated the presence of living bacteria in the female bladder (Hilt et al. 2014). In short, the detection of these commensal bacteria in the bladder is the first step to focus on how infection and inflammation contribute to UUI.

It has increasingly been appreciated that there are differences in the microbial flora of patients with and without UUI (Pearce et al. 2014, 2015) and decreased diversity in the urinary microbiome is associated with more severe UUI symptoms (Karstens et al. 2016). Furthermore, medication response was found to be related to the urinary microbiota composition, where responders at baseline were more likely to have less diverse bacterial microbiomes than non-responders (Thomas-White et al. 2015). Overall, it is becoming clear that characteristics of the urinary microbiota play a role in UUI.

In addition to evidence associating the urinary microbiome with urogenital health and disease, it is long known that the nearby vaginal microbiome also contributes to urogenital health and disease. The vaginal microbiome, or vaginal flora, is known to play a protective role in preventing diseases such as bacterial vaginosis, sexually transmitted infections (STIs), and urinary tract infections (UTIs) (Ma, Forney, and Ravel 2012). More specifically, four *Lactobacillus* species (*L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii*) have been shown to dominate the vaginal flora in healthy, reproductive-age women (Ravel et al. 2011). The role of *Lactobacillus* species in the vaginal microbiota is also becoming clearer, whereby they tightly regulate the vaginal environment by producing lactic acid after consuming host-provided glycogen (Nunn and Forney 2016).

Despite research supporting the role of the microbiome in urogenital conditions, little is known about the relationship between the urinary microbiome and vaginal microbiome. Individually, the microbiota of the vagina and the urinary tract are known to play a complex role in determining urogenital health (MacIntyre, Sykes, and Bennett 2017). The overall composition of the vaginal and urinary microbiomes are more similar to each other in bacterial composition compared to the microbiomes of other human body-sites (i.e., the gut microbiome) (Thomas-White et al. 2018; Komesu et al. 2020). Furthermore, the functional diversity of the urinary and vaginal bacterial genomes are also more similar (Thomas-White et al. 2018). However, the characteristics of how these two microbiomes interact as an ecological environment and how their interaction may influence the development of disease are not well understood.

The vaginal microbiota and the urinary microbiota are studied using similar approaches, but these methods have limitations in their inferential power and downstream

clinical application. Traditional approaches to characterizing the microbiome include quantifying the number or distribution of types of bacteria per sample (alpha diversity) and quantifying differences between samples (beta diversity) (Finotello, Mastrorilli, and Di Camillo 2016). Also included is differential abundance, which identifies bacteria with the greatest relative change between groups of interest (Paulson et al. 2013). However, these approaches are a starting point to developing hypotheses about how emergent changes in the normal microbial flora influence the development of diseases, such as UUI (Shade 2017).

Investigations to date have focused on studying the presence and type of microbial flora present. However, newer approaches now allow for systems-based approaches to investigate how urogenital microbial networks influence the development and response to treatment of UUI. Microbes are not merely a collection of independent organisms. Rather, they engage in ecological interactions that affect the stability and dynamics of the complex microbial community. We can start modeling the complexity of these interactions as a system using network-based approaches. These approaches aim to create a more comprehensive picture of microbial variation associated with UUI. This framework also allows us to consider the impact of bacteria and bacterial subgroups in women with UUI using the context of the microbial community. Investigating the role of the urinary and vaginal bacteria in UUI using innovative computational techniques may help identify modifiable risk factors that can be a target for future interventions. With this background, this thesis aims to address these questions:

1. **How can we more effectively use microbial abundance data to understand the relationship between the vaginal and urinary microbial communities associated with urgency urinary incontinence?**

2. **How are traditional microbial analyses represented within the microbial network as it relates to urgency urinary incontinence?**

3. **How do interactions within the urinary and vaginal microbiome influence the development of urgency urinary incontinence?**

In the next sections, we will review important developments in urgency urinary incontinence research, both of the urinary and vaginal microbiomes, and microbial network analyses. In Chapter 2, we identify differences in the microbiome of women with and without urgency urinary incontinence using traditional microbiome analytical techniques. In Chapter 3, we introduce our network model and its application to the urinary microbiome and the vaginal microbiome to identify co-occurring groups of bacteria associated with urgency urinary incontinence. Last, we present a discussion and conclusion in Chapter 4, followed by suggested future work in Chapter 5.

1.1 Statement of aims

Specific Aim 1. Identify differences in the microbiome of women with and without urgency urinary incontinence using traditional microbiome measures.

We will investigate the differences in the urinary microbiome and vaginal microbiome of women with and without UUI. We hypothesize that the diversity and abundance of bacteria will be different between women with and without UUI.

Specific Aim 2. Characterize the site-specific microbial community network structures of the female urogenital tract and characterize its relationships with urgency urinary incontinence in women

We will investigate the interactions within the urinary and vaginal microbiome by creating a network-based framework to understand the relationship between the site-specific microbiomes of the female urogenital tract and urgency urinary incontinence in women. This framework uses co-occurrence correlations to define bacterial relationships and will allow us to compare the currently accepted microbiome data analysis methods for the vaginal microbiome (Aim 2A) and urinary microbiome (Aim 2B). This framework will also enable us to assess microbiome communities with an ecological perspective, an understanding of which will generate experimentally testable hypotheses on the bacterial interactions that contribute to health.

1.2 Aim 1 background

Urgency urinary incontinence (UUI) is a highly prevalent condition among women, the risk of which increases with age, and ultimately lowers their quality of life. This stigmatized condition is currently treated through a tiered treatment plan with increasing invasiveness with each treatment trial. Despite the progress in better managing this condition, the etiology of the disease and effective patient stratification are still poorly understood.

1.2.1 Current understanding of urgency urinary incontinence in women

Urgency urinary incontinence is a chronic condition that predominantly affects women. The International Urogynecological Association (IUGA) and the International Continence Society (ICS) define this condition as, "[an] involuntary leakage accompanied by

or immediately preceded by urgency[1]" (Haylen et al. 2009). The underlying condition of urinary incontinence affects both sexes. For men, urinary incontinence is often because of an enlarged prostate or prostate cancer. In contrast, urinary incontinence in women occurs because of issues surrounding the bladder or pelvic floor muscles (Aoki et al. 2017). These issues in women are often related to childbirth or menopause. These life events that are unique to women motivate the innovation of treatment options for this chronic condition.

The prevalence of urgency urinary incontinence in women is a global problem and reports estimate it to gradually increase over time. A number of studies across multiple countries estimated female urinary incontinence prevalence. Although there is variation in estimates, Milsom et al. (2013) summarizes that an at least weekly leakage occurs in approximately 10% of all adult women, while occasional leakage affects between 25% and 45% of adult women. One common risk factor that is consistently associated across studies is age. Across eighteen reports spanning from 1966 to 2011, the rate of new cases was less than 2 per 1,000 person-years before age 40 years, but tended to increase there after (Stewart et al. 2014). Moreover, urinary incontinence affects societies financially, costing the US an estimated $19.5 billion in 2000 (Hu et al. 2003). Estimates suggest the number of US women with urinary incontinence will increase from 18.3 million in 2010 to 28.4 million by 2050 (Wu et al. 2009). These statistics demonstrate that this condition is not uncommon among women.

[1] Haylen et al. (2009) defines "urgency" as the "complaint of a sudden, compelling desire to pass urine which is difficult to defer"

Despite this observed prevalence of urinary incontinence, stigma around it has crippling effects on one's quality of life and blocks individuals from receiving the clinical help they may need. Stigma around medical conditions can lead to worse outcomes because of the fear of discrimination from peers and discourage care-seeking (Link and Phelan 2006). One study to better understand the stigma around urinary incontinence was conducted using the Boston Area Community Health (BACH) Survey. The BACH survey is a population-based, random sample epidemiological survey of urologic symptoms with a random subsample of 151 men and women aimed to differentiate the stigma associated with both frequency and urgency from just urinary incontinence. Their results show that there is not only stigma around urinary incontinence, but also urinary frequency and urgency (Elstad et al. 2010). The women felt stigmatized for having an "unclean body or compromised social identity." Furthermore, a study of 201 women at St. George's and St. James's Hospitals, London reported the women had reluctance to discuss their possible urinary incontinence problems with their primary care provider because of embarrassment, which further delayed the appropriate treatment for their symptoms (Norton et al. 1988). These unique issues to women, the high prevalence of urinary incontinence in women, and stigma around urinary incontinence all motivate the reason for the ongoing research to better understand the disease etiologies and develop novel treatment options for UUI.

There are three known etiologies for urinary incontinence: detrusor overactivity, poor detrusor compliance, and urothelium hypersensitivity (Aoki et al. 2017). Detrusor overactivity is defined as, "a urodynamic observation characterized by involuntary detrusor contractions during the filling phase which may be spontaneous or provoked" (Abrams et

al. 2002). In contrast, a bladder with low detrusor compliance means it cannot stretch and contract, which results in leakage from the increased pressure within the bladder and can also cause discomfort. Last, a bladder with urothelium hypersensitivity will react to stimuli. The urothelial lining is not a passive barrier, but is able to detect thermal, mechanical, and chemical changes. This last etiology involving the urothelium has shifted the focus of ongoing research to the effects of urothelial inflammation and infection on UUI. The next section explores current treatment plans that target these known etiologies.

1.2.2 Current medical treatment guidelines

The differential diagnosis of UUI primarily depends on urinary incontinence associated with symptoms of urgency. Robust self-completion questionnaires are widely recognized as a valid way to measure a person's signs and symptoms to more accurately diagnosis UUI[2]. There are two broad groups of questionnaires: general surveys and urgency urinary incontinence specific. The general surveys measure lower urinary symptoms (LUTS). Some examples include the Urinary Distress Inventory (UDI) (assesses lower urinary tract dysfunction) and Incontinence Impact Questionnaire (IIQ) (assess urinary incontinence's impact on health-related quality of life) (Shumaker et al. 1994). Overactive bladder[3] symptom-specific surveys include the OAB-q (assesses all overactive bladder symptoms and their impact on health-related quality of life) (Coyne et al. 2002) and International Consultation on Incontinence Questionnaire (ICIQ) (assess urinary incontinence and its impact on the quality of life) (Avery et al. 2004). Despite equipping

[2] Other important measurement tools not mentioned include urodynamics and urine culture, both of which can exclude differential diagnoses.

[3] A similar condition that gets diagnosed with urgency urinary incontinence is overactive bladder; urgency urinary incontinence is (Aoki et al. 2017).

researchers and health professionals with a variety of validated assessment tools for urinary incontinence symptoms, no ideal questionnaire exists (Shy and Fletcher 2013). Nonetheless, the variety of questionnaires give clinicians the ability to assess and customize a patient's assessment to better treat and manage their symptoms.

An additional tool to measure patient symptoms is a bladder diary. Patients can track their symptoms in bladder diaries, also known as voiding diaries, for three or seven days (Dmochowski et al. 2005). For each day, the patient records information for hourly intervals on details such as fluid intake, number of voids, and the level of urgency they felt. The observations of the diary can then facilitate discussions between the health professional and the patient to personalize their health plan. These diaries are invaluable in part because of its temporal nature and intentional record keeping making it a more objective measure of their condition's severity (Stav, Dwyer, and Rosamilia 2009). Importantly, this record keeping also allows patients to track symptom improvement, thus shedding light on therapeutic effectiveness or failure. In sum, bladder diaries offer an integral assessment and tracking tool in evaluating a patient's symptoms. After completing a thorough symptom assessment, healthcare professionals can proceed with multiple lines of treatment, each with varying levels of invasiveness and possibility of irreversible side effects.

There are three general lines of treatment (Corcos et al. 2017). First-line treatments are non-invasive and reversible, which includes behavioral therapies, lifestyle changes, and patient education. There are two main treatments for behavioral therapy: bladder training (e.g., timed voiding) and pelvic floor muscle therapy (e.g., urgency suppression). Lifestyle changes that patients may make include managing fluid and caffeine intake and weight loss.

Patient education empowers them to engage with their treatment, which may affect their motivation and ultimately influence their health outcome. The second line of treatment involves pharmacological management of antimuscarinics[4] (to target muscarinic acetylcholine receptors) and beta-3 andrenoceptor agonists (to target β_3 adrenergic receptors). These pharmacological agents act to increase bladder muscle function, with the goal of improving quality of life and decreasing a patient's symptoms. The last and third-line treatments include onabutulinumtoxinA and neuromodulation. Intra-detrusor injections of onabutulinumtoxinA, more commonly known as Botox, paralyze the detrusor smooth muscle enough to reduce the number of urgency episodes (Chapple et al. 2013; Apostolidis, Dasgupta, and Fowler 2006). Neuromodulation treatments are artificial nerve stimulations that include peripheral tibial nerve stimulation (PTNS) and sacral neuromodulation (SNM). More specifically, the International Neuromodulation Society defines these neuromodulation methods as, "the alternation of nerve activity through the delivery of electrical stimulation of chemical agents to targeted sites of the body" ("International Neuromodulation Society," n.d.). These three lines of treatment give clinicians the flexibility to personalize treatments based on an individual patient's needs and symptom presentation.

1.2.3 Treatment limitations for physicians and patients

Despite the range of treatments, each option has considerable inconveniences and clinical limitations, which warrant further research into alternative therapeutics and

[4] Antimuscarinics are a subset of a larger class of anticholinergic drugs, which also include the distinctly separate class of nicotinic receptor antagonists, and should be noted to avoid confusion (Montastruc et al. 2010)

treatment options. The first-line treatments (e.g., behavioral therapies) are the least invasive, but they require behavior changes that are difficult for long-term compliance among patients (Borello-France et al. 2010). Second-line treatments (e.g., antimuscarinics) have a separate problem. Compliance among patients is dependent on which drug-specific side effects they can tolerate (e.g., dry mouth and impaired cognitive function); this is further complicated with the fact that randomized trials have found that no single drug outperforms the rest (Gormley et al. 2015). Moreover, for older individuals, careful consideration should be taken when prescribing antimuscarinics because of the possible worsening of cognitive function in an already at-risk population for cognitive decline (Ouslander 1995). Last, although neuromodulation treatments are effective and regarded as safe, the side effects include unwanted bleeding, infection/inflammation, and pain at the device insertion site (Corcos et al. 2017). The tiered treatment course, which increases with invasiveness, could be made more efficient if the most effective treatment could be more directly prescribed. In theory, this ideal scenario would increase overall patient satisfaction from not experiencing a number of unnecessary side effects and increase physician efficiency in promptly designing the best treatment.

1.2.4 The Human Microbiome Project to facilitate progress in host-microbe understanding

The development of DNA-based analyses from high-throughput sequencing has expanded the ability to characterize not just the disease-causing bacteria. The completion of the Human Genome Project promoted enthusiasm for a deeper and genome-based understanding of human health and disease. Immediately after the completion of the human genome, researchers reminded the research community that microbes also play a vital role in human health and disease (Relman and Falkow 2001; Davies 2001). Individual

smaller initiatives have since been conducted to better understand these bacteria on humans. However, at the time, there had not been a concerted effort to systematically characterize and understand the human microbiome. Since then, many initiatives worldwide have been conducted.

The Human Microbiome Project (HMP) was one of the first coordinated efforts to characterize the healthy human microbiome occupying the body. Previously, there was no comprehensive effort in careful sampling across the multiple human body sites at a population level. The National Institutes of Health initiated this project in 2007 (Phillips 2008; Proctor 2012) with the following goals: demonstrate feasibility of characterizing the human microbiome, understand the microbiome's influence on disease, and develop the infrastructure for future microbiome research (The NIH HMP Working Group et al. 2009). The project also created important computational and technological resources to sustain future microbiome research (Proctor 2012). For example, bioinformatics software for 16S rRNA gene sequencing and whole metagenome sequencing assembly was developed[5] along with centralized databases to host reference genomes for the human microbiome.[6] The project recruited 300 healthy volunteers to participate, where 279 were sampled twice and 100 were sampled for a third time. Each participant was then sampled up to 18 sites around the body. The major results of the project show the range of microbial variation in a Western population and how distinct body sites differed more within an individual than

[5] Tools and technology can be found at https://www.hmpdacc.org/hmp/resources/.

[6] Data can be found at https://portal.hmpdacc.org/.

between the same body sites among different individuals. This project has left a lasting impact on the research community, catalyzing a new field for decades to come.

Since the HMP has finished, studies suggest that body site-specific diseases are independent of the concentration of their resident bacteria. A naive assumption about infections is that more bacteria may lead to more disease. But from the HMP, we have learned, for example, that the vagina generally contains less resident bacteria compared to both the oral cavity and gut bacteria (Human Microbiome Project Consortium 2012). However, some notable findings show that each body habitat can have an associated disease with changes in its microbiome. Some examples include the interaction between obesity and the gut microbiota (Delzenne and Cani 2011), chronic obstructive pulmonary disease (COPD) and the respiratory microbiome (Dickson, Erb-Downward, and Huffnagle 2013), and urogenital infections and the vaginal microbiome (White et al. 2011). Each of these disease-associations altogether not only support the association between bacteria and disease, but also the independence of disease on the concentration of bacteria at the body area. This further supports the need to understand how each bacterium's role plays in human health and disease and to go beyond associations to also understand causal mechanisms.

1.2.5 Urinary microbiome, vaginal microbiome, and urgency urinary incontinence

Although the bladder was originally thought to be sterile and excluded from the HMP, there has been progress in changing the paradigm that the bladder is sterile. With emerging evidence of bacteria residing in the bladder in the absence of an acute infection, the microbiome of the healthy bladder may play an important role in preventing the

pathophysiology of lower urinary tract symptoms. Similarly, our understanding of lower urinary tract symptoms is not isolated from bacteria in the bladder. Robust evidence shows a link between the vaginal flora and urinary tract infections, which suggests a possible interaction between the vaginal flora and bacteria of the bladder. This highlights the need to explore the structure of a combined urogenital microbiome.

1.2.6 From neglect to recognition of the urinary microbiome

The bladder was notably not included in the HMP because of the long-held belief that the bladder is sterile (Thomas-White et al. 2016). The exclusion from the HMP slowed research on the bladder compared to other body sites. As mentioned above in section 1.2.4, these numerous examples show that the human microbiome changes with normal health and disease. For example, the vaginal microbiota of reproductive women varies with a number of host factors such as age and ethnicity (Ravel et al. 2011). Careful sampling of bacteria is also necessary because contamination from the environment can confound research findings. As a result of the technological developments under the HMP, sample collection methods were carefully designed and optimized to maximize their use for downstream analyses (Aagaard et al. 2013). Similarly, computational groups also developed computational methods to handle these novel sources of data (Gevers et al. 2012). These advancements developed collaboration networks, validated and agreed upon protocols, and robust computational techniques. Although these methods were all used to study bacterial communities, this did not guarantee direct applicability to other types of samples. The exclusion of the bladder from the HMP left researchers uncertain as to whether these established technologies and computational methods apply to study the microbial potential in the urine without additional considerations.

Decades-old hints of latent bacteria in the bladder and the difficulty in studying them were recently rediscovered, which ultimately helped reignite interest in the scientific and medical implications of bacteria in the healthy bladder. In 1979, Rosalind Maskell[7] and colleagues investigated the strange phenomenon called "urethral syndrome," where patients would present with UTI symptoms but without significant bacteriuria[8]. Maskell, Allen, and Pead showed that incubating urine cultures for more than 24 hours under 7% CO_2 (instead of atmospheric air) revealed a number of slow-growing organisms (Maskell, Allen, and Pead 1979). The presence of these microbes contradict the at-the-time paradigm of a sterile bladder. Such a finding should not be surprising given the adaptability of microorganisms to exist virtually anywhere there is liquid water present (Rothschild and Mancinelli 2001). Maskell and her colleagues' findings suggested at least one technological barrier (i.e., optimized growth media conditions) to better understand these more "fastidious" bacteria in the bladder.

Leaning on the pioneering work of Maskell and colleagues, a separate group recently identified two major barriers in comprehensively identifying bacteria in the bladder: first, avoiding contamination from the vagina, and second, ensuring that the extracted bacterial DNA originated from live bacteria (Thomas-White et al. 2016). The vagina contains its own microbiome and the urethra that carries the urine is located in front of the vaginal opening. Typical urine specimens to be tested are collected using a technique called clean-catch,

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[7] Maskell spent much of her research career on understanding urinary tract infections, postulating the early existence of bacteria in the bladder that require special culturing techniques https://doi.org/10.1136/bmj.i6147.

[8] Bacteriuria is defined as the presence of bacteria in urine.

midstream voided urine.[9] Although measures are taken to avoid contamination, close proximity of the urethra to the vagina increases the risk of contamination of the urine with resident vaginal bacteria. For this reason, Wolfe et al. (2012) investigated the suitable urine collection method through a study of patients undergoing gynecologic surgery. They found clean-catch urine to be inferior to another urine collection method of transurethral catheterization. As the gold standard for detecting what is in the bladder, the suprapubic aspirate collection technique[10] that directly samples from the bladder was used as a comparison. Their results showed that the microbial composition of bladder samples between the suprapubic aspirate and transurethral catheter are more similar to each other than clean-catch urine (Wolfe et al. 2012). These results show that transurethral catheterization is a suitable method to avoid vulvovaginal contamination when sampling urine from the bladder.

After the technological advance to adequately sample urine from the bladder without contamination, two new questions arise: can we be sure the bacteria from the bladder are alive and is there a way to engineer the conditions to culture these bacteria. Pioneering work by Hilt et al. (2014) developed the next necessary technological advance to answer both of these questions. Their group developed what is called an expanded quantitative urine culture (EQUC). Traditionally, the clinical microbiology protocol for culturing urine streaks 0.001 mL of urine onto a 5% blood agar plate and is then incubated

[9] The protocol for clean-catch, midstream voided urine is as follows. The area between the labia is first cleaned with sterile wipes before the subject is then asked to briefly urinate (in order to flush out any resident microorganisms and secretions). The subject is then asked to continue the flow of urine so a urine cup can collect a urine specimen. More on the topic can be found at https://medlineplus.gov/ency/article/007487.htm.

[10] https://medlineplus.gov/ency/patientinstructions/000145.htm.

aerobically at 35°C for 24 hours. In contrast, the EQUC protocol notably uses 100 times as much urine (0.1 mL). The growth medium is also a modified blood agar plate with chocolate, colistin, and nalidixic acid agars, which is then incubated in 5% CO_2 at 35°C for 48 hours. Out of the 65 urine specimens, there were 52 specimens (52/65 [80%]) that were reported as no growth by the traditional clinical microbiology protocol. However, the new protocol was able to grow bacterial species in 48 of the previously no-growth specimens (48/52 [92%]). Furthermore, the bacteria grown in EQUC culture were compared and matched with bacterial DNA sequences by 16S rRNA amplicon sequencing. This confirmed that the bacteria identified through 16S rRNA sequencing are alive and not just passing through into the bladder. The development of EQUC and the validated use of urethral catheters both laid the foundation for standardized study protocols in further studying the urinary microbiome.

1.2.7 Urinary microbiome and urgency urinary incontinence

This paradigm shift to the bladder not being sterile has created additional research opportunities to explore its connection with chronic urinary conditions. Currently, chronic urinary conditions such as overactive bladder syndrome (OAB) and UUI are poorly understood. For UUI, current treatments target abnormal neuromuscular signaling and/or functioning. However, medications for these treatments are ineffective in nearly half of subjects with UUI (Nitti et al. 2010; Hartmann et al. 2009). Now that we can characterize the bacteria in the bladder, this offers a new source of variation to better understand the heterogeneity in individuals with chronic urinary conditions.

Early studies to explore the relationship between the urinary microbiome and chronic urinary conditions started with UUI. As a part of a larger study focusing only on

women who planned to undergo treatment for UUI, Brubaker et al. (2014) used qPCR to categorize women as positive and negative qPCR for bacterial DNA, a general measure for the presence and absence of the urinary microbiota. These women were also evaluated for post-operative UTI[11]. The study found that subjects with a positive qPCR reading had more UUI episodes but less of a risk for a UTI compared to the women with negative qPCR readings (Brubaker et al. 2014). The implication of these results reveal the variation in the urinary microbiota of women affected by UUI and its potential to influence clinical outcomes after treatment. To further understand the differences in the urinary microbiota of women with UUI, Pearce et al. (2014) aimed to characterize the bacteria in urine between 58 women with UUI and 60 women without UUI. Two notable results from this study reveal differences in observed bacteria genera[12] between cohorts and bacterial composition based on a subsample of cohort samples. Although there was no statistical difference between the UUI and non-UUI control groups, the researchers observed six groups after clustering of the urinary microbiome samples. These groupings, called urotypes[13], were based on the most dominant bacterial taxa in that sample (e.g., *Lactobacillus*) and the six dominant taxa were: *Lactobacillus*, *Gardnerella*, *Enterobacteriaceae*, *Staphylococcus*, *Sneathia*, and diverse. Their results suggest that this heterogeneity in the urinary microbiome across women is linked with demographic

[11] UTI was defined "either as $> 10^5$ CFU/mL or as any treatment with antibiotics for a UTI (suspected or documented) at any point between randomization and 6 months".

[12] Nine genera (*Actinobaculum*, *Actinomyces*, *Aerococcus*, *Arthrobacter*, *Corynebacterium*, *Gardnerella*, *Oligella*, *Staphylococcus*, and *Streptococcus*) were cultured more frequently in the UUI cohort, which is calculated by person presence rather than by abundance counts.

[13] Classification of human microbiome samples into groups was first done with the gut microbiome where they've termed their groups as "enterotypes".

information and symptom severity because their statistical analyses show age, body mass index (BMI), and baseline UUI episodes per day were different across urotypes. To further investigate a possible interaction between the urinary microbiota and clinical treatment response, Thomas-White et al. (2015) recruited women taking oral medications for UUI. These women were categorized into non-responders, 5 mg solifenacin responders, and 10 mg solifenacin responders[14]. The authors then observed the frequency of urinary bacteria across these categories. Although their study did not look at microbial diversity (e.g., Shannon diversity), their results showed variation in the detected bacteria with respect to drug response, suggesting a possible drug response stratification based on urinary bacteria. Further efforts to associate the urinary bacteria with clinical symptoms show that an increase in UUI symptom severity is associated with decreased microbial diversity in women with UUI (Karstens et al. 2016). Moreover, decreased microbial diversity and richness of the urinary microbiota in women with OAB is associated with increased levels of self-rated depression scores (Wu et al. 2017). Altogether, these results suggest an underlying microbial community structure to the urinary microbiota that changes with disease, knowledge of which may be useful in understanding UUI and OAB to assign more effective treatment options.

1.2.8 Summary

In summary, urgency urinary incontinence is a highly prevalent condition that debilitates the quality of life of those suffering with this condition. This complex condition

[14] The primary outcome was treatment response after 12 weeks. After 4 weeks, "response" was based on whether the women reported improved symptom control as measured on the Patient Global Symptom Control (PGSC) questionnaire. Non-responders were increased to 10 mg solifenacin. Participants who did not respond at 10 mg at 12 weeks were categorized as non-responders.

has a range of treatment options with varying levels of invasiveness, but not without their own limitations and side effects. The human microbiome is shown to vary with health and disease, including the recent recognition of the commensal urinary microbiome. Changes in the urinary microbiome have been associated with urgency urinary incontinence. In contrast, evidence is currently lacking as to whether changes in the vaginal microbiome are associated with urgency urinary incontinence symptoms. Furthermore, the relationship on how the microbial communities of the bladder and the vagina both change with UUI is still a growing area of research.

1.3 Aim 2 background

In reality, microbial communities are complex collections of different microbes that interact dynamically (Coyte, Schluter, and Foster 2015; Gilbert and Lynch 2019). These collective interactions have the potential to elicit emergent effects that contribute to urinary tract symptoms such as UUI. The emerging area of microbial networks, which is the field of network science applied to the microbiome, has the potential to gain novel insights into the connection between the underlying structure of microbial community interactions and diseases such as UUI (Layeghifard, Hwang, and Guttman 2017; Röttjers and Faust 2018).

Previous computational methods fail to capture the underlying microbial complexity to prescribe effective treatments. To understand the relationship between the microbiome and disease, researchers have relied on quantifying the number and distribution of bacteria (alpha diversity), quantifying bacteria-wise differences (beta diversity), or identifying differential abundant bacteria between states of health and disease. Although these methods have advanced our knowledge of the human microbiome in human health and

disease, broad applications of restoring the diversity of bacteria have mixed results. For example, studies aiming to restore the gut microbiome to a healthy state using a fecal microbiome transplant show not all recipients of the treatment experienced restored gut health (Li et al. 2016; Paramsothy et al. 2017). These results rely on the underlying hypothesis that resetting the microbiome back to a healthy state can be done solely by replacing the microbiome with other microbiomes of a perceived healthy status, effectively replenishing the missing microbes that kept the microbiome healthy. These mixed results suggest that this hypothesis fails to account for the complexity in microbial systems to make effective changes.

In recent years, there has been considerable interest in applying microbial networks to human disease, which have shown potential in improving our understanding of the relationship between the microbiome and disease. In contrast to diversity-centric methods, ecological interactions more directly motivate the theory behind microbial networks. These precise ecological interactions are unknown and invisible. However, microbial networks aim to infer this invisible interaction structure, from which higher-level inferences about these microbial systems can be made. Despite the interest in applying microbial networks in other microbiomes, microbial networks remain relatively unexplored in biomedical areas of research involving the urinary microbiome and vaginal microbiome. Before more advanced modeling of these microbial communities can be reasonably explored and

understood[15], the next step to understanding these microbial relationships is to understand the network structures and effects of the microbiomes.

1.3.1 Current limitations in established methods

Microbial diversity and differential abundance are mainstay methods in microbiome science, but the knowledge gained from these methods is starting to plateau. Johnson and Burnet (2016) remind us that diversity is but one way to understand an ecosystem and that there are other factors to consider, such as stability and structure of an ecosystem(Johnson and Burnet 2016). Diversity measures have their own assumptions of the data and thus direct comparisons with each other is not straightforward. Despite the utility in the previously mentioned methods, effective inference from diversity measures requires extensive sampling and knowledge on how each diversity metric gives weight to organisms that differ in abundance (Bent 2008; Willis 2019). Living organisms rarely live in isolation, but rather, they exist in the same environment and influence each other (see section 1.3.2). Microbial diversity abstracts the complex microbial community into a single property. Fundamentally, accounting for interactions or community dynamics is not a direct assumption in these measures of the microbiome.

1.3.2 Microbial ecology to study microbial communities

Microbial ecology is the study of microorganisms in nature and their role among humans. Traditionally, we study microorganisms as individual species or strains after

[15] More advanced modeling would include causal inferences on adding or removing select individual or groups of bacteria and exploring those effects on the rest of the microbiome. Some methods include, but are not limited to, Bayesian networks, system of differential equations, and structural equation modeling. An example of such as model can be found in Angulo, Moog, and Liu (2019) (https://doi.org/10.1038/s41467-019-08890-y).

isolating them from their native ecosystems. Microbial communities, however, are multi-species collections that interact and live among each other. Precise definitions of communities vary from tight ecological interactions among organisms to species that co-occur within the same physical and chemical environments (Konopka 2009). Nonetheless, the concept around "communities" provides a mental framework to study multiple species of microorganisms rather than single species.

Using ecological principles in microbiology, we can start asking and answering questions that were previously not possible. Although it has been long known that microorganisms play an important role in the living world (Gilbert and Neufeld 2014), here we will focus on their interactions among each other and with their hosts.

From a host-microbe perspective, the questions we can ask revolve around how these interactions occur and what benefits result in the fitness and health of the host (Antwis et al. 2017). The co-existence between a host and its microbes motivate questions about the underlying mechanisms that shape these interactions. For example, we can ask, "What are the primary mechanisms within a host that mediate microbe-microbe and host-microbe interactions?" More pertinent to our work here is to ask how these interactions influence our health. Ultimately, the long-term goal is to use our understanding of the host-microbe interactions to impact disease prevention and treatment (Zmora et al. 2016).

From a microbial community perspective, the questions we can ask revolve around microbial interactions and their collective functional potential. The interest in studying microbial interactions is important to understand ecosystem dynamics, such as ecosystem stability and functional potential (Konopka 2009). Understanding these dynamics open up the potential for targeted manipulation of these microbial systems by the precise

introduction of smaller microbial communities or targeted removed of key microbes. All of which can further the vision of personalized and precision medicine.

It is now the opportune moment to capitalize on the philosophy of holism and the deeper principles of ecology to complement the scientific knowledge built from reductionist methods in microbiology. Ecology and systems thinking are the driving principles in opening new opportunities for using the human microbiome to restore and maintain human health (Dethlefsen, McFall-Ngai, and Relman 2007).

A common theme in microbial ecology is to use a systems approach to understanding microbial communities. These themes include: studying microbial origins and evolution, the taxonomic relatedness among bacteria, the roles bacteria play in their ecosystems, the interactions between other organisms, interactions with the environment, and the capacity to degrade chemical substances (Bertrand et al. 2014).

On the theme of interactions, ecology provides us a framework for classifying different interactions between organisms. Lidicker proposed such a system that defined three types of interaction effects on an organism: positive, negative, or neutral (Lidicker 1979). These three types describe the overall effect that one organism experiences from an interaction. Example interpretations of these interactions include mutualism (both organisms have positive interactions) and competition (both organisms have negative interactions).

In particular, the theme of interactions between other microorganisms is particularly suitable for network analysis. With a low number of microorganisms, we can enumerate all possible pairwise combinations. However, as the number of microorganisms

increases, the number of pairwise associations becomes exponentially more difficult to organize and computationally analyze.

1.3.3 Microbial co-occurrence networks

Solving the research limitations of the current analytical methods will require novel methods to better understand all aspects of microbial communities. As noted in section 1.3.1, the current methods of diversity and differential abundance now provide limited additional insight into the complexities of microbial communities. The complexity originates from fact that bacteria will interact in direct and indirect mechanisms. One-step up from focusing on individual bacteria is to observe pairwise associations. Enumerating all pairwise comparisons of the bacteria in a community, however, increases exponentially, which will prove difficult to scale and interpret.

Using principles of ecology and systems science, networks provide a promising approach to overcome the scale and difficulty in interpretations. Fundamentally, networks are a collection of objects and their connections with each other. These networks have a long history of research, which gives us analytical tools for inference on networks. Consequently, networks are also used in biology, ranging from constructing food webs to gene regulatory networks. The only difference between these biological networks is how you define the objects and the kind of connections between them.

1.3.4 Growing evidence for connected urogenital microbiome

Recent conjecture suggests an integrated urogenital microbiome consisting of the urinary and vaginal microbiomes. Traditionally, the urinary microbiome and vaginal microbiome are studied independently of each other. Komesu and colleagues proposed methodology to study the vaginal and urinary microbiomes in women with mixed urinary

incontinence (Komesu et al. 2016). Although the larger overarching study focuses on the urinary microbiota, the uncertain origins of the urinary microbes motivate this secondary exploration between the urinary and vaginal microbiomes. A review by MacIntyre, Sykes, and Bennett postulates the connection between the two microbiomes based on their close physical proximity and how each microbiota environment is highly regulated on their own (MacIntyre, Sykes, and Bennett 2017). Recently, Thomas-White et al. (2018) aimed to culture bacterial strains from urine because of the lack of reference strains for the urinary microbiota. Without pre-existing reference genomes to compare with, they compared their data to reference genomes of well-studied body sites. These other body sites were the gut bacteria and, notably, the vaginal bacteria. Their results show the urinary bacteria to have similar functional characteristics (based on conserved protein domains) and the existence of the same strains of bacteria in both the urine and vaginal microbiota in the same women. Similarly, Veit-Rubin et al. (2018) presented results at the International Continence Society conference on the relationship between the urinary, urothelial, and vaginal microbiome in OAB.[16] They categorized women into cohorts of high and low bacterial abundance. From this grouping, they found patients with low abundance vaginal microbiome had increased *Parvimonas spp* in their urinary microbiome. Conversely, patients with a low abundance in their urinary microbiome had increased *Escherichia coli* in their vaginal microbiome. Moreover, they found *Prevotella spp* on the urothelium to have a protective role against urinary incontinence. Although presented as a conference abstract, these results suggest a relationship among the urogenital microbiome that may have clinical diagnostic and

[16] Note, this is only a conference abstract and was not peer-reviewed.

therapeutic potential. Altogether, the evidence suggesting a combined urogenital microbiome is growing, but there is much to be learned of its community dynamics and structure.

The vaginal microbiome has long been known to be associated with health and disease. Typically, the vaginal microbiome is studied by taking a swab of the vaginal epithelium (Ravel et al. 2011; Hyman et al. 2005) located in the lower genital tract. Before high-throughput sequencing methods, characterizing the vaginal flora exclusively used culture-based methods (Donders 2010). From these low-throughput methods, we have come to understand that a majority of the bacteria on the vagina exist in a mutualistic relationship, and provide a first-line of defense from infection by pathogenic bacteria. Members of the genus *Lactobacillus* encompass the majority of the bacteria in the vagina and are well known for maintaining vaginal health. Its dominance in the vaginal microbiome drove research to identify the protective effects of *Lactobacillus* through its production of lactic acid and resulting low pH. Major findings of the vaginal microbiome include our understanding of the dominant *Lactobacillus* groups, the crucial role of estrogen and glycogen in supporting lactobacilli, and rethinking the definition of a healthy and normal vaginal microbiome (Nunn and Forney 2016). Modern molecular techniques have revealed four dominant species of lactobacilli to dominant the vaginal microbiome: *L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*. In reality, little is known about how these lactobacilli and associated microbial communities differ between women and how these bacteria functionally interact with their host humans.

2 Urinary and vaginal microbial differences and urgency urinary incontinence

2.1 Abstract

It has increasingly been appreciated that there are differences in the microbial flora of women with and without urgency urinary incontinence (UUI). Compositional changes in the urinary microbiome are known to be associated with UUI. However, the relationship between the vaginal microbiome and UUI and how that variation relates to the urinary microbiome is still unknown. Additionally, there are a lack of studies exploring the relationship between the vaginal microbiome and UUI. Here we collected catheterized urine and vaginal swabs from women with UUI (n=20) and without UUI (n=30) and show that trends in alpha diversity are similar between the urinary and vaginal microbiome. After adjusting for age, body mass index, menopause status, and estrogen use, there were no differences in the urinary and vaginal microbiomes between women with UUI and without UUI. These results are partially in contrast with previous studies, which show microbial diversity changes in the urinary microbiome of women with UUI. There is still evidence lacking to show a relationship between the changes in vaginal microbiome composition and having UUI. We are surprised by the results here that contrast with the general consensus that the urinary microbiome differs in women with UUI relative to women without UUI. There is still more work to be done to understand the relationship between UUI and the vaginal microbiome and the relationship between the urinary microbiome and the vaginal microbiome. Ultimately, understanding how UUI symptoms, the urinary microbiome, and vaginal microbiome coexist will improve our understanding to best treat women with UUI and other lower urinary tract symptoms.

2.2 Introduction

Historically, the bladder was thought to be sterile in the absence of an acute infection. However, with more widespread use of high-throughput sequencing and enhanced culturing techniques, bacteria are being recognized as a commensal part of the normal bladder (Wolfe et al. 2012). Additionally, the development of an alternative culturing method, called expanded quantitative urine culture (EQUC), has further demonstrated the presence of living bacteria in the female bladder (Hilt et al. 2014).

It has increasingly been appreciated that there are differences in the microbial flora of women with and without UUI (Pearce et al. 2014, 2015). For example, decreased diversity in the urinary microbiome is associated with more severe UUI symptoms (Karstens et al. 2016). Furthermore, medication response was found to be related to the urinary microbiota composition, where responders at baseline were more likely to have less diverse bacterial microbiomes than non-responders (Thomas-White et al. 2015). Overall, it is becoming clear that characteristics of the urinary microbiota play a role in UUI.

In addition to evidence associating the urinary microbiome with urogenital health and disease, it is long known that the nearby vaginal microbiome also contributes to urogenital health and disease. The vaginal microbiome is known to play a protective role in preventing diseases such as bacterial vaginosis, sexually transmitted infections (STIs), and urinary tract infections (UTIs) (Ma, Forney, and Ravel 2012). More specifically, four *Lactobacillus* species (*L. iners*, *L. crispatus*, *L. gasseri*, and *L. jensenii*) are shown to dominate in healthy, reproductive-age women (Ravel et al. 2011). The role of *Lactobacillus* species in the vaginal microbiota is also becoming clearer, whereby they tightly regulate the vaginal

environment by producing lactic acid after consuming host-provided glycogen (Nunn and Forney 2016).

Despite research supporting the role of the microbiome in urogenital conditions, little is known about the relationship between the urinary microbiome and vaginal microbiome. Individually, the microbiota of the vagina and the urinary tract are known to play a complex role in determining urogenital health (MacIntyre, Sykes, and Bennett 2017). Furthermore, it is shown the bacterial genomes and composition between the vaginal and urinary microbiomes are more similar compared to other human microbiomes (e.g., the gut microbiome) (Thomas-White et al. 2018; Komesu et al. 2020) However, it is not well understood how these two microbiota interact as an ecological environment and how their interaction may influence the development of disease.

In this study, we explore the relationship between UUI and the microbiome. We compare the urinary and vaginal microbiomes across women with and without UUI.

2.3 Methods

2.3.1 Study population and design

This was a case-control study conducted at Oregon Health & Science University (OHSU) between 2016 and 2019. Study approval was obtained from OHSU's Institutional Review Board (IRB 00010729). Participants were women between the ages of 45 and 85 and were recruited both from the general population in the Portland Area as well as through urogynecology clinical providers from OHSU, Kaiser Permanente NW, and affiliated Portland area hospitals. Participants were prescreened over the phone and those who met the inclusion criteria completed their study visits at OHSU's Women's Health Research Unit (WHRU). This study is a part of a larger effort to understand overactive bladder syndrome

in women. For the purposes of this study, we focused on a subpopulation of women with urgency urinary incontinence and recruited 20 women with urgency urinary incontinence (cases) and 30 women with normal bladder function (controls). Case participants included females with daily urge-predominant incontinence confirmed on a 3-day voiding diary[17] with urge-predominant leakage as determined by Perceived Urgency Scale score greater than or equal to 3 ("Severe urgency that I could not postpone voiding" and "I leaked before arriving at the toilet") for >50% of the total incontinence episodes on the diary. Control participants include female participants without a history of any urge urinary incontinence symptoms or frequent stress incontinence symptoms (more than once a week) based on a screening questionnaire and confirmation on a 3-day voiding diary. Participants were excluded if they had any of the following: a baseline need for intermittent self-catheterization, known neurological diseases that could affect bladder function (stroke, multiple sclerosis, brain or spinal cord injury, myasthenia gravis), current pregnancy or lactation, history of pelvic radiation, current pelvic or bladder malignancy, symptomatic urinary tract infection detected on screening urinalysis and confirmed with culture (growth of $>10^5$ colonies per mL), symptomatic pelvic organ prolapse (sensation of vaginal bulge), or prior or current diagnosis of painful bladder syndrome.

All participants provided written consent and completed a demographic and health questionnaire, as well as a three-day bladder diary. Participants were asked to score their urinary urgency on the bladder diary using the Patient Perception of Intensity Urgency Scale (PPIUS). Participants also completed the International Consultation on Incontinence

[17] "Voiding diary" is synonymous with "bladder diary".

Questionnaire (ICIQ) (Avery et al. 2004), Pelvic Floor Distress Inventory Urogenital Distress Inventory (UDI) (Barber, Walters, and Bump 2005), and Overactive Bladder Questionnaire (OABq) (Coyne et al. 2002). These are validated questionnaires to assess urinary incontinence symptoms, impact of pelvic floor disorders on daily function, quality of life, symptom bother, and health-related quality of life, respectively. Participants were asked about using the following vaginal products: douches, vaginal medications or suppositories, feminine sprays, genital wipes, contraceptive spermicides, and personal lubricants. Vaginal product use was collapsed to "any vaginal product use" because none of these products were used by a majority of the participants. During a study visit, a trained and licensed practitioner collected the participant's urine from the bladder using an aseptic technique with a urethral catheter. The total volume of the bladder was emptied and urine specimens were aliquoted into sterile 50 mL conical tubes and stored at −20°C until further processing. All urine specimens were handled in a sterile biosafety cabinet subsequent to collection.

The bladder behaviors were summarized as an average of averages, first summarized per woman and then per group (UUI or control). For example, urge leaks are the number of leaks when an urge to urinate was difficult to defer and resulted in a leak. The number of times this occurred is recorded on each of the three days. This one subject's bladder diary is then averaged across the three days. This average is then aggregated across subjects per cohort and averaged. This is done for the other bladder behaviors. Stress leaks are the number of leaks that result after sneezing, coughing, or other physical exertions. Night voids, also known as nocturia, is the complaint that an individual has to wake at night

one or more times for voiding. Voids is the number of times per day a person urinates and empties their bladder.

2.3.2 DNA extraction and PCR amplification

Microbial DNA from urine was extracted from microbial pellets formed from the centrifugation of 20-45mL of urine at 10,000 g for 30 min twice. DNA extraction was performed using the cultured cells protocol supplied with the DNeasy Blood and Tissue Kit (QIAGEN, Germany). Microbial DNA from vaginal swabs was extracted by vortexing swab heads in PowerBead tubes before centrifugation at 10,000 g for 30 seconds at room temperature following the MO BIO PowerSoil DNA isolation kit protocol (QIAGEN, Germany).

Bacterial DNA was amplified by PCR using Golay barcoded primers which target the V4 region of 16S rRNA genes (Caporaso et al. 2012). Template DNA was amplified in triplicate using the GoTaq Hot Start Polymerase kit (Promega, USA). One microliter of template DNA and 1µL of a unique barcoded reverse primer were added to 48µL of master mix containing 1x colorless reaction buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2mM forward primer, and 1.25 U of polymerase enzyme. The reaction volumes were placed in a thermocycler and run through the following conditions: 94°C for 3 min (initial denaturation), followed by 35 cycles of 94°C for 45 sec (denaturation); 55°C, 40 sec (annealing); 72°C, 1.5 min (extension); with a final extension at 72°C for 10 min.

Ten microliters of each product was used to verify the amplification by gel electrophoresis on a 2% agarose gel. Replicates yielding visible bands at 382 bp were pooled together and purified following the QIAquick PCR Purification kit (QIAGEN, Germany) provided protocol. Purified products were again quantified and quality checked

at A260/A280 nm (Nanodrop, Thermo Fisher Scientific, USA). Products were diluted to 10ng/μL and 5μL of each sample were pooled together for sequencing on the Illumina MiSeq sequencer (Illumina, USA).

2.3.3 Sequence processing, taxonomic assignment, and phylogenetic tree building

Illumina sequence reads for catheterized urine specimens and vaginal swab samples were processed using DADA2 (version 1.4.0) to yield amplicon sequence variants (ASVs) (Callahan et al. 2016). The paired-ended reads for sample reads were trimmed 10 bases from the 5' end for both forward and reverse reads. Subsequently, forward and reverse reads were truncated to 240 and 160 bases, respectively. Chimeric sequences were identified and removed by taking a consensus across samples using the `removeBimeraDenovo` function with default parameters in the `dada2` R package (version 1.4.0). Sequences were aligned to the SILVA reference database (version 132) (Quast et al. 2012) and taxonomy was assigned to individual ASVs using the RDP classifier as implemented in the `assignTaxonomy` function with default parameters in the `dada2` R package (version 1.4.0) (Wang et al. 2007). To increase our power to detect patterns, we agglomerated our ASVs to the genus taxonomic rank. For alpha and beta diversity analyses, the vaginal and urinary microbiome sequences variants were normalized by rarefaction without replacement to 15,000 reads per sample and 2,500 reads per sample, respectively. Performed separately for vaginal and urine samples, ASVs that contributed greater than 5% of the total ASVs of at least one sample were considered for further analysis. Identification and removal of contaminant sequences was performed on urinary microbiome samples using the `decontam` R package (version 1.4.0) (Davis et al. 2017). The `frequency` classification method of `decontam` to identify contaminants was used at a threshold of 0.3.

Phylogenetic trees were constructed by generating a neighbor-joining tree based on a multiple sequence alignment as implemented in the `AlignSeqs` function with default parameters in the `DECIPHER` R package (version 2.14.0) (Wright 2016). This multiple sequence alignment was then fit to a Generalized time-reversible (GTR) model with a Gamma rate variation (GTR+G+I) maximum likelihood using the neighbor-joining tree as implemented in the `pml` function and optimized with `optim.pml` in the `phangorn` R package (version 2.5.5) (Schliep 2010). For the urinary microbiome, the genera of experimentally cultured reference strains, as detailed in Thomas-White (2018), were retained if they were removed through decontam.

2.3.4 Statistical analyses

Differences in clinical and demographic characteristics between cases and controls were tested using Student's t-tests for normal and continuous characteristics, Mann-Whitney U for non-normal and continuous characteristics, and Fisher's exact test for categorical data. The Shapiro-Wilk test was used as the test for normality prior to testing. All analyses were performed in R (version 3.6.1) (R Core Team 2019). Data management, descriptive statistics, and visualizations of the microbiome data were performed using the `phyloseq` R package (version 1.28.0) (McMurdie and Holmes 2013), the `tableone` R package (version 0.12.0) (Yoshida and Bartel 2020), and the `ggplot2` R package (version 3.3.2) (Wickham 2016), respectively.

Stacked bar plots based on sequence relative abundance were produced for the vaginal and urinary microbiome samples. Distance matrices for participants using clinical information and microbiome data were calculated using Gower's distance (accounts for accounting for both numerical and categorical information) (Gower 1971) and weighted

UniFrac distance (Lozupone et al. 2007), respectively. Complete linkage was used for hierarchical clustering as implemented in the `hclust` function using the Ward criterion (as implemented in `hclust` using the `ward.D2` method) in R (version 3.6.1) (Murtagh and Legendre 2014). A dendrogram was used to visualize the hierarchical clustering relationships via the `dendextend` R package (version 1.13.4) (Galili 2015). The dendrogram was cut based on the Silhouette metric, a measure assessing the similarity of within-cluster points with other cluster points. The Silhouette metric was calculated for 2 to 6 potential number of clusters. For the UUI subtype clustering based on clinical observations, the numerical data used were: age, body mass index (kg/m^2), UDI, OABq symptom scores, OABq health-related quality of life scores, ICIQ, average daily number of urge leaks, average daily number of night voids, average daily number of stress leaks, days since last vaginal intercourse, average number of voids, and average number of urges. The categorical data used were: menopausal status, estrogen use, history of incontinence surgery, history of hysterectomy, history of prolapse surgery, history of IBS, history of anxiety, pelvic or vaginal surgery, vaginal delivery, history of recurrent UTI, estrogen status, Patient Global Perception of Severity of Urinary Symptoms, and Patient Perception of Bladder Condition.

Alpha and beta diversity were calculated for the UUI case and control samples. Alpha diversity definitions used were the observed number of taxa, Pielou's evenness index (Pielou 1966), and inverse Simpson index. We tested the relationship of the alpha diversity measures with clinical characteristics as covariates using a generalized linear model. The observed number of taxa and inverse Simpson index were calculated using the `diversity` function in the `vegan` R package (version 2.5.6) (Oksanen et al. 2019) and Pielou's evenness index was calculated using the `evenness` function in the `microbiome` R package (version

1.6.0) (Lahti and Shetty, n.d.). Clinical covariates that were statistically different between UUI cases and controls were considered as covariates in downstream analyses. Beta diversity between subject samples was calculated using the weighted UniFrac distance measure (Lozupone et al. 2007) using the `distance` function in the `phyloseq` R package (version 1.28.0) (McMurdie and Holmes 2013), visualized using principal coordinates analysis (PCoA), and PERMANOVA was used to test ordination significance using the `adonis` function in the `vegan` R package (version 2.5.6) (Oksanen et al. 2019; Anderson 2001).

We identified candidate urinary and vaginal genera with differential abundance between UUI and health controls using a negative binomial generalized linear model (GLM) as implemented in `DESeq2` (version 1.24.0) (Love, Huber, and Anders 2014). The significance of the fitted coefficients is tested using a Wald test. To increase our power, we filtered out genera having a non-zero number of reads in less than three samples. The false discovery rate (FDR) of multiple testing was adjusted using the Benjamini-Hochberg procedure at a critical level of p-value < 0.1 (Benjamini and Hochberg 1995).

2.4 Results

Our study included 20 women with UUI and 30 healthy women between the ages of 41 and 81 (Table 1). Women with UUI were older (p-value = 0.04), had a higher body mass index (p-value = 0.005), and more often had a history of recurrent urinary tract infections (p-value = 0.007). Women with UUI and healthy women did not differ in menopause status, estrogen use, and race (p-value > 0.05). Table 1 also displays participant demographics that did not differ between our cohort groups, which include: had a vaginal delivery, history of diabetes, currently smoking, and history of pelvic floor surgery.

	UUI (n=20)	Control (n=30)	P-value
Age (years)[a]	**64.2±10.5**	**57.9±10.4**	**0.04**
Body mass index (kg/m²)[b]	**29.25 [25.93, 32.8]**	**25.4 [23.2, 28.3]**	**0.005**
Menopause status			0.317
Premenopausal	3 (15.0%)	9 (30%)	
Postmenopausal	17 (85.0%)	21 (70%)	
Any Estrogen use	9 (45.0%)	7 (23.3%)	0.220
Race			0.680
White	18 (90.0%)	27 (90.0%)	
Non White	2 (10.0%)	3 (10.0%)	
Vaginal delivery (Yes)	11 (55%)	17 (46.7%)	1.000
Number of vaginal deliveries	2.91±2.55	1.88±0.99	0.144
History of diabetes	4 (20%)	6 (6.7%)	0.143
Smoking (current)	1 (5%)	0 (0%)	0.400
Has history of recurrent UTI	**5 (25.0%)**	**0 (0.0%)**	**0.007**
History of Anxiety	4 (20.0%)	4 (13.4%)	0.266
History of IBS	4 (20.0%)	2 (10.0%)	0.279
History of Pelvic Floor Surgery	7 (35.0%)	11 (36.7%)	1.000

Table 1: Participant demographics. Student's t-test was performed on continuous, normally distributed data and displayed with mean and standard deviation. The Kruskal-Wallis test was performed on continuous, non-normally distributed data and displayed with median and IQR. The Fisher's exact test was performed on categorical data and counts reported as number of individuals with corresponding demographic or condition. Bold rows are statistically significant between cohorts. SD = standard deviation, IQR = interquartile range, a = normal distribution, b = non-normal distribution, c = categorical data.

As expected, the women with UUI had different scores on the symptom severity and pelvic floor questionnaires (UDI, OAB-q, ICIQ, $p < 0.001$), all indicating a significant impact of urinary tract symptoms (Table 2). Additionally, women with UUI were more prevalent to have daily urge leaks (p-value < 0.001) (Table 3). We observed no significant difference in the prevalence of daily stress leaks (p-value > 0.05), prevalence of daily night voids, (p-value > 0.05), and average number of voids (p-value > 0.05).

	UUI (n=20)	Control (n=30)	P-value
Urogenital distress inventory (UDI-6 Short Form)	**5.50 [4.75, 9.00]**	**0.00 [0.00, 0.00]**	**<0.001**
OAB-q symptom bother	**45.00 [40.00, 70.00]**	**6.67 [3.33, 15.00]**	**<0.001**
OAB-q health-related quality of life	**66.92 [45.77, 78.85]**	**98.46 [95.77, 100.00]**	**<0.001**
International Consultation on Incontinence Questionnaire (ICIQ)	**10.50 [8.00, 14.25]**	**3.00 [0.00, 3.00]**	**<0.001**

Table 2: Participant bladder symptoms. This table summarizes bladder symptoms, assessed by validated pelvic floor questionnaires and notes statistical differences between the two groups, urgency urinary incontinence (UUI) cases and controls. Bold rows are statistically significant between cohorts. Statistics performed by Kruskal-Wallis, comparing each groups to controls.

	UUI (n=20)	Control (n=30)	P-value
Daily urge leaks[a]	**12 (60.0%)**	**0.00 (0.00%)**	**<0.001**
Daily stress leaks[a]	3 (15.0%)	1 (3.4%)	0.291
Night voids[a]	9 (45.0%)	5 (17.2%)	0.054
Number of voids[b]	8.89±3.46	7.56±2.31	0.129

Table 3: Summary of bladder habits over a 3-day bladder diary. Counts for daily urge leaks, daily stress leaks, and night voids represent the number of participants who had at least a daily occurrence of the bladder observation. Bold rows are statistically significant between cohorts. a = non-normal distribution, b = normal distribution

The urinary microbiome data were clustered in a dendrogram generated via hierarchical clustering using the weighted UniFrac distance between urine samples (fig. 1). We observed two groups, which were defined when optimizing on the highest Silhouette metric (S = 0.548). One cluster is a mixture of bacteria primarily from the Firmicutes and Bacteroidetes phyla, and the second cluster is a mixture of *Lactobacillus* and *Gardnerella* genera. Notable genera in the Firmicutes and Bacteroidetes dominated cluster are *Escherichia/Shigella*, *Bacteroides*, and *Lactobacillus*.

We tested the relationship between the clinical observations of our participants and their urinary microbiomes. We found no relationship between urinary microbiome variation with cohort status (p-value = 0.76), vaginal product use (p-value = 1.0), or

estrogen use (p-value = 1.0). However, being post-menopausal did have a significant association with the urinary microbiome, where the Firmicutes-Bacteroidetes dominant cluster was predominantly post-menopausal (29 post-menopausal, 4 pre-menopausal) and the women in the *Lactobacillus/Gardnerella* dominant cluster have an even distribution of menopausal status (9 post-menopausal, 8 pre-menopausal) (p-value = 0.01).

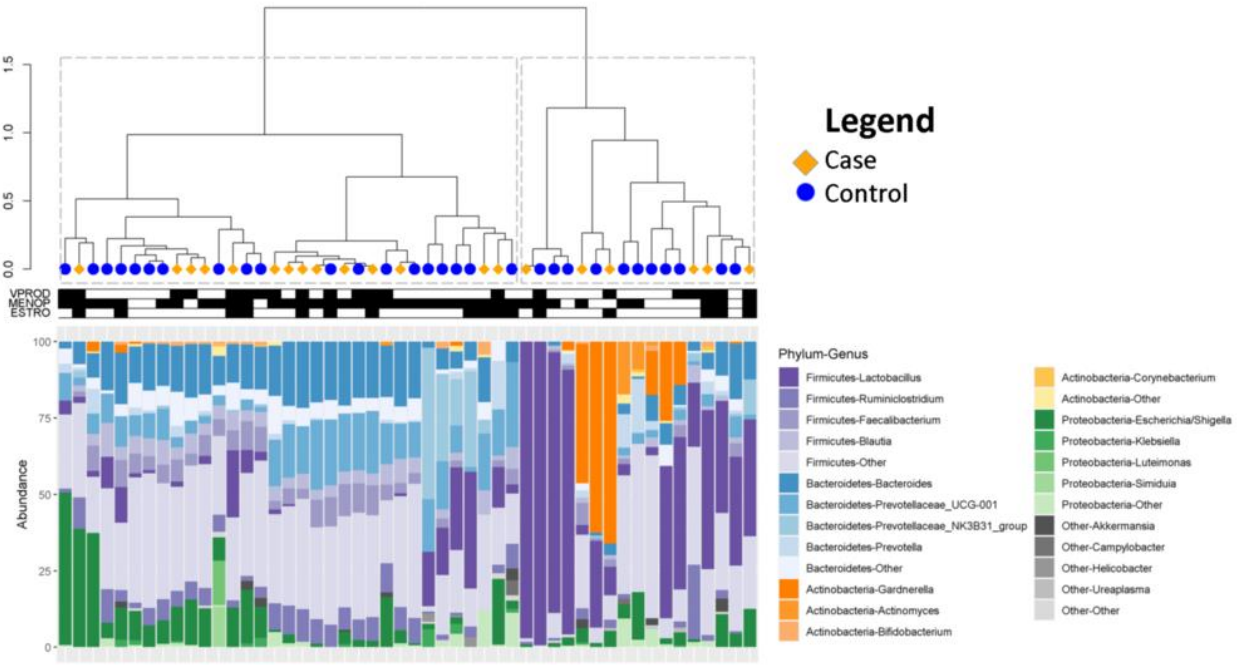

Figure 1: Clustering of urinary microbiome is associated with menopausal status. Hierarchical clustering was performed using the Ward's minimum variance method on weighted UniFrac distances between samples. Dotted lines outline clusters that were chosen based on the Silhouette metric. Statistics performed by Fisher's exact test, comparing clinical observations of participants with their clustering group. Cases are labeled with yellow diamonds and controls are labeled as blue squares. Black squares underneath the dendrogram indicates the participant either claimed to use any vaginal product (VPROD), is post-menopausal (MENOP), or claimed to use any estrogen (ESTRO). White squares indicate the complement of the status, and a white square for MENOP indicates being premenopausal.

We similarly clustered the vaginal microbiome samples via hierarchical clustering using the weighted UniFrac distance (fig. 2). We also observed two groups when optimizing on the highest Silhouette metric (S = 0.611). One cluster is dominated by the *Lactobacillus* genus, and the second cluster is a mixture of *Lactobacillus*, *Gardnerella*, and *Escherichia/Shigella* genera. Participants with vaginal product use had a significant

association with the clustering groups (p-value = 0.02). The distribution of vaginal product use was more evenly used among participants in the *Lactobacillus*-dominated cluster (14 no, 16 yes) versus the participants in the non-*Lactobacillus* dominant cluster (16 no, 4 yes). We found no relationship between cohort status (p-value = 1.0), being post-menopausal (p-value = 0.74), or estrogen use (p-value = 0.22).

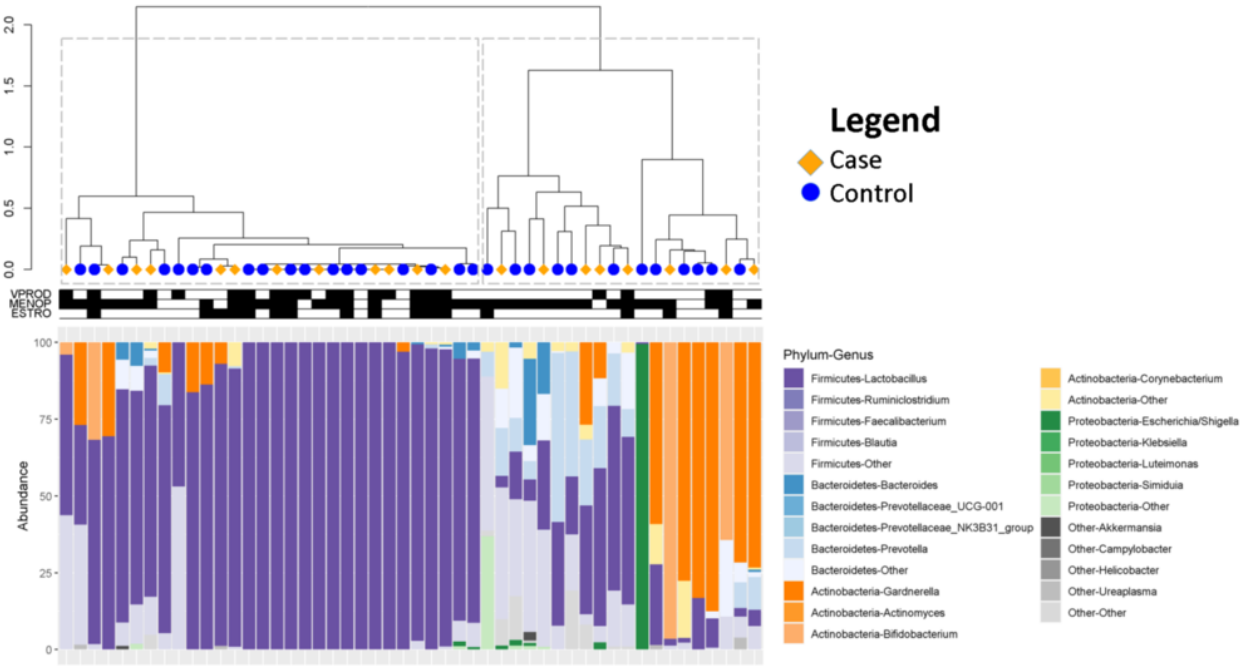

Figure 2: Clustering of vaginal microbiome is associated with any vaginal product use. Hierarchical clustering (top) was performed using the Ward's minimum variance method on weighted UniFrac distances between samples. Stacked bar plots (bottom) show relative abundance of vaginal microbiome of women with and without UUI. Dotted lines outline clusters that were chosen based on the Silhouette metric. Statistics performed by Fisher's exact test, comparing clinical observations of participants with their clustering group. Cases are labeled with yellow diamonds and controls are labeled as blue squares. Black squares underneath the dendrogram indicates the participant either claimed to use any vaginal product (VPROD), is post-menopausal (MENOP), or claimed to use any estrogen (ESTRO). White squares indicate the complement of the status, and a white square for MENOP indicates being premenopausal.

On our study, we did not detect any significant differences in alpha diversity measures between our UUI and non-UUI in either the urinary or vaginal microbiomes. After adjusting for age, menopause-estrogen status, and BMI, the alpha diversity of the urinary microbiome does not differ between women with UUI and without UUI (fig. 3). Menopause-

estrogen status was divided into three groups: post-menopausal and supplementing with estrogen, post-menopausal and not supplementing with estrogen, and pre-menopausal. Similarly, for the vaginal microbiome, the alpha diversity also does not differ between women with UUI and without UUI (fig. 4).

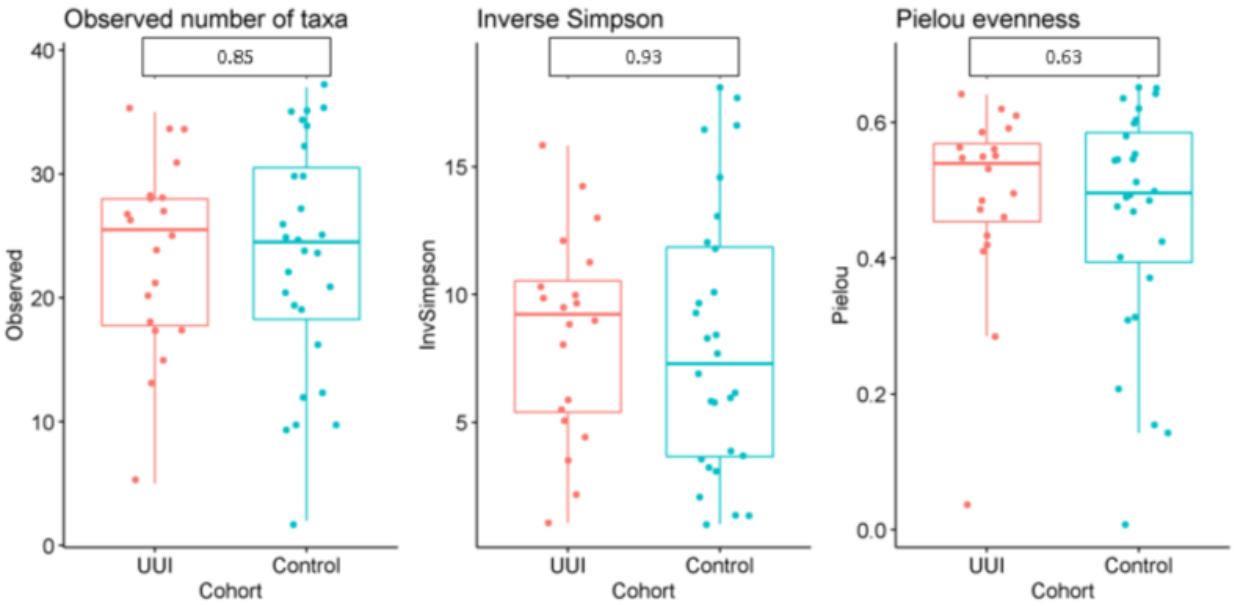

Figure 3: Women with and without UUI do not differ in urinary microbiome diversity. Alpha diversity is visualized using box-and-whisker plots and measured using observed number of taxa, inverse Simpson, and Pielou evenness. A generalized linear model was used to adjust for age, BMI, and menopause-estrogen status.

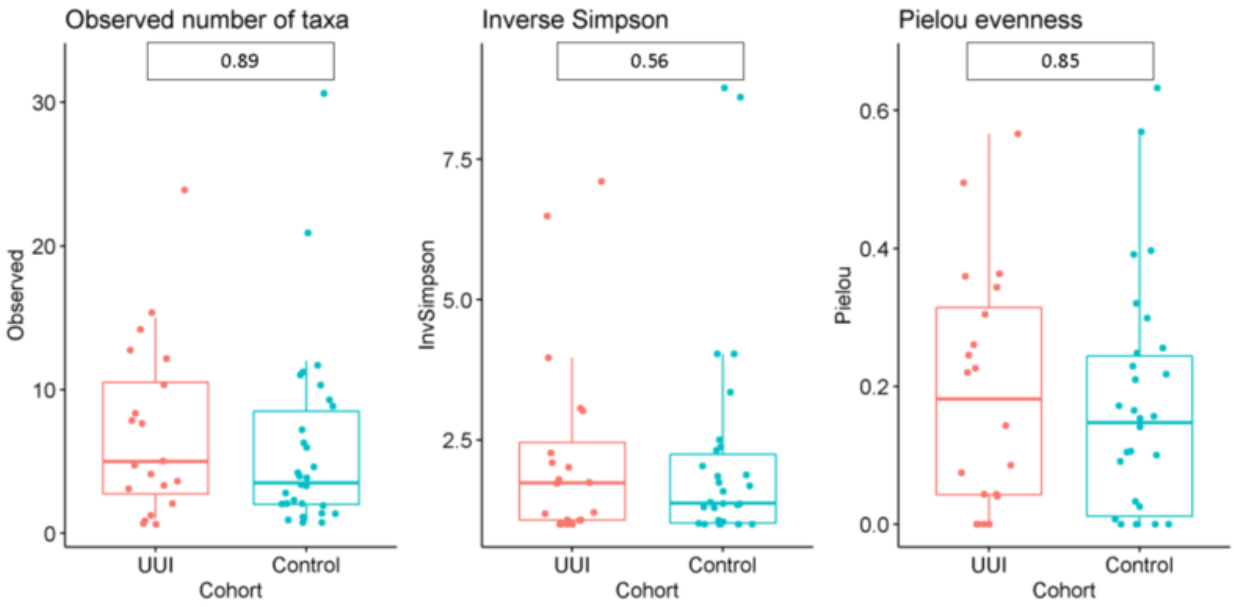

Figure 4: Women with and without UUI do not differ in vaginal microbiome diversity. Alpha diversity is visualized using box-and-whisker plots and measured using observed number of taxa, inverse Simpson, and Pielou evenness. A generalized linear model was used to adjust for age, BMI, and menopause-estrogen status.

We did not detect any significant differences in beta diversity across multiple measures between UUI and non-UUI participants' urinary or vaginal microbiomes. For the urinary microbiome, the beta diversity variation does not differ between women with UUI and without UUI (fig. 5). A majority of the microbial composition variation is captured in the two axes of the principal coordinate plot (69.8% of the total distance). After adjusting for age, BMI, and menopause-estrogen status, a PERMANOVA analysis shows no difference between the urinary microbiome composition of women with UUI and without UUI (p-value = 0.23). Similarly, for the vaginal microbiome, the beta diversity similarly does not differ between women with UUI and without UUI (fig. 6). A majority of the microbial composition variation is captured in the two axes of the principal coordinate plot (74.7% of the total distance). After adjusting for age, BMI, and menopause-estrogen status, a PERMANOVA

analysis shows no difference between the vaginal microbiome composition of women with UUI and without UUI (p-value = 0.71).

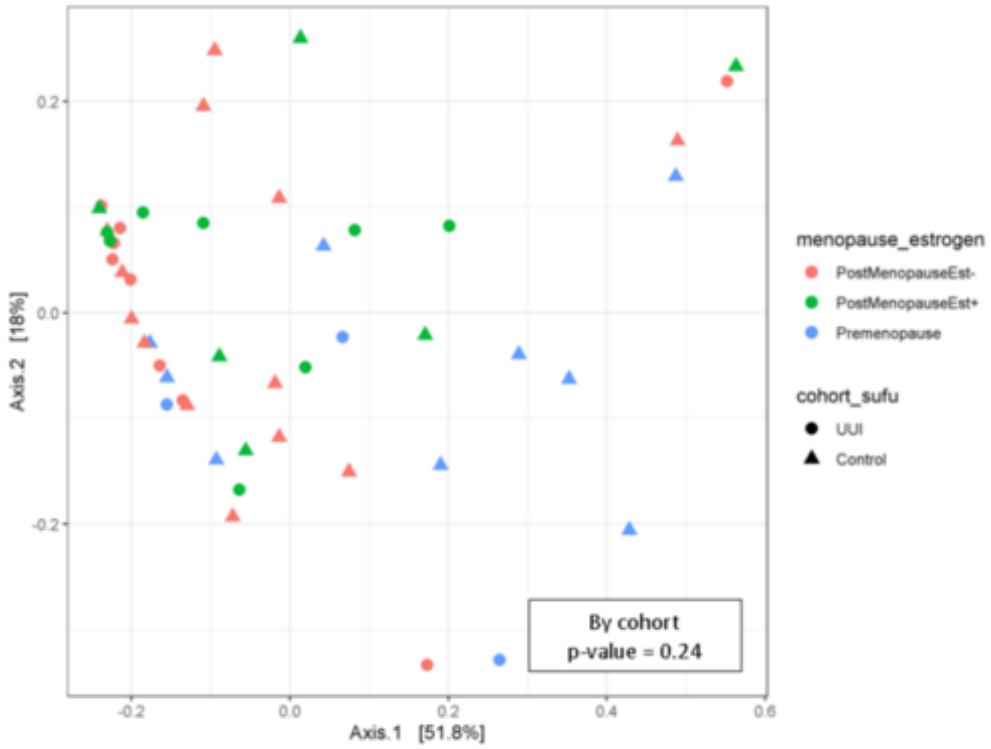

Figure 5: Variation of the urinary microbiome between women does not differ between UUI cases and controls. The ordination plot is a principal coordinate analysis (PCoA) on weighted UniFrac distance. Permutation analysis of variance (PERMANOVA) was used to test the relationship between microbiome composition and cohort status. Statistical analysis was adjusted for age, body-mass index, and menopause-estrogen status. Beta diversity was not significantly associated with cohort (p-value = 0.24). Shapes represents the cohort, where circles are women with UUI and triangles are women without UUI. Color represents a combination of menopause status and estrogen use status.

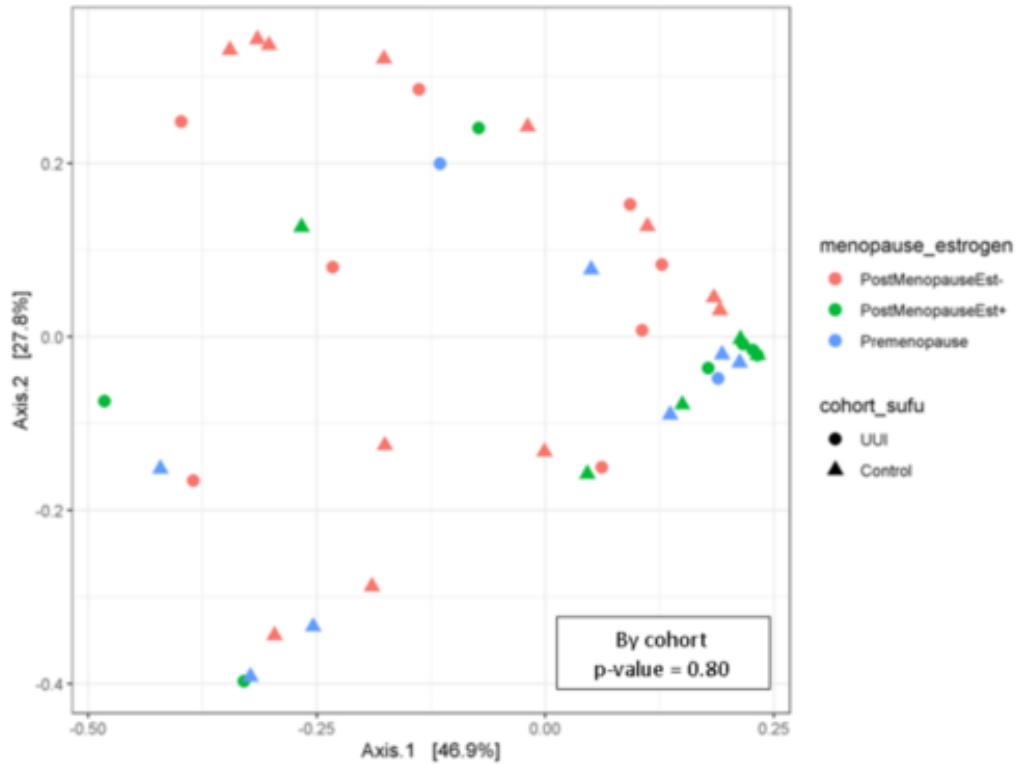

Figure 6: Variation of the vaginal microbiome between women does not differ between UUI cases and controls. The ordination plot is a principal coordinate analysis (PCoA) on weighted UniFrac distance. Permutation analysis of variance (PERMANOVA) was used to test the relationship between microbiome composition and cohort status. Statistical analysis was adjusted for age, body-mass index, and menopause-estrogen status. Beta diversity was not significantly associated with cohort (p-value = 0.80). Shapes represents the cohort where circles are women with UUI and triangles are women without UUI. Color represents a combination of menopause status and estrogen use status.

We performed differential abundance of genera in the urinary microbiome and vaginal microbiome (results not shown). After adjusting for clinical covariates age, BMI, menopause status, and estrogen use and correcting for multiple testing using Benjamini-Hochberg, no genera in either the urinary microbiome or vaginal microbiome was found to be differentially abundant between women with UUI and without UUI.

In order to explore potential subtypes within UUI, we only clustered the participants with UUI and identified three clusters (fig. 7). Out of all 25 clinical variables used in clustering, these three clusters of participants differed in age ($p = 0.02$), menopausal-status ($p = 0.001$), estrogen use ($p < 0.001$), and any vaginal product use ($p < 0.001$) (Table 4).

Two of the groups (both n=9) were generally older (median ages 64 and 68), had a lower BMI (median BMI 27.07 and 30.98), and were predominantly post-menopausal (100% and 88.9%) compared to the third group. The third group of two participants were younger (median age 43.50 years), had a higher BMI (median BMI 35.69), and were pre-menopausal.

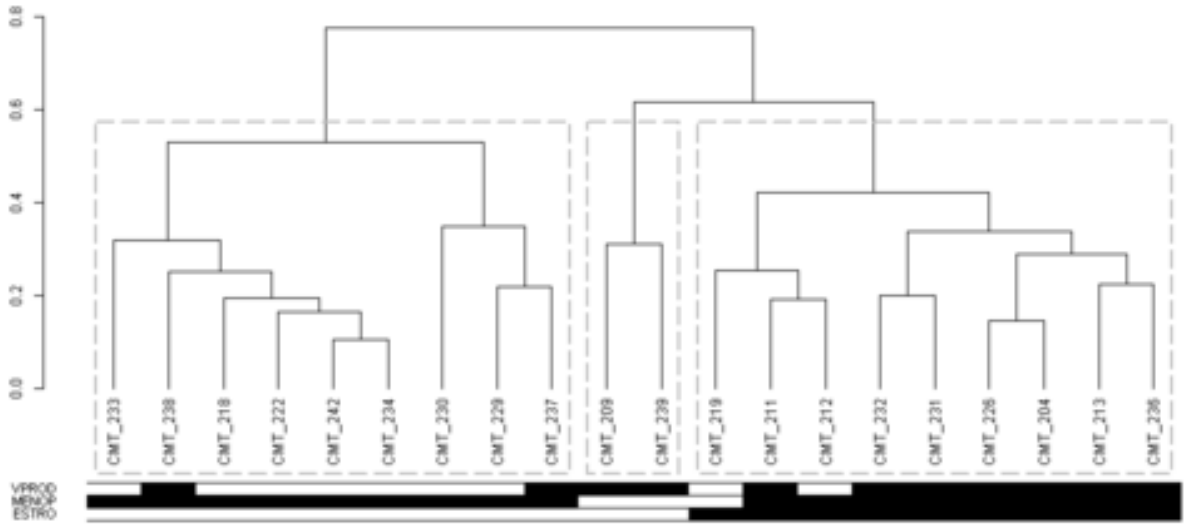

Figure 7: Participants with UUI clustered based on clinical observations. Hierarchical clustering, using the Ward criterion, was used to cluster subjects. The distance matrix used the Gower's distance measure to account for both numerical and categorical information. Black squares underneath the dendrogram indicates the participant either claimed to use any vaginal product (VP), over the age of 60 year (AGE60), is post-menopausal (MP), or claimed to use any estrogen (EST). White squares indicate the complement of the status, and a white square for MENOP indicates being premenopausal.

	C1 (n=9)	C2 (n=9)	C3 (n=2)	P-value
Age (median, [IQR])	**64 [60, 64]**	**68 [67, 76]**	**43.50 [43.24, 43.75]**	**0.02**
BMI (median, [IQR])	27.07 [25.79, 30.04]	30.89 [27.95, 32.92]	35.69 [32.46, 38.92]	0.366
Menopause status (%)				**0.001**
Post-Menopause	9 (100%)	8 (88.9%)	0 (0%)	
Pre-Menopause	0 (0%)	1 (11.1%)	2 (100%)	
Estrogen use (%)				**<0.001**
No	9 (100%)	0 (0%)	2 (100%)	
Yes	0 (0%)	9 (100%)	0 (0%)	
Vaginal product use (%)				**<0.001**
No	7 (77.8%)	2 (22.2%)	0 (0%)	
Yes	2 (22.2%)	7 (77.8%)	2 (2%)	

Table 4: Subject clustering based on clinical observations. Statistics performed by Kruskal-Wallis, comparing UUI cases to controls and displayed with the median and IQR. The Fisher's exact test was performed on categorical data and counts reported as number of individuals with corresponding demographic or condition. Bold rows are statistically significant between cohorts. IQR = interquartile range, BMI = body mass index.

To compare the relationship between having UUI and the urinary and vaginal microbiomes, we re-clustered the urinary and vaginal microbiome samples for only the UUI participants and compared the clusters with our groupings based on the clinical observations (Table 5). Each pair of clustered groups were not statistically different from random clustering (clinical/urinary p-value = 1.00; clinical/vaginal p-value = 1.00; urinary/vaginal p-value = 1.00). In other words, participants did not cluster into the same groups from one clustering to another using a different participant observation type (clinical observations, urinary microbiome, and vaginal microbiome).

	Urinary microbiome	
Clinical clustering	UM1	UM2
C1	8	2
C2	7	1
C3	2	0

	Vaginal microbiome	
Clinical clustering	VM1	VM2
C1	5	4
C2	6	3
C3	1	1

	Urinary microbiome	
Vaginal clustering	UM1	UM2
VM1	10	2
VM2	7	1

Tables 5: Pairwise clustering comparisons among clustering groupings based on clinical observations, urinary microbiome, and vaginal microbiome. CX represents the different clusters identified clustering the clinical variables where "X" is a unique identifier for the cluster. Similarly, UMX and VMX are the cluster groups identified in the urinary microbiome and vaginal microbiome, respectively, with "X" being the unique identifier. Clinical/urinary comparison (p-value = 1.00); clinical/vaginal comparison (p-value = 1.00); urinary/vaginal comparison (p-value = 1.00).

2.5 Discussion

In this pilot study, we characterized the urinary microbiome and vaginal microbiome of women with normal bladder function and women with UUI. Surprisingly, our results partially contradict previous results reported in the literature comparing control and UUI urinary microbiomes. Namely, we did not find that the urinary microbiome composition changes with UUI status. In addition, although the evidence for linking UUI and the vaginal microbiome is sparse, we similarly did not find evidence of a relationship between variation in the vaginal microbiomes of our control and UUI groups.

Women in the UUI group had a higher body mass index (BMI); this result is consistent with previous literature that obesity is associated with urgency urinary incontinence. Other risk factors associated with UUI are parity, previous hysterectomy or

pelvic surgery, pulmonary disease, diabetes mellitus, and nursing home admission or dementia (Coyne et al. 2013).

The difference in bladder behaviors between cohorts is consistent with our definition of UUI. The significant difference in the number of urge leaks is thus expected. Stress leaks are associated with another common urinary incontinence subtype, stress incontinence. This type of incontinence is leakage as a result of physical exertion. Often, these two subtypes, stress incontinence and urgency urinary incontinence coexist. Despite this fact, we did not see a significant difference in the number of stress leaks between women with UUI and without UUI (p-value = 0.291). Although night voiding is a common symptom among people with UUI, we do not see a significant difference in night voids in our UUI cohort (p-value = 0.054).

We were surprised to see that vaginal product use (douches, vaginal medications or suppositories, feminine sprays, genital wipes, contraceptive spermicides, and personal lubricants) was associated with our vaginal microbiome clustering analysis. There is a growing number of studies looking at the effect of vaginal product use on the vaginal microbiome. Recent studies have found that vaginal products can help inhibit uropathogenic bacteria (Hung et al. 2020). This may explain the *Lactobacillus* dominant cluster having more participants who used vaginal products.

Based on the clustering analysis, variation in the urinary microbiome composition was not associated with UUI status, vaginal product use, or estrogen use (fig. 1). However, urinary microbiome variation is associated with menopausal status. This relationship appears to be stronger than the effect of having UUI because the clustering based on the urinary microbiome is not associated with UUI status. The Firmicutes-Bacteroidetes

dominant cluster is pre-dominantly post-menopausal women, so that lack of a dominant *Lactobacillus* is consistent with previous findings (Curtiss et al. 2018).

No difference in all three alpha diversity measures between cohort microbiomes suggests that UUI is not associated to variation in either the urinary and vaginal microbiomes. Similarly, there was no statistically significant clustering of participant samples into women with UUI and without. This evidence is consistent with previous findings. Karstens et al. (2016) and Pearce et al. (2014) both were unable to detect large differences in sequence-based microbial diversity. Our study sample population size was greater than Karstens et al. (2016), but less than Pearce et al. (2014). In light of a smaller sample size, our results further suggest that the amount of microbial diversity in the urinary microbiome of women with UUI may not differ from women without UUI. Despite previous studies finding no significant difference in the microbial diversity of urine in women with UUI, individual bacterial differences were identified (Karstens et al. 2016; Pearce et al. 2014). Some possible explanations for the discrepant results are that our study had a smaller sample size, our study population is from the Pacific Northwest compare to the mid-west, our predominantly non-Hispanic Caucasian sample population, or our UUI participants were being treated with estrogen (Pearce et al. 2015, 2014; Thomas-White et al. 2015; Karstens et al. 2016).

2.6 Limitations

It is plausible that a number of limitations may influence our results. First, the demographics of this study can only generalize to Caucasian women around the ages of 60. Also, the UUI and non-UUI cohorts differ in BMI. We are thus unable to say whether BMI is related to potential microbial differences rather than the urinary symptomology because

increased BMI is a known risk factor for UUI. Future studies, with larger sample sizes and matched clinical covariates and greater ethnic diversity, will be required to evaluate these important differences. There is still a lack of studies that describe the female urinary microbiome in large, well-characterized populations. The vaginal microbiome is much more characterized in comparison. A limitation for survey data is that the data presented here is cross-sectional while longitudinal studies of the stability of these scores is unknown. Limitations of the stacked bar plot are that only large and dominant bacterial differences can be observed among samples. Differences in lower abundant bacteria among participant samples are difficult to identify. This is especially the case where most of the UUI cases and controls do not associate into a single cluster. This further suggests that there does not appear to be an association between the composition of the vaginal microbiome and UUI.

Another limitation with this analysis is that measures of microbial diversity reduce the complexities of a microbial community. Each microbial sample is summarized into a single number, which is taken as a measure of the health of a microbial system. However, this may not be the case. Plus, this ignores the ecological interactions that exist in a community, which will affect the microbial dynamics. Microbial diversity is a starting point to further understanding the extent of the microbiome's effects on human health and disease.

2.7 Conclusion

We characterized the vaginal microbiome and urinary microbiome of pre-dominantly post-menopausal women to understand the connection between the vaginal and urinary microbiome with urgency urinary incontinence (UUI). The lack of clustering by UUI status suggests that there is not a global relationship between UUI and vaginal

microbiome composition or urinary microbiome composition. The urinary microbiome clusters significantly by menopause status, unlike the vaginal microbiome. However, the vaginal microbiome clusters significantly by vaginal product use. By their clinical observations, women with UUI cluster significantly by age, menopause status, estrogen use, and any vaginal product use. Finally, the lack of concordance between clustering the participants by clinical information, urinary microbiome, and vaginal microbiome suggests little relationship linking each of them together. Thus, our study does not provide evidence for a relationship between the variation in composition of the vaginal microbiome and having UUI. Our results are consistent with previous studies on the lack of evidence supporting changes in the diversity of urinary microbiome associated with UUI. However, our results contrast previous studies that identified differences in individual bacterial abundances in the urinary microbiome of women with UUI. There is still work to be done to further explore the relationship between UUI and the vaginal microbiome, along with the relationship between the urinary microbiome and the vaginal microbiome. Ultimately, understanding how UUI symptoms, the urinary microbiome, and vaginal microbiome coexist will improve our understanding to best treat women with UUI and other lower urinary tract symptoms.

3 Urinary and vaginal microbial co-occurrence and urgency urinary incontinence

3.1 Abstract

The state of the vaginal microbiome is known to contribute significantly to women's health. We're also increasing our understanding of the role the commensal urinary microbiome has on lower urinary tract symptoms. Markers of microbiome health and dysbiosis rely on diversity summary measures and identifying differentially abundant bacteria. These methods have expanded our understanding of how changes in these microbial communities are associated with disease. However, these current microbiome analytical methods do not fully account for the ecological interactions of constituent members of the microbial community, which may uncover subtle microbial community differences that are missed through microbial diversity indicators and differential abundance methods. Here we show that microbial co-occurrence networks can uncover known and emerging uropathogens in both the urinary microbiome and vaginal microbiome that are missed with traditional analytical methods. We found that the urinary microbiome of women with UUI have unique correlations between *Lactobacillus* and known genera associated with urinary tract infections and bacterial vaginosis such as *Campylobacter*, *Corynebacterium*, *Actinotigum*, *Aerococcus*, *Prevotella*, and *Escherichia/Shigella*. Similarly, we identified the genera *Aerococcus* as a central constituent bacteria in the vaginal microbiome co-occurrence network. The genera *Aerococcus* was also found to co-occur with *Gardnerella* and *Prevotella*, which are two bacteria known to cause bacterial vaginosis. Our results demonstrate the potential of using microbial co-occurrence networks to understand lower urinary tract symptoms and their relationship with the urinary and vaginal microbiomes. We anticipate our work to be a starting point for more

sophisticated and robust microbial network models of the urinary and vaginal microbiome ecology.

3.2 Introduction

Organisms exist with each other through known ecological interactions, such as mutualistic (win-win) and competitive (lose-lose) situations (Lidicker 1979). These interaction classifications also exist for microbial communities (Konopka 2009). The aggregate of these microbial community interactions has recently been explored and modeled using microbial co-occurrence networks in the human microbiome (Faust et al. 2012). These microbial networks allow us to uniquely identify key bacteria using the structure of the co-occurrence network to gain information about the community as a whole. Here we explore model the microbial co-occurrence of the urinary and vaginal microbiome in women with and without UUI.

3.3 Methods

The study population and design, DNA extraction and PCR amplification, and sequence processing, taxonomic assignment, and phylogenetic tree building are the same as those described in section 2.3.1, section 2.3.2, and section 2.3.3, respectively.

3.3.1 Network analysis

The abundance count table was processed using SparCC for composition-aware variation. SparCC accounts for the compositional nature of 16S rRNA data by performing a linear Pearson correlation on log-ratio transformed data (Friedman and Alm 2012). This transformation is beneficial because it retains the true abundance values as a ratio, which are independent of other taxa included in the data, and the transformation can take any value rather than being constrained to a fixed abundance. The SparCC method was

performed as implemented in the `sparcc` function with default parameters in the `SpiecEasi` package (version 1.0.7) (Kurtz et al. 2019). Network analyses were performed using the R packages `tidygraph` (version 1.2.0) (Pedersen 2020b), with the underlying functionality of `igraph` (version 1.2.5) (Csardi and Nepusz 2006), and visualized using the R package `ggraph` (version 2.0.3) (Pedersen 2020a). Community detection was performed using the InfoMap community detection algorithm, which minimizes the expected description length of a random walker along the network, as implemented in `cluster_infomap` in the R package `igraph` (version 1.2.5) (Rosvall and Bergstrom 2008; Csardi and Nepusz 2006).

A permutation analysis was used on all UUI case and control vaginal microbiome data to determine a correlation threshold by shuffling the sample labels for each genera in a pairwise comparison prior to calculating correlations. A similar permutation was performed separately on the urinary microbiome data. This permutation analysis generates a null distribution of correlations from which to identify a threshold of correlations for downstream analyses. A permutation of 1000 trials was performed and a threshold of the top 5% of the null distribution was used to determine a correlation cut off for each the vaginal microbiome (correlations > 0.23) and urinary microbiome data (correlations > 0.22). Only positive correlations were considered for the network analysis.

3.4 Results

We inferred two urinary microbial interaction networks by analyzing 20 women with UUI and 30 women without UUI (fig. 8, Table 6). The UUI network had fewer genera in the connected network (93 genera in UUI versus 135 genera in controls) and fewer unique bacterial co-occurrences (624 associations in UUI versus 763 associations in controls). The

basic structure of the urinary microbiome network showed 4 clustered subgroups of bacteria for women with UUI and 9 clusters for women without UUI. Visually and quantitatively using modularity and connectance, we see that the control urinary microbial network is more clustered into smaller microbial groups, which may suggest more specialized microbial niches. We also observe a difference in how the bacterial phyla associate with each other in the network (p-value < 0.001) (fig. 9).

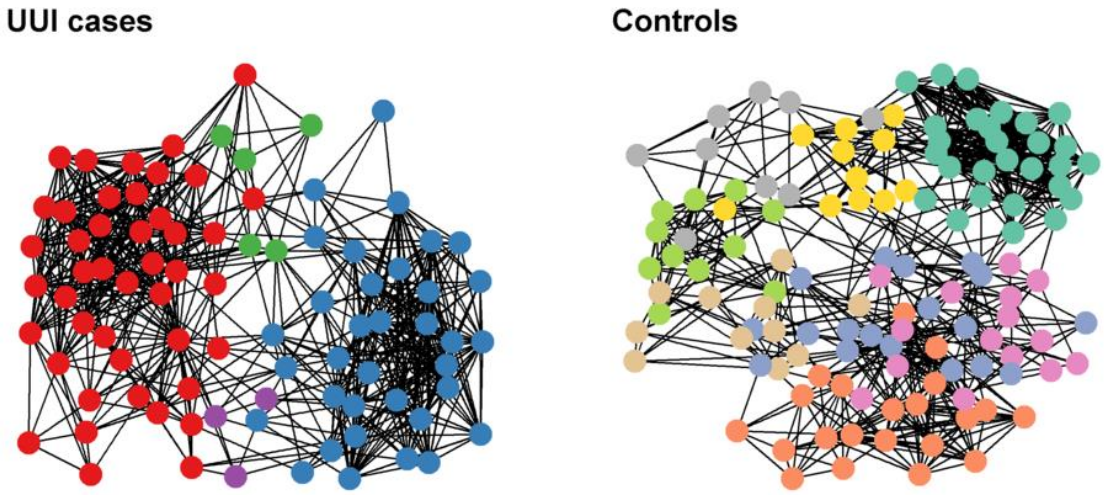

Figure 8: Network visualization of urinary microbiome in UUI and healthy controls. This shows the urinary microbiome co-occurrence network, one for the case subjects (left) and one for the control subjects (right). The bacterial genera here are shown as circles, colored by communities of bacteria identified using the InfoMap algorithm, and connected by the SparCC correlation. Each edge represents a significant co-occurrence relationship greater than a correlation of 0.22 defined by a permutation analysis. Only positive relationships are shown.

	Interpretation	Case	Control
Number of nodes	Space of co-occurring bacteria to consider	93	135
Number of edges	Number of co-occurrence relationships	624	763
Modularity	Measure of community detection	0.46	0.60
Normalized connectance	Complexity of system	0.15	0.09

Table 6: Network statistics for urinary microbiome networks across cohorts.

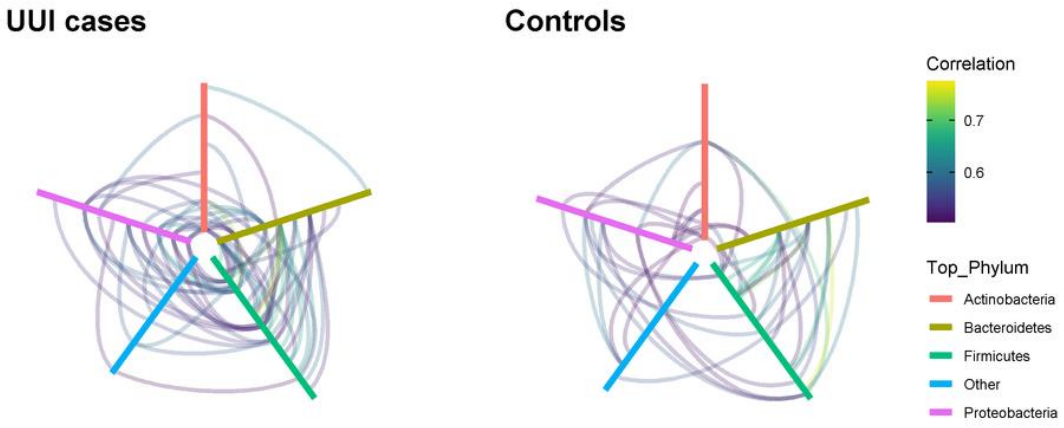

Figure 9: Urinary bacterial phyla associate differently in UUI versus healthy controls. This hive plot orients the different phyla on the axes. Only the top four phyla are shown and the remaining phyla are collapsed into "Other". Correlations limited to greater than 0.5 for visualization purposes.

We explored key urinary bacteria in each of the UUI and control urinary microbial networks using the betweenness centrality measure (Table 7). We found the *Lactobacillus* genera to be more central in both UUI and controls. Because of the previously known prevalence of *Lactobacillus* in the commensal urinary microbiome, our observation of *Lactobacillus* here as a key bacteria to the microbial community structure is a promising positive control for our network-based approach.

Because *Lactobacillus* is central to both UUI and non-UUI networks, we explored the microbial associations between the *Lactobacillus* genera and other genera in each cohort

(fig. 10). We found 25 unique genera in UUI and 16 unique genera in the controls that associated with *Lactobacillus* in their respective networks. Both networks shared a total of 10 common genera that associated with *Lactobacillus*. Among the unique associated genera in UUI, we found that at least a quarter of them are known uropathogens associated with urinary tract infections (Table 8).

	Cohort	Phylum	Genus	Betweenness
1	UUI	Firmicutes	Lactobacillus	0.19
2	UUI	Firmicutes	Agathobacter	0.07
3	UUI	Firmicutes	Incertae_Sedis	0.06
4	UUI	Actinobacteria	Bifidobacterium	0.04
5	UUI	Proteobacteria	Azomonas	0.04
1	Control	Firmicutes	Lactobacillus	0.12
2	Control	Firmicutes	Veillonella	0.10
3	Control	Firmicutes	Agathobacter	0.07
4	Control	Actinobacteria	Corynebacterium	0.07
5	Control	Firmicutes	Psuedobutyrivirbio	0.07

Table 7: Network statistics for urinary microbiome networks across cohorts. Betweenness = betweenness centrality, UUI = urgency urinary incontinence.

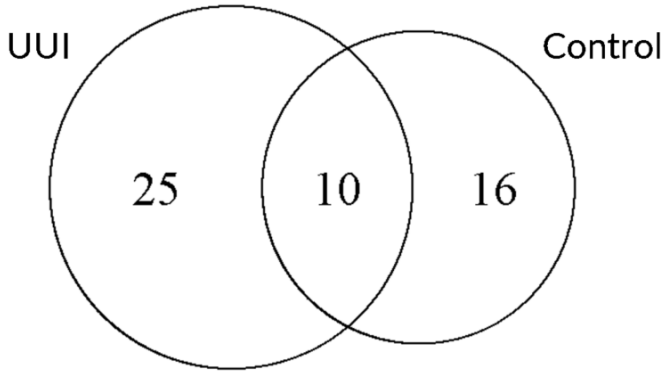

Figure 10: Overlap of bacteria correlated with *Lactobacillus* in UUI and controls.

Correlation	Phylum	Genus
0.49	Proteobacteria	Campylobacter
0.40	Actinobacteria	Corynebacterium
0.38	Actinobacteria	Actinotignum
0.35	Tenericutes	Ureaplasma
0.34	Firmicutes	Dialister
0.32	Firmicutes	Aerococcus
0.26	Bacteroidetes	Prevotella
0.25	Proteobacteria	Escherichia/Shigella

Table 8: Notable urinary bacteria correlated with Lactobacillus in UUI are associated with urinary tract infections.

Additionally, we inferred two vaginal microbiome-wide microbial interaction networks of the 20 women with UUI and 30 women without UUI (fig. 11, Table 9). The UUI network had similar number of genera in the connected network (60 genera in UUI versus 58 genera in controls) but more unique bacterial co-occurrences (396 associations in UUI versus 195 associations in controls). The number of clusters in the vaginal microbiome network were similar between cohorts, where the UUI network clustered into 5 subgroups of bacteria and 6 clusters for women without UUI. Visually and quantitatively using modularity and connectance, we see that the control urinary microbial network is more clustered into smaller microbial groups, which may suggest more specialized microbial niches. We also observed a difference in how the bacterial phyla associate with each other in the network (p-value < 0.001) (fig. 12).

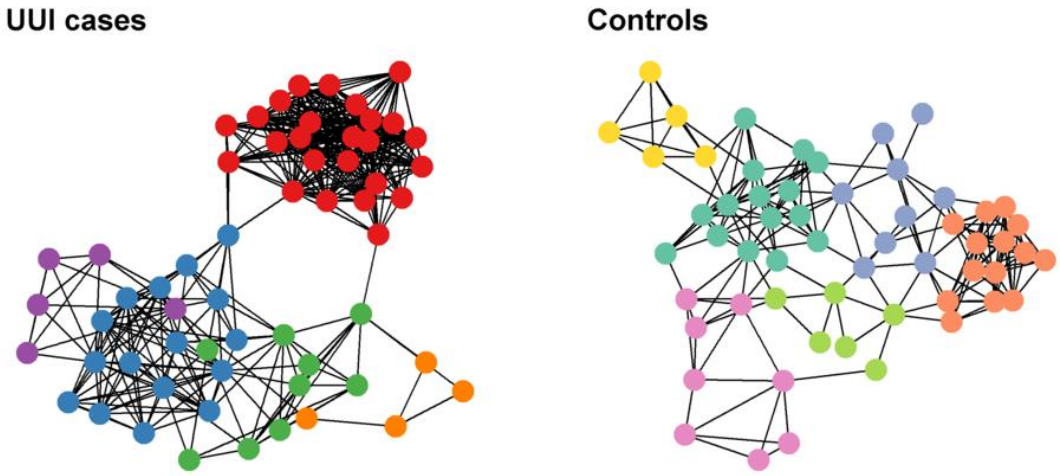

Figure 11: Network visualization of vaginal microbiome in UUI and healthy controls. This shows the vaginal microbiome co-occurrence network, one for the case subjects (left) and one for the control subjects (right). The bacterial genera here are shown as circles, colored by communities of bacteria identified using the InfoMap algorithm, and connected by the SparCC correlation. Each edge represents a significant co-occurrence relationship greater than a correlation of 0.23 defined by a permutation analysis. Only positive relationships are shown.

Interpretation		Case	Control
Number of nodes	Space of co-occurring bacteria to consider	60	58
Number of edges	Number of co-occurrence relationships	396	195
Modularity	Measure of community detection	0.50	0.59
Normalized connectance	Complexity of system	0.23	0.12

Table 9: Network statistics for vaginal microbiome networks across cohorts.

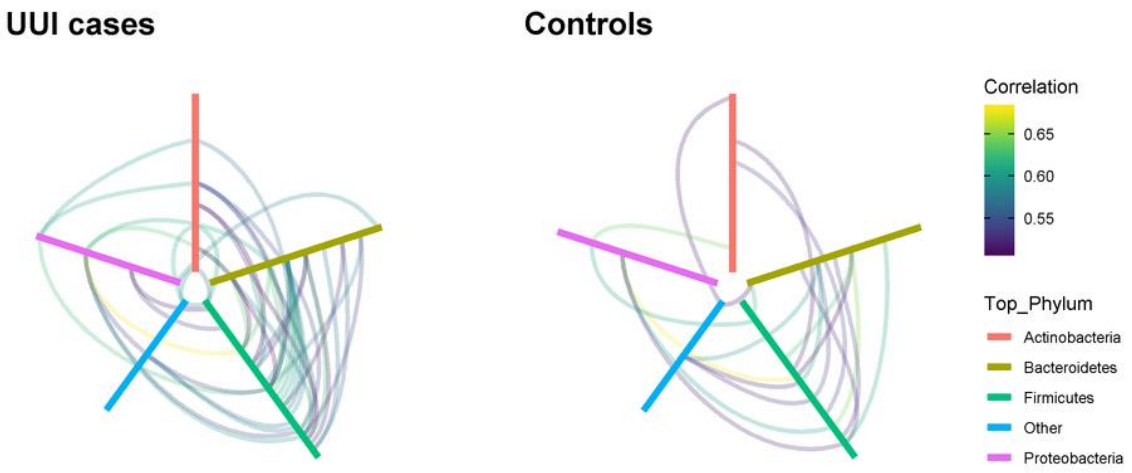

Figure 12: Vaginal bacterial phyla associate differently in UUI versus healthy controls. This hive plot orients the different phyla on the axes. Only the top four phyla are shown and the remaining phyla are collapsed into "Other". Correlations limited to greater than 0.5 for visualization purposes.

We explored central vaginal bacteria in each of the UUI and control vaginal microbial networks using the betweenness centrality measure (Table 10). Unlike the urinary microbiome network (Table 6), we did not find *Lactobacillus* as being central to both the UUI and control networks, but only for the control network. Because of the dominance and clinical importance of *Lactobacillus* in the commensal vaginal microbiome, observing *Lactobacillus* as a central bacteria to the microbial community structure is another promising positive control for our network-based approach.

Because the *Aerococcus* genus is central to our vaginal microbial network in UUI and it is a known uropathogen associated with urinary tract infections (Zhang et al. 2000; Hilt et al. 2020), we explored its surrounding bacterial connections in our network (Table 11). We found that the two top average relative abundant genera that *Aerococcus* was associated with, *Gardnerella* and *Prevotella*, are both together associated with bacterial vaginosis (Randis and Ratner 2019).

Cohort	Phylum	Genus	Betweenness
UUI	Firmicutes	Aerococcus	0.33
UUI	Firmicutes	Streptococcus	0.19
UUI	Proteobacteria	Mannheimia	0.15
UUI	Bacteroidetes	Bacteroides	0.15
UUI	Bacteroidetes	Prevotellaceae_UCG-001	0.15
Control	Firmicutes	Lactobacillus	0.20
Control	Fusobacteria	Fusobacterium	0.14
Control	Firmicutes	Faecalibacterium	0.13
Control	Firmicutes	Parvimonas	0.13
Control	Firmicutes	Staphylococcus	0.12

Table 10: Network statistics for vaginal microbiome networks across cohorts. Betweenness = betweenness centrality, UUI = urgency urinary incontinence.

Phylum, Genus	Average relative abundance (%)
Actinobacteria, Gardnerella	7.47
Bacteroidetes, Prevotella	4.01
Bacteroidetes, Bacteroides	1.35
Bacteroidetes, Prevotellaceae_UCG-001	0.89
Firmicutes, Ruminiclostridium_6	0.45
Firmicutes, Staphylococcus	0.27
Fusobacteria, Streptobacillus	0.15
Actinobacteria, Actinomyces	0.10
Firmicutes, Helcococcus	0.04

Table 11: Connections with Aerococcus in the vaginal microbiome of UUI cases. Average relative abundance was calculated based on UUI case samples.

3.5 Discussion

We constructed microbial co-occurrence networks for both the urinary and vaginal microbiomes of women with UUI and without. We found that the control networks had more bacteria clustered in smaller groups than in the UUI case networks. These networks being more clustered into these smaller groups suggests a putative structure among the

clustered bacteria which may have evolutionary and functional synergy. In other words, microbes that are closely related may compete more for resources, while a more modular community with complementary functions would exhibit mutualistic behaviors that are worth exploring further.

In both the urinary and vaginal microbiome networks, we found *Lactobacillus* to be a central microbial player in the network structure. This is consistent with previous findings (Pearce et al. 2014; Ravel et al. 2011). For the urinary microbiome of women with UUI, we found positive correlations between *Lactobacillus* and a number of bacteria that are known to or are gaining evidence to be associated with urinary tract infections. Adjacent to the urinary microbiome, we found the genus *Aerococcus* as most central to the microbial community structure of the vaginal microbiome of women with UUI. It is notable that *Aerococcus* has recently been isolated from the bladders of women with urinary tract symptoms (Hilt et al. 2020). Although *Aerococcus* was not directly found in the bladder, the vaginal microbiome is recognized as a key anatomical site for the pathogenesis of urinary tract infections (Stapleton 2016) and is shown to have similar functional capacities with the urinary microbiome (Thomas-White, et al. 2018). This further suggests the possible transmission of the vaginal microbiome to seed the urinary microbiome with uropathogens that are associated with urgency urinary incontinence.

3.6 Limitations

It is plausible that a number of limitations could have influenced the results obtained. Small sample sizes reduce our power to identify results and increase the amount of noise in our data. This is especially true for microbiome data that is sparse, meaning most of the microbes are not present across all the samples. This sparsity can cause issues when

calculating correlations because low number of samples per pairwise correlation can artificially inflate the strength of a correlation with a small number of samples.

Compositional data analysis is an up-and-coming area of research to be applied to the microbiome (Gloor et al. 2017). The work here presents and uses a compositional aware network method, SparCC, to generate the microbial networks. Although this is a good first step, there needs to be further work on studying the influence of compositional data on such low biomass microbiomes. Current studies of compositional data methods are typically performed on high biomass microbiomes, such as the gut microbiome. However, we don't fully understand the implications of these methods being done on a differently structured microbiome, such as the urinary microbiome or vaginal microbiome.

Additional limitations include the lack of functional information annotated on these bacteria and the lack of temporal information. Bacterial function would enrich this analysis to better understand relationships among the bacteria and how or why they may co-occur with each other. This strengthens the hypothesis that those co-occurring bacteria complement each other evolutionarily or functionally.

3.7 Conclusion

We modeled the vaginal microbiome and urinary microbiomes of pre-dominantly post-menopausal women to understand microbial associations within the vaginal microbiome and within urinary microbiome to identify important bacteria to the microbiome structure that are associated with urgency urinary incontinence (UUI). There is still work to be done to further explore the range of possibilities to use network-based analyses to deepen our understanding of bacterial associations and their relationship with UUI. Ultimately, understanding how UUI symptoms, the urinary microbiome community

structure, and the vaginal microbiome community structure will improve our understanding to best treat women with UUI and other lower urinary tract symptoms.

4 Discussion and conclusions

We characterized the vaginal microbiome and urinary microbiomes using traditional bioinformatic methods and explored the utility of microbial co-occurrence networks to understand urgency urinary incontinence in women. The initial evidence from the traditional analysis suggests that there is no link with either the urinary microbiome or vaginal microbiome and urgency urinary incontinence. The reason for this rather contradictory result is still not entirely clear, but may be due to our study population having more estrogen treatments compared to other studies. Despite these contradictory results, our network analysis shows promising insights into other differences not apparent through traditional microbial diversity or differential abundance methods. In the urinary microbiome of women with UUI, we found bacteria associated with *Lactobacillus* to be known uropathogens for urinary tract infections. Moreover, we found the vaginal microbiome of women with UUI to have a known uropathogen associated with urinary tract infections, *Aerococcus*, to be central to the microbial community. Our results demonstrate the potential of using microbial co-occurrence networks to understand lower urinary tract symptoms and their relationship with the urinary and vaginal microbiomes. We anticipate our work to be a starting point for more sophisticated and robust microbial network models of the urinary and vaginal microbiome ecological states.

5 Future work

There are a number of directions this work can take. These next steps include: connecting the urinary and vaginal networks to create an integrated urogenital network, robust testing the microbial co-occurrence networks in low biomass environments, and incorporating negative correlations into the network analysis. After these steps are evaluated, an orthogonal direction to explore is temporal networks in how these microbial networks change over time. And ultimately, these results will need to be experimentally validated in the lab, starting with small triplets of bacteria unique to UUI that may influence UUI symptoms.

5.1 Interconnected urogenital microbial co-occurrence network

We have two networks, a urinary and vaginal microbiome co-occurrence network. It may be fruitful to explore the interconnected nature of the urogenital microbiome by constructing a single network comprised of both the urinary and vaginal microbiome. This can be limited to just interactions between the two microbiomes, which can be modeled using a bipartite graph. To make this mode tractable, focusing on putative clusters in each microbiome can reduce the computational overhead of this analysis. Removing this restriction can be explored to identify co-occurring urinary and vaginal bacteria. While these two sets of bacteria might not interact, they still co-occur with each other and may have a biological link between them. A first step in understanding the relationship between these two microbiomes is to perform a Mantel test[18] to test the correlations between the distance matrices constructed for each of these microbiomes. This tests whether the

[18] See https://mb3is.megx.net/gustame/hypothesis-tests/the-mantel-test.

variation in one microbiome (e.g., urinary microbiome) is associated with the variation in the other microbiome (e.g., vaginal microbiome). Furthermore, a canonical correlation analysis (CCA)[19] can be performed to understand the relationship between the microbiome and clinical information, similar to work shown in Komesu et al. (2020).

5.2 Explore other correlation methods applied to low-biomass microbiomes

Although the work presented here works with low-biomass microbiomes, this was not an in-depth benchmarking of network methods applied to low-biomass environments. There are many network methods to choose from and there may be more appropriate methods to be applied. Additionally, most of these network methods have been tested and developed with higher biomass microbiomes. Some of those assumptions may not apply to lower biomass microbiomes. Lower biomass microbiomes have increased sparsity, which will need to be explored and addressed.

5.3 Negative correlations

Negative correlations were unexplored in this work. These correlations can be thought of co-exclusion patterns. A simple measure to explore is the utility of the positive-to-negative ratio of correlations (Ma 2017). Another measure and technique that can be explored is the utility of co-exclusion-specific tools (Albayrak et al. 2018).

5.4 Temporal networks

The microbiome is always changing. It is known that the vaginal microbiome fluctuates with hormonal cycle, contraceptives, diet, and exercise (Song et al. 2020). A

[19] See https://mb3is.megx.net/gustame/constrained-analyses/cca.

natural extension of the work presented in Chapter 3 is to explore network variations over time using temporal networks (Li et al. 2017). There already is R code and instruction to explore temporal networks (Brey 2018).

5.5 Construct networks based on urotype and community state type

The urinary and vaginal microbiome have been shown to have subtypes. These subtypes are based on dominant bacteria in an individual's microbiome. In the vaginal microbiome these subtypes, or community state types, have clinical and research utility such as being a good predictive indicator of bacterial vaginosis (Seta et al. 2019). Similarly, the urinary microbiome can be classified into subtypes, or urotypes, which indicate some susceptibility to diseases like bacterial vaginosis (Gottschick et al. 2017). Based on this information, it may be worthwhile to explore the network variation among these different subtypes and what those implications may be.

5.6 Focus on disease and pathogen networks

The work presented in Chapter 3 is a first application in using networks to the urinary microbiome and growing applications to the vaginal microbiome. Poudel et al. (2016) have proposed a framework for identifying candidate microbial assemblages for disease. This framework consists of four network analyses: a general network analysis (similar to the work presented in Chapter 3), host-focused analysis to relate features of the microbial network with host-based information, pathogen-focused analysis to *a priori* focus on known pathogens in the network, and a disease-focused network (similar to the work presented in Chapter 3). Work that can be done here is to formalize this framework for human microbiome networks because the original paper was directed to plant pathology and management.

5.7 Consensus network from multiple methods

There are a plethora of network methods available (Jiang et al. 2019). However, it is difficult to know which set of networks is the most accurate. Crowdsourcing construction of gene regulation networks have proved successful (Prill et al. 2011). This approach can also be applied to microbial network construction to identify a biologically meaningful and tractable model of microbial communities.

5.8 *In vitro* validation of microbial interactions

We now have putative interactions and communities from the urinary and vaginal microbiomes. These are *in silico* results and will still need to be experimentally validated. We can combine *in silico* and *in vitro* results to validate our findings. Because of the unique and fastidious nature of the urinary microbiome, it may prove to be difficult to appropriately engineer a culture medium to effectively grow these bacteria. It may be advantageous to explore computational methods to hypothesize and optimize appropriate media for urogenital microbe growth using databases such as a Known Media Database (KOMODO) (Oberhardt et al. 2015).

Ideally, the results of these work will be experimentally validated *in vitro* by taking pairs or triples of bacteria to grow together in order to observe their ecological interactions. Previous work shows exploring ecological interactions by measuring metabolic output using small numbers of bacteria together (Medlock et al. 2018). Similarly, there are model microbiome communities that can be used to explore constrained microbiome interactions for results presented here (Lozano et al. 2019).

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