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Evaluation of the Urinary Microbiota of Women With Uncomplicated Stress Urinary Incontinence

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Abstract

Background—Female urinary microbiota (FUM) are associated with urgency urinary incontinence (UUI) and response to UUI medication. FUM of women with stress urinary incontinence (SUI) has not been described.

Objective—Study the cross-sectional relationships between FUM features and demographic and clinical characteristics of women undergoing SUI surgery.

Design, Setting, and Participants—Pre-operative urine specimens were collected from women without urinary tract infection and were available from 197 women (174 voided, 23 catheterized) enrolled in a multi-center prospective randomized trial, the Value of Urodynamic Evaluation (ValUE) study. Demographic and clinical variables were obtained including SUI and UUI symptoms, menopausal status, and hormone use.

Outcome Measurements and Statistical Analysis—The bacterial composition of the urine was qualitatively assessed by sequencing the bacterial 16S rRNA gene. Phylogenetic relatedness and microbial alpha diversity were compared to demographics and symptoms using generalized estimating equation models.

Results—The majority of 197 urine samples (86%) had detectable bacterial DNA. Bacterial diversity was significantly associated with higher BMI ($p=0.02$), increased Medical, Epidemiologic, and Social Aspects of Aging (MESA) urge index score ($p=0.04$), and hormonal status ($p<0.001$). No associations were detected with SUI symptoms. Increased diversity was also associated with a concomitant lower frequency of *Lactobacillus* in hormone-negative women.

Conclusions—Women undergoing SUI surgery have detectable urinary microbiota. This cross-sectional analysis revealed that increased diversity of the microbiota was associated with UUI symptoms, hormonal status and BMI. In contrast, the FUM was not associated with stress urinary incontinence symptoms.

Keywords

Microbiome; Stress Urinary Incontinence; Urgency Urinary Incontinence; Estrogen; Bladder

INTRODUCTION

The influence of the human microbiota on health and disease is increasingly appreciated in a variety of medical fields [1] These microbial communities are often described by their predominant organism, the diversity of organisms within the community and the amount of those organisms [2–4].

The female urinary microbiota (FUM), composed of resident bladder bacteria, was recently recognized when bacterial DNA and low levels of live bacteria were detected in catheterized urine specimens considered “sterile” by standard urine culture [5–7]. Enhanced urine culture techniques have provided clear evidence that FUM microbes are alive; unlike standard urine culture protocols, these enhanced culture techniques provide the appropriate conditions for growth for a wide range of microbes [6, 8]. The living microbial community within the female bladder may provide insight into a variety of common urinary disorders, including

urinary incontinence and urinary tract infections. The presence and response to urgency urinary incontinence (UUI) treatment appears related to FUM diversity and/or composition in adult women with UUI [2, 9]. There is also an association between the FUM and risk of urinary tract infection (UTI) following urinary tract surgery [10] or instrumentation [5, 11]. However, there is a lack of information regarding the FUM of adult women with stress urinary incontinence (SUI). The two most common forms of bothersome urinary incontinence (UUI and SUI) often coexist in adult women, especially those seeking surgical treatment for SUI. Information concerning the FUM has the potential to further develop the phenotype of adult women affected by urinary incontinence, with the hope of improving the targeting of treatment in order to improve overall outcomes.

The National Institutes of Health sponsored a large, multi-center, clinical trial of women with uncomplicated SUI planning surgery and previously established a biorepository of urine samples collected for various scientific purposes [12]. In this sub-study, we describe the FUM analysis using 16S rRNA gene sequencing to characterize the cross-sectional relationships between FUM parameters and demographic and clinical characteristics of adult women undergoing surgery for SUI.

MATERIALS AND METHODS

Subject Recruitment and Urine Collection

The Value of Urodynamic Evaluation (ValUE) study was an Institutional Review Board (IRB) approved, multi-center prospective randomized trial comparing surgical outcomes using 2 strategies for pre-surgical testing: multichannel urodynamic testing versus standardized basic office evaluation. [12, 13]. Briefly, adult women were eligible if they reported symptoms of SUI ≥3 months, had a post-void residual <150 mL, a negative urinalysis/standard urine culture, clinical assessment of urethral mobility, desire for SUI surgery, a positive provocative stress urinary test and a qualifying Medical, Epidemiologic, and Social Aspects of Aging (MESA) questionnaire [13, 14] subscale score (stress > urge). Demographic and clinical characteristics were obtained by self-report including hormonal status, which was categorized by the study team into the hormone group that most appropriately described the patient's hormone use: pre-menopausal, post-menopausal (with or without self-reported, current exogenous hormone use) or uncertain about status.

Participants in the main study provided written consent to contribute a single baseline urine specimen to the biorepository. Urine specimens were collected prior to surgery by a standard protocol and tested by dipstick to exclude UTI at study entry. Specimens were centrifuged at 500–1500g for 10 minutes and the supernatant dispensed into ten 2cc Eppendorf tubes, which were frozen at −80°C until shipped on dry ice to the National Institute of Diabetes and Digestive and Kidney diseases (NIDDK) biorepository. Available baseline urine specimens with sufficient volume for the planned studies were shipped to Loyola University Chicago on dry ice, and stored at −80°C until processed for sequence analysis. Samples from 197 of the 630 (31%) ValUE participants were used in this analysis; most (174) samples had been obtained by clean catch, with the remaining 23 by catheterization. Analyses for this report were approved by The Loyola University Chicago IRB.

16S rRNA gene sequencing

Microbial composition was determined by sequencing the variable 4 (V4) region of the bacterial 16S rRNA gene, as described [2, 6, 9, 11]. The V4 region is ~250 bp, ideal for MiSeq sequence technology (Illumina), and sufficient to classify most bacteria to the family or genus level [15–18]. DNA isolation was performed in a laminar flow hood to avoid contamination. Genomic DNA was extracted from 1 ml of urine, using validated protocols [2, 6, 19]. The V4 region was amplified by a two-step polymerase chain reaction (PCR), using modified universal primers 515F and 806R, as described [2, 6]. Extraction negative controls (no urine) and PCR negative controls (no template) were included to assess contribution of extraneous DNA from reagents. Final PCR products were purified from unincorporated nucleotides and primers using Qiaquick PCR purification kit (Qiagen, Valencia, CA) and Agencourt AMPure XP-PCR magnetic beads (Beckman coulter). Purified samples were normalized to equal DNA concentration, as determined by Nanodrop spectroscopy (Thermo Scientific, Waltham, MA). The sample library and PhiX sequencing control library (Illumina) were denatured and added to the 2x250 bp sequencing reagent cartridge, according to manufacturer's instructions (Illumina).

Sequence processing

Each specimen was sequenced in duplicate and classified by phylogenetic diversity as measured by Bray-Curtis dissimilarity. A phylogenetic tree was generated and compared to percent total classified reads (relative abundance) at each taxonomic level (phyla, class, order, family, genus). For a genus level example, see Figure 1.

Each major branch or clade (termed urotype) in the phylogenetic tree was named for the predominant classified taxon (e.g., *Lactobacillus*). When there was no predominant taxon, we used the term "non-predominant" to describe the urotype [2, 9, 20]. MiSeq sequence reads were processed following mothur's MiSeq SOP at http://www.mothur.org/wiki/MiSeq_SOP [18], with minor modifications. Mothur software (version 1.34.4) [21] was used to process raw reads and, using default mothur parameters, to remove low quality and chimeric sequences. Taxonomic classification from phylum to genus level of sequence reads was performed by the RDP Classifier (version 2.5) [22] using the default 0.8 confidence threshold. The sampling depth for this analytic set was set at 2000 reads; the Kolmogorov–Smirnov test confirmed that when subsampling depth exceeded 2000 reads, the distribution of subsampled and original reads distribution had >95.9% similarity amongst all samples.

Most (171/197) samples had detectable DNA with 338,000 and 340,000 reads subsampled for replicates 1 and 2, respectively. The 26 samples without detectable DNA following PCR amplification were classified as "below the detection threshold." Due to read depths less than 2000, two samples from replica 1 and one sample from replica 2 were also classified as "below the detection threshold", for a total of 28 in replica 1 and 27 in replica 2. Using mothur's built-in average-linkage clustering algorithm, the cleaned high-quality sequences were clustered into species level operational taxonomic units (OTUs) based on the commonly used 97% similarity cutoff, resulted in 2579 and 3082 OTUs for replicas 1 and 2, respectively. We used the resultant OTU count table and the R package vegan [23, 24] to determine the Chao1 richness estimate, Pielou evenness index, and Shannon diversity index,

which accounts for both richness and evenness, two measures of microbial diversity. Richness is a measure of the total number of unique taxa within a given individual, but does not take into account the distribution of those taxa. In contrast, evenness is a measure of distribution, or equality of representation, of taxa within an environment. Samples "below the detection threshold" lack diversity measurements; these were excluded from subsequent diversity comparisons.

Statistical analysis

Generalized Estimating Equations (GEE), extensions of Generalized Linear Models that account for correlation between replicas, were used to describe associations between demographic and clinical factors with diversity measurements after adjusting for genus urotype. A gamma distribution with a log link was assumed for Shannon, Chao, and Peilou diversity measurements, due to their skewed nature. To be inclusive, we did not make adjustments for Type I error in the GEE analyses when determining potential clinical and demographic associations with microbiota characteristics. Only results from the lowest detected resolution level (i.e., genus) are reported. There was insufficient sample size and power to compare urotypes between catheterized and voided samples. All statistical analyses were conducted using SAS v9.4 (SAS Software Cary, NC) and statistical significance was assessed at the α=0.05 level.

RESULTS

The demographic and clinical characteristics of the 197 participants we studied (Table 1) were similar to those of the overall trial population [12]. Most of these participants were non-Hispanic Caucasian (79%) and currently married (74%). The mean age of the subset was 51 (SD:9.7) years. Forty-two percent of women were pre-menopausal, 31% post-menopausal without current exogenous hormone use, and 18% were using exogenous hormones; the remaining 10% were "unsure" of their status. Consistent with the entrance requirements for the trial [12], women reported stress predominant UI with a median MESA stress index score of 78 (IQR:59–89) and 76% reporting urinary leakage every day and/or night. Concomitant urinary symptoms were common at time of trial enrollment; the median MESA urgency urinary incontinence index score was 33 (IQR:17–50). As only 21 participants had an urgency index of zero, dichotomous group comparisons by urgency index were not performed.

Figure 1 displays a representative phylogenetic tree and histogram for replica 1 of the remaining samples classified at the genus level. Together, the phylogenetic tree and histogram show how samples cluster into urotypes. Supplementary Figure S1 displays similar representations for each replica at other taxonomic levels.

Replicas 1 and 2 yielded similar results (Table 2). The only major difference involved samples of the *Lactobacillus* urotype, the most common urotype in both replicas (replica 1: 46%, 90/197; replica 2: 37%, 72/197). The difference in *Lactobacillus* urotype frequency between replicas was mirrored by an inverse difference in the frequency of the "non-predominant" urotype (replica 1: 5%, 10/197; replica 2: 12%, 23/197). This inverse relationship was primarily caused by phylogenetic reorganization and therefore re-

classification of the most diverse samples in the *Lactobacillus* urotype as members of the "non-predominant" urotype.

The results of the GEE analyses before and after adjustment for urotype were similar, thus only adjusted results are presented (Table 3). We used two types of microbial diversity measurements: richness (total number of unique taxa) and evenness (equality of representation of taxa within an environment). Richness, as estimated by Chao1, was significantly associated only with urine pH (p=0.03). In contrast, richness and evenness, as measured together by the Shannon index, were significantly associated with MESA urge index score (p=0.04), BMI (p=0.02) and hormonal status (p<0.001) (Table 3). For a 10% increase in MESA urge index score, the Shannon index increased by 0.03 units (p=0.04). On average, a 10-unit increase in BMI was associated with a 0.1-unit increase in Shannon diversity (p=0.02). Post-menopausal women not on exogenous hormones had a Shannon index 0.23 units higher than did pre-menopausal women (p=0.004).

Because these parameters did not associate with richness (Chao1), we tested evenness alone, as measured by Peilou diversity (Table 3). Evenness was significantly associated with MESA urge index score (p=0.04), BMI (p=0.02) and estrogen status (p<0.001). For a 10% increase in the MESA urge index score, the Pielou diversity increased by 0.03 units (p=0.04). On average, a 10-unit increase in BMI was associated with a 0.1-unit increase in Peilou diversity. Finally, post-menopausal women had a 0.21 unit higher diversity measurement compared to pre-menopausal women (p=0.02). Since these results were similar to those observed with the Shannon index, we conclude that increased community evenness associates with UUI symptoms, BMI and especially hormonal status.

Because community evenness associates strongly with hormonal status, we constructed a visual comparison of microbial diversity subdivided by hormonal use (Figure 2). Compared to other groups, the hormone-positive women (pre-menopausal and post-menopausal on exogenous hormones) had a higher frequency of *Lactobacillus* or *Gardnerella* urotypes (66%) and a lower frequency of 'non-predominant' urotypes, while the hormone-negative women (post-menopausal not on exogenous hormones) had a lower frequency of *Lactobacillus* or *Gardnerella* urotypes (38%) and greater frequency of 'non-predominant' urotypes (p<0.001).

DISCUSSION

Main findings

In this study of women undergoing surgery for uncomplicated SUI, the presence of UUI symptoms appears related to increased microbial evenness, indicating that the FUM of women with UUI symptoms was less likely to be predominated by a single microbe. A similar relationship was observed between microbial evenness and both hormone status and BMI. The clinical impact of these findings is significant, given the common coexistence of UUI in women who undergo surgical SUI treatment. While considerable uncertainly persists about the effect of SUI surgery on pre-existing UUI symptoms and each individual patient's risk of developing *de novo* UUI symptoms following SUI surgery, the microbiota may provide useful information for clinical phenotyping of patients prior to surgery. Without

comparison to an age and hormone-status matched continent control group, we cannot conclude that women with “pure” SUI are like continent women. However, it does appear the presence and characteristics (i.e. evenness) of the FUM relate to UUI in this clinically relevant cohort of women undergoing SUI surgery. Our findings will require further research to validate and clarify the physiological mechanisms.

We observed this association between microbial evenness and UUI symptoms despite the possibility of vulvo-vaginal contamination in these voided urine samples. The FUM detected in the pre-surgical voided urine samples of this cohort was similar to those assessed in catheterized samples obtained from other cohorts of women [2, 9, 11]. To ensure appropriate data interpretation of voided sample data, therefore, we recommend prior information of FUM composition from women of a similar cohort using catheter collection methods that obtain urine directly from the bladder.

Research implications

Our finding of a relationship between microbial evenness to hormonal status may provide clues for further study. Menopausal women not on exogenous hormones had increased microbial evenness and their FUM was less likely to be predominated by a single microbe. These results suggest that predominance (most often by *Lactobacillus* species) is typical of hormone-positive women and that loss of that predominance might be associated with UUI symptoms. Increased microbial diversity, in particular community evenness, positively correlated with UUI and BMI symptoms, but not with SUI symptoms. BMI is considered a contributing factor to urgency symptoms [25], and the FUM of UUI women have been reported to be associated with BMI [2]. Obesity is also associated with an increase in circulating estrogens, emphasizing the complex nature of vaginal and urinary health [26, 27]. Further study of BMI and the FUM are needed.

It is well known that estrogen receptors are found throughout the lower urinary tract, supporting the likelihood that estrogen has a role in optimizing bladder function [28–30]. Intriguingly, the use of intravaginal estrogen has been reported to improve the lower urinary tract symptoms of urinary urgency, frequency, SUI, UUI and UTI [31]. Direct effects of estrogen on the bladder could include maintenance of bladder structural and functional integrity via bladder wall thickness, expression of vascular epithelial growth factor (VEGF), and effects on β3-adrenoreceptor activity, as described in rats [32, 33]. Given that use of intravaginal estrogen correlated with an increase in *Lactobacillus* species and a decrease in anaerobic bacteria [34], it might also influence the FUM.

Although we did not assess the vaginal microbiota of VaLUE subjects, we note the FUM’s similarity to published data regarding the vaginal microbiota. We acknowledge the possibility that the vulvo-vaginal flora contributes to bacterial diversity. However, it is biologically plausible that both these adjacent anatomical sites experience microbial alterations related to estrogen status. The bladder is a low biomass niche that contains many organisms similar to those of the vagina, including *Lactobacillus*, *Gardnerella*, and a diverse set of anaerobes [2, 6, 7]. The relationship between these two biological niches deserves further study.

Strengths and Limitations

Our study has a number of strengths, including rigorous participant characterization, multi-site recruitment, cutting-edge sequencing techniques and state-of-the art analytic approaches. The study has limitations associated with the cross-sectional study design. The study would have benefitted from controls matched for age, BMI and estrogen status, paired vaginal samples and/or longitudinal sampling, further details regarding hormone status, a higher proportion of participant samples, increased proportion of catheterized specimens and concurrent enhanced urine cultures [6]. Also, the known relationship between estrogen status and aging may mask a biological relationship that is rightfully attributed to only one of these variables. A larger study that includes women with various forms of urinary incontinence and matched controls will be needed.

CONCLUSIONS

Women undergoing SUI surgery have detectable urinary microbiota that may be of value for phenotyping patients. This cross-sectional analysis revealed that diversity of the microbiota was associated with UUI symptoms, hormonal status and BMI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary of Terms

DNA Amplicon Sequencing

Sequencing is a process of determining the precise order of nucleotides within a DNA molecule. Amplicon sequencing refers to the sequencing of a short stretch of DNA amplified from the genome or sample

16S rRNA gene sequencing

Sequencing the 16S ribosomal RNA gene is a common amplicon sequencing method used to accurately classify bacteria present in a given sample

Sequencing Reads
The individual sequences obtained from a given sample

OTU
Operational taxonomic unit is used to classify groups of closely related sequencing reads

RDP classifier
A naive Bayesian classifier that rapidly and accurately provides taxonomic assignments from domain to genus

Phylogenetic tree
A phylogenetic tree is a branching diagram that shows the evolutionary or community relationships between samples

Alpha Diversity
A measurement of diversity of a single site or sample. Compared to Beta diversity which is the measurement between sites or samples. Alpha diversity include measurement of species richness and evenness. Richness is a measure of the total number of unique taxa within a given individual, but does not take into account the distribution of those taxa. In contrast, evenness is a measure of distribution, or equality of representation, of taxa within an environment

Pielou evenness index
An alpha diversity measurement for evenness only

Chao 1 richness
An alpha diversity measurement for richness only

Shannon diversity index
An alpha diversity measurement for both richness and evenness

Urotype
A urotype is defined as a group on the phylogenetic tree (i.e., clade) that is predominated by a single organism, or labeled as “non-predominant” when no single organism predominates

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SUMMARY

In pre-surgical SUI patients, urinary microbiota are associated with UUI symptoms, hormonal status and BMI, but not SUI-specific symptoms.

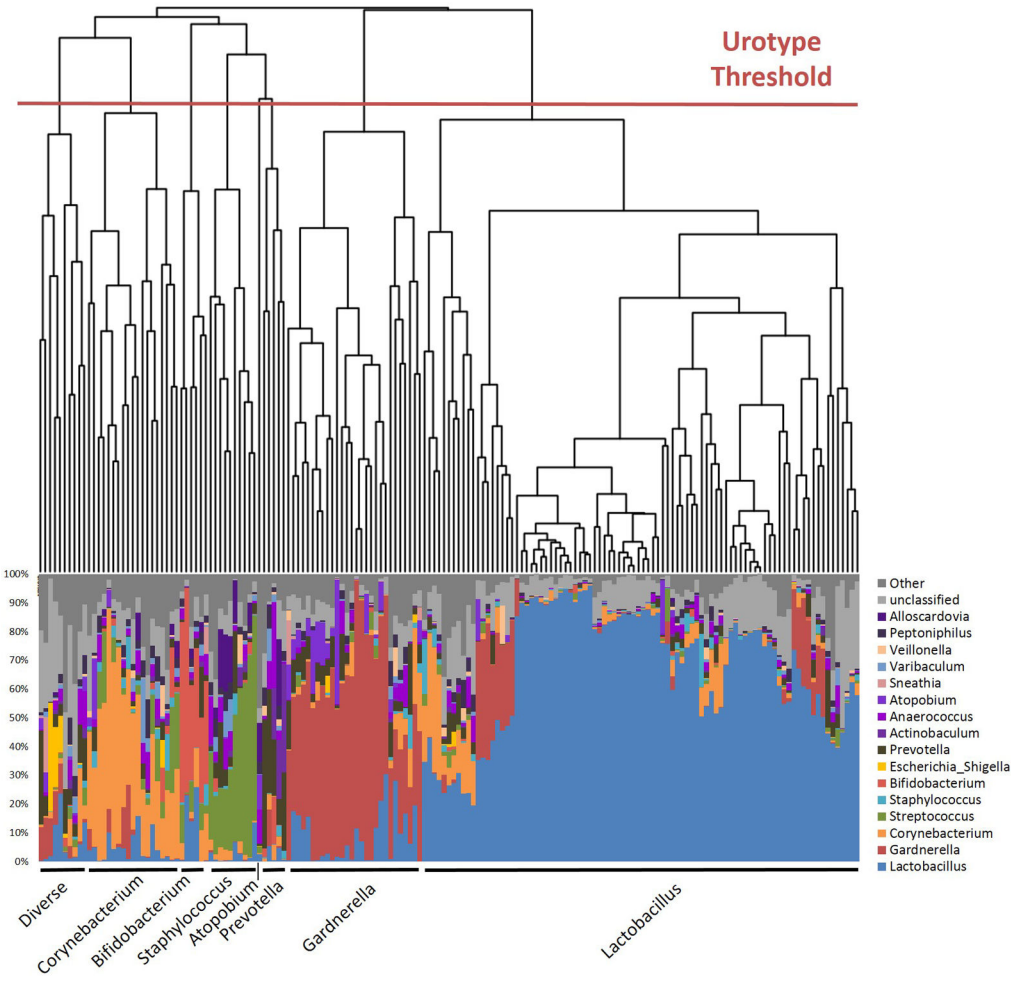

Figure 1. Using phylogenetic similarity to determine similar profiles (aka urotypes)

For each taxonomic level (Phylum, Class, Order, Family, Genus), all samples were compared to each other using Bray-Curtis similarity, which produces a phylogenetic tree, or dendrogram, in which shorter branches link similar samples, and longer branches link more dissimilar samples. Therefore, each tree can be divided into groups or clades. When aligned to relative sequencing abundance, the clades of each tree separate by the identity of the predominant organism. Below is one example, the genus classification from the first replica. The urotype indicates the clades that fall into the same urotype. Each urotype is named for the predominant genus. If no one genus is predominant, then the urotype is considered non-predominant. All corresponding graphs for each replica and each taxonomic level can be found in supplementary Figure S1.

Genus Identification from the first sequencing replica set

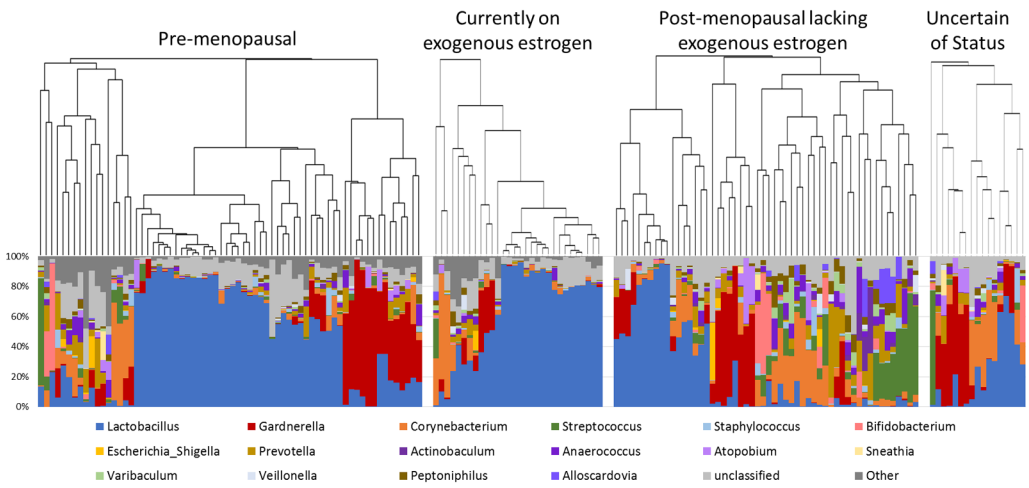

Figure 2. Phylogenetic diversity and urotype distribution between estrogen status

Relative abundance of the microbial community at the genus level for each of the 4 estrogen groups. Each bar is a separate individual with the percent of total classified reads to the genus level represented on the y-axis. Phylogenetic relatedness as measured by Bray-Curtis dissimilarity is depicted in the dendrograms above each group.

The full cohort is separated by hormone status: pre-menopausal, post-menopausal (with or without self-reported, current exogenous hormones) or uncertain about hormonal status. Estrogen positive groups (pre-menopausal and those currently on exogenous estrogen) have a greater prevalence of Lactobacillus-predominant individuals (blue) than the estrogen negative group. The estrogen negative group has a greater number of non-predominant (multi-colored) profiles compared to the estrogen positive populations.

Table 1

Baseline Clinical and Demographic Characteristics of VALUE Participants Assessed for Urinary Microbiota

Demographics	**N=197**
Age [Mean (SD)]	51 (9.7)
Body Mass Index (kg/m^2) [Mean (SD)]	29 (5)
Race/Ethnicity *	
Hispanic	23 (12%)
Non-Hispanic Caucasian	156 (79%)
African-American	9(5%)
Other	9(5%)
Education	
Less High School	6 (3%)
High School/GED	39 (20%)
Some College	58 (29%)
Completed 4 Years of College	53 (27%)
Graduate/Professional Degrees	41(21%)
Self Reported Hormonal Status *	
Pre-Menopausal	82 (42%)
Post-Menopausal lacking exogenous hormones	61 (31%)
Post-Menopausal or Uncertain about status on Exogenous hormones	35 (18%)
Uncertain about status lackingexogenous hormones	19 (10%)
Ever Pregnant	190 (96%)
Number of Pregnancies, Mdn(Range)	3 (0–10)
Vaginal Parity, Mdn (Range)	2 (0–7)
History of Smoking	66 (33%)
Currently Smoking	20 (10%)
Currently Married	145 (74%)
Prior Pelvic Surgeries	151 (77%)
Prior Non-surgical Treatment	122 (62%)
Symptom Severity	
MESA score [a]	
Stress Index, Mdn(IQR)	78(59–89)
Urge Index, Mdn(IQR)	33 (17–50)
Frequency of Urine Leakage	
Less than once a month	0
A few times a month	11 (6%)
A few times a week	36 (18%)
Every day and/or night	150 (76%)
Voiding Phase Dysfunction	7 (4%)

Demographics	**N=197**
Suspected Intrinsic Sphincter Deficiency	39 (20%)
Urine Measures	**N=167**
Specific Gravity, Mdn(IQR)	1 (1.01–1.02)
Urine pH, Mdn(IQR)	6 (5–7)
Glucose positive n (%)	6 (4%)
Blood	
Negative	110 (70%)
Trace (Non–hemolyzed)	11 (7%)
Moderate (Non-hemolyzed)	5 (3%)
Trace	11 (7%)
Small (+)	6 (4%)
Moderate (++)	6 (4%)
Large (+++)	9 (6%)
Protein	25 (16%)
DIVERSITY OUTCOME MEASURES[Mean (SD)][b]	
Shannon	1.86 (0.97)
Chao	124.08 (59.35)
Pielou	0.43 (0.19)

Total number of SUI participants is 197.

Mean (SD) or N(%) reported unless otherwise specified.

Mdn=Median; SD=Standard deviation; IQR = Interquartile Range;

[a]Stress Index and Urge Indices were calculated using the MESA questionnaire.

[*]Race/Ethnicity and Hormonal status per subject report

[b]Least Squares Means- adjusted for correlation between both replicas

Table 2

Urotype frequency across all taxonomic levels and between replicas

Using Bray-Curtis dissimilarity, each dataset was divided into clades (row). A urotype is defined as a clade that is predominated by a single organism, or labeled as “non-predominant” when no single organism predominates. Here, the frequency of urotype identification is shown for each taxonomic classification for both replicas. Urine is a low biomass niche and some samples had so little DNA that the signal could not be distinguished from background, these samples are labeled as “below detection level”.

A. Replica 1: Frequency of Sequencing Urotype

Phylum		Class		Order		Family		Genus	
Below Detection Level	14% (28/197)	Below Detection Level	14% (28/197)	Below Detection Level	14% (28/197)	Below Detection Level	14% (28/197)	Below Detection Level	14% (28/197)
				Non-predominant	4.6% (9/197)	Non-predominant	11% (22/197)	Non- predominant	5.1% (10/197)
Firmicutes	41% (80/197)	Bacilli	43% (84/197)	Lactobacillales	48% (94/197)	Lactobacillaceae	35% (68/197)	Lactobacillus	46% (90/197)
Firmicutes/Actinobacteria	15% (29/197)	Bacilli/Mixed	4.1% (8/197)			Streptococcaceae	2.0% (4/197)	Streptococcus	4.6% (9/197)
Actinobacteria	6.6% (13/197)	Actinobacteria	23% (46/197)	Bifidobacteriales	15% (30/197)	Bifidobacteriaceae	24% (48/197)	Bifidobacterium	3.6% (7/197)
Actinobacteria/Mixed	18% (35/197)	Actinobacteria/Mixed	6.1% (12/197)					Gardnerella	14% (28/197)
				Actinomycetales	10% (20/197)	Corynebacteriaceae	9.1% (18/197)	Corynebacterium	10% (19/197)
								Atopobium	0.5% (1/197)
Bacteroidetes	3.0% (6/197)	Bacteroidetes	2.0% (4/197)	Bacteroidales		Prevotellaceae	2.0% (4/197)	Prevotella	2.5% (5/197)
Proteobacteria	1.0% (2/197)	Gammaproteobacteria	1% (2/197)	Enterobacteriales	0.5% (1/197)	Enterobacteriacea	2.5% (5/197)		
Firmicutes		Clostridia	6.1% (12/197)	Clostridia	7.6% (15/197)				
Fusobacterium	2.0% (4/197)								

B. Replica 2: Frequency of Sequencing Urotype

Phylum		Class		Order		Family		Genus	
Below Detection Level	14% (27/197)	Below Detection Level	14% (27/197)	Below Detection Level	14% (27/197)	Below Detection Level	14% (27/197)	Below Detection Level	13% (27/197)
				Non-predominant	12% (24/197)	Non-predominant	12% (23/197)	Non- predominant	12% (23/197)
Firmicutes	53% (104/197)	Bacilli	39% (77/197)	Lactobacillales	44% (87/197)	Lactobacillaceae	38% (74/197)	Lactobacillus	37% (72/197)
Firmicutes/Actinobacteria	17% (34/197)	Bacilli-Mixed	8.1% (16/197)			Streptococcaceae	12% (23/197)	Streptococcus	2.5% (5/197)

B. Replica 2: Frequency of Sequencing Urotype

Phylum		Class		Order		Family		Genus	
						Clostridiales	7.1% (14/197)		
Actinobacteria	13% (25/197)	Actinobacteria	24% (48/197)	Bifidobacteriales	16% (31/197)	Bifidobacteriaceae	23% (45/197)	Bifidobacterium	2.0% (4/197)
Actinobacteria/Mixed		Actinobacteria/Mixed	13% (26/197)					Gardnerella	18% (35/197)
				Actinomycetales	14% (28/197)	Corynebacteriaceae	4.1% (8/197)	Corynebacterium	7.1% (14/197)
Bacteroidetes	2.0% (4/197)					Prevotellaceae	2.5% (5/197)	Prevotella	1.5% (3/197)
Proteobacteria	1.5% (3/197)	Gammaproteobacteria	0.5% (1/197)			Enterobacteriacea	0.5% (1/197)		
		Betaproteobacteria	1.0% (2/197)						

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Table 3

Association between Microbiota Characteristics and Demographic

Factors or Symptom Severity adjusted for Genus urotype Generalized Estimating Equations (GEE), assuming a gamma distribution and log link, were used to assess the association between each demographic or symptom measurement for three separate microbial diversity measurements after adjusting for urotype at the genus level. P-values are not adjusted for type I error. Diversity measurements include Shannon diversity (richness and evenness), Chao diversity (richness only) and Peilou (evenness only).

	Community Diversity Gamma GEE Models Adjusted for Genus Urotype								
	Shannon			**Chao1**			**Peilou**		
Demographics	**Beta**	**SE**	**p-value**	**Beta**	**SE**	**p-value**	**Beta**	**SE**	**p-value**
BMI	0.01	0.006	**0.02**	0.006	0.005	0.18	0.01	0.005	**0.02**
Race/Ethnicity *			0.53			0.25			0.52
Hispanic	0.07	0.11	0.55	0.05	0.08	0.58	0.04	0.09	0.66
Non-Hispanic Caucasian	Ref								
African-American	−0.15	0.22	0.51	0.08	0.09	0.36	−0.17	0.20	0.40
Other	−0.30	0.30	0.27	−0.28	0.15	0.05	−0.25	0.23	0.28
Education			0.51			0.31			0.59
Less High School	0.039	0.19	0.84	0.18	0.08	0.02	−0.01	0.16	0.96
High School/GED	−0.03	0.11	0.77	0.11	0.09	0.23	−0.03	0.09	0.70
Some College	Ref								
Completed 4 Years of College	−0.12	0.09	0.18	0.003	0.07	0.97	−0.10	0.08	0.21
Graduate/Professional Degrees	−0.16	0.11	0.15	0.07	0.08	0.34	−0.14	0.10	0.17
Hormonal Status *			**<0.001**			0.32			**<0.001**
Pre-Menopausal	Ref								
Post-Menopausal lacking exogenous hormones	0.23	0.08	**0.004**	0.08	0.07	0.25	0.21	0.07	**0.02**
Post-menopausal or Uncertain about exogenous hormonal status	−0.28	0.14	0.05	−0.07	0.08	0.39	−0.26	0.12	**0.04**
Uncertain about exogenous hormonal status	0.21	0.09	**0.02**	−0.03	0.10	0.73	0.22	0.07	0.003
Ever Pregnant	−0.21	0.15	0.16	−0.04	0.10	0.73	−0.18	0.12	0.22
Smoking History	−0.16	0.07	0.04	−0.07	0.06	0.21	−0.13	0.06	0.06
Currently Married	0.10	0.08	0.21	−0.04	0.06	0.54	0.11	0.07	0.11
Prior Pelvic Surgeries	−0.14	0.07	0.08	−0.02	0.06	0.72	−0.13	0.07	0.07

	Community Diversity Gamma GEE Models Adjusted for Genus Urotype								
	Shannon			Chao1			Peilou		
Demographics	**Beta**	**SE**	**p-value**	**Beta**	**SE**	**p-value**	**Beta**	**SE**	**p-value**
Prior Non-surgical Treatment	0.03	0.08	0.66	−0.02	0.06	0.73	0.04	0.07	0.54
Symptom Severity									
MESA score[a]									
Stress Index	0.002	0.002	0.28	0.0003	0.002	0.87	0.002	0.002	0.31
Urge Index	0.003	0.002	**0.04**	0.002	0.001	0.18	0.003	0.001	**0.04**
Frequency of Leakage			0.08			0.61			0.04
A few times a month	−0.38	0.24	0.11	−0.05	0.09	0.53	−0.37	0.21	0.07
A few times a week	−0.15	0.10	0.12	0.05	0.07	0.30	−0.15	0.08	0.06
Every day and/or night	Ref								
Urine Characteristics									
Protein	0.18	0.10	0.09	0.08	0.08	0.38	0.14	0.08	0.09
Specific Gravity	−0.18	0.01	0.32	−0.10	0.01	0.31	−0.14	0.008	0.32
Urine pH	−0.06	0.04	0.11	−0.07	0.03	**0.03**	−0.05	0.04	0.13
Glucose	−0.07	0.19	0.70	−0.13	0.11	0.25	−0.03	0.15	0.82
Blood			0.40			0.69			0.29
Negative	Ref								
Trace (Non-hemolyzed)	−0.08	0.16	0.60	−0.06	0.14	0.66	−0.06	0.14	0.69
Moderate (Non-hemolyzed)	−0.001	0.25	0.10	0.0853	0.13	0.50	0.008	0.24	0.97
Trace	0.18	0.11	0.09	0.16	0.10	0.10	0.18	0.10	0.08
Small (+)	0.21	0.13	0.11	−0.10	0.12	0.43	0.23	0.09	0.01
Moderate (++)	0.39	0.18	0.03	0.13	0.15	0.39	0.32	0.14	0.03
Large (+++)	0.10	0.13	0.45	0.01	0.07	0.84	0.10	0.11	0.38

[a] MESA Questionnaire - SUI and UUI index scores each ranging from 0–100 with higher scores indicating greater symptom severity)

[*] Race/Ethnicity and Hormonal status per subject report