The healthy urinary microbiome in asymptomatic participants in the MAPP Network Study: Relation to gender, age, and menopausal status

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Abstract

Introduction: To understand the role of the urinary microbiome in disease states and interpret non-culture-based diagnostic urine testing of midstream urine specimens, we must have a better understanding of the urinary microbiome in asymptomatic healthy individuals. We examined the impact of gender, age, and menopausal status on the healthy human urinary microbiome in asymptomatic control subjects enrolled in the multi-institution National Institute of Diabetes and Digestive and Kidney Diseases

Key Messages

- A better understanding of the normal healthy urinary microbiome will allow improved interpretation of non-culture bacterial identification (polymerase chain reaction, next generation sequencing) in patients with complex or complicated urinary symptoms.
- The urinary microbiome of healthy asymptomatic subjects differed between genders
- The genera Bifidobacterium, Staphylococcus, Lactobacillus, and Corynebacterium were more predominant in females, while for males the most prevalent organisms included only Propionibacterium.
- For diagnostic and prognostic testing, non-invasive urine sampling by midstream urine specimen sampling provides knowledge and characterization of the healthy urinary microbiome in typical clinical specimens is essential to be able to develop useful testing protocols and parameters in clinical practice.
(NIDDK) Multidisciplinary Approach to the Study of Chronic Pelvic Pain Network (MAPP) study.

**Methods:** Asymptomatic healthy controls, recruited to be age- and sex-matched to patients in the Trans-MAPP Epidemiology and Phenotyping Study, provided midstream urine collection for polymerase chain reaction (PCR)-electrospray ionization mass spectrometry identification of urinary microbiota. The microbiomes of male and female participants were described and analyzed for differences in composition and diversity at the species and genus level by sex, age, and, in females, by menopausal status.

**Results:** Sixty-six total species were detected with a mean of 1.2 species (standard deviation [SD] 1.1) per male (n=97; mean age=43) and 2.3 (SD 1.3) per female (n=110, mean age=38) in asymptomatic healthy controls. Species and genera diversity analyses showed significantly greater richness and diversity in females. With regard to species, *Bifidobacterium subtile*, *Lactobacillus crispatus*, and *Lactobacillus johnsonii* were more predominant in females. The genera Bifidobacterium, Staphylococcus, Lactobacillus, and Corynebacterium were more predominant in females, while for males the most prevalent organisms included Staphylococcus and Propionibacterium; only Propionibacterium approached a significant difference between genders. No significant difference in the presence and/or diversity of micro-organisms with menopausal status could be observed. Sex-specific age trends, particularly diversity, were larger for females than males.

**Conclusions:** These results suggest the urinary microbiome of healthy asymptomatic subjects differed between genders and age in females, but not menopausal status. Gender differences may be attributable to the detection of urethral/vaginal organisms in females and prostate organisms in males. These findings will better allow us to interpret the results of microbiome reports in the midstream urine specimens of patients with urinary symptoms.

**Introduction**

Outside the setting of a urinary tract infection (UTI), the urinary tract was historically believed to be a sterile environment. This traditional dogma was based on standard microbial assessment consisting of gram staining and culturing urine in standardized media. Both of these microbiological evaluations are over a century old and still employed in modern microbiology laboratories. This belief persisted until only a decade or so ago, to the point that the Microbiome Project neglected to include the urinary tract in its strategic plans. With newer microbial evaluation technology, specifically non-culture techniques, this has been proven not to be the case; in fact, the genitourinary tract is not sterile.¹

A recent search of the published world literature on bacteria described in the urinary tract by culture and metagenomic techniques found evidence of 562 bacterial species.² In fact, the
human urinary repertoire of bacteria reported included 225 (40.0%) species described as causative agents of UTI. The majority of studies published during the last decade examined the urinary microbiome in the context of specific urological symptoms (interstitial cystitis, male and female lower urinary tract symptom, and chronic prostatitis). With regard to the microbiome in these disease states, no single micro-organism has been found to be responsible for incontinence, interstitial cystitis, chronic prostatitis/chronic pelvic pain syndrome or any other studied conditions. However, there are strong signals that an altered microbiome may be implicated. Some bacteria appear to be more predominant in certain urinary disease states, while in others, global shifts in bacterial communities (urotypes) may be implicated.

Learning from the models of the microbiome at other body surfaces, the urinary microbiome is hypothesized to play a crucial role in keeping the human host and their urinary tract healthy. To truly understand the role of the urinary microbiome in disease states, we must have a better understanding of the microbiome in healthy subjects with no urologic disease. Recent studies performed primarily in women have revealed complex and diverse bacterial populations, but with some genera/species consistently appearing dominant (for example, Lactobacilli in pre-menopausal women). In this study, we used culture-independent molecular bacterial detection to examine the midstream urine results from the control cohort of healthy asymptomatic subjects enrolled in the multi-institution NIDDK Multidisciplinary Approach to the Study of Chronic Pelvic Pain (MAPP) study for which the primary objective was to describe the microbiome of patients with interstitial cystitis/bladder pain syndrome and chronic prostatitis/chronic pelvic pain syndrome. Our study further examined the role of gender, age, and menopausal status on the healthy human urinary microbiome.

Methods

Participants and specimens
The trans-MAPP Research Network’s initial central clinical study, the Trans-MAPP epidemiology/phenotyping (EP) Study, recruited participants with IC/BPS (primarily females) and CP/CPPS (males) as well as asymptomatic control subjects, for comprehensive baseline phenotyping with standardized data acquisition and analysis and biological sample collection across network sites (all received IRB/REB approval). Asymptomatic healthy controls were recruited to be age- and sex-matched to patients. To ensure a clearly defined healthy control subgroup, potential control participants were excluded if they reported any pain in the pelvic or bladder region or chronic pain in more than one non-urologic body region. For females, data on self-reported menopausal status was collected. Further details of the study design, including descriptions of the study population, enrollment criteria and disease-specific questionnaires, are available. The current study details the analysis of midstream urine specimens collected at the in-clinic baseline visit from male and female asymptomatic, healthy control subjects.
Subjects with a positive urine culture (i.e., traditional uropathogen(s) detected in midstream collected urine employing traditional culture technique) were excluded from the analysis. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

**Specimen handling**
Following in-clinic collection of urine specimens using standardized collection kits at 6 of the MAPP Network Discovery sites, specimens were transferred to 50 mL conical tubes and immediately frozen at −80 °C, then shipped to the central MAPP network tissue analysis and technology core (TATC). The specimens were then thawed, thoroughly mixed, and aliquoted into 1 and 3 mL aliquots and refrozen at −80 °C until use. Three milliliter frozen aliquots were transferred to the Center for Genomic Sciences at Drexel University College of Medicine in Philadelphia, PA, for microbial analyses.

**DNA extraction and Ibis eubacterial and fungal domain assays on the PLEX-ID**
The Plex-ID molecular diagnostic platform and infectious agent diagnostic kits (Abbott Molecular, Des Plaines, IL, USA) were employed to identify bacterial genera and species in the specimens. In brief, total DNA was extracted from all urine samples, and microbial (i.e., bacterial and fungal) DNAs were amplified by polymerase chain reaction (PCR) using the 16 primer pair BAC (Bacteria, Antibiotic resistance genes and Candida) detection systems developed by Ibis as described. The individual amplicons were “weighed” using the PLEX-ID instrumentation by electrospray ionization—time-of-flight—mass spectrometry (ESI-TOF-MS) which reports out the molecular mass. The amplicon masses were then used to determine their exact base compositions, as a particular mass can only be produced by a single combination of the four nucleotides (A, C, G, T). The taxonomic identities of the amplicons were then revealed using a database containing base composition data on virtually all bacterial/fungal species sequenced to date. Comparison of this technique with other non-culture methods and details of the methodology are beyond the scope of this paper and have been reported elsewhere (details previously described).

**Statistical analysis**
The microbiomes of male and female participants were described and analyzed at the species and genus level. Wilcoxon’s rank sum test was used to evaluate differences in diversity measures (Chao1 and Shannon’s index) at the species and genus level by sex and, in females, by menopausal status. The association of continuous age with microbial diversity (Chao1) was assessed overall and separately by sex using negative binomial regression to account for overdispersion. The association of Shannon’s index with continuous age was assessed by the
two-part gamma model, where a logistic regression was used to model the probability that Shannon’s Index was greater than 0, and a gamma model was used to assess age trends among individuals with Shannon>0. Differences in the representation of individual taxa were tested using logistic regression for presence or absence and Wilcoxon’s rank-sum test for relative abundance. Differences in the overall microbial composition between groups were assessed by permutational multivariate analysis (PERMANOVA). This procedure is a nonparametric analogue of multivariate ANOVA that uses resampling for inference. The abundance of particular taxa for each subject is converted into a numerical matrix from which distance matrices are calculated and compared between groups according to a selected distance measure. The Bray–Curtis and Jaccard distances were chosen as the basis of this analysis, using samples that contained at least one detected species. In the univariate analyses comparing one species at a time between participant groups, we restricted comparisons to species (or genera) present in 10 or more participants to reduce the likelihood of underpowered analyses. Tests of individual taxa were adjusted for multiple comparisons by controlling the false discovery rate (FDR). To identify regional variations in urinary microbial communities, we used the Multivariate Association with Linear Models (MaAsLin) method to discover potential associations between location of sample collection and microbial abundance, controlling for confounding variables of age, gender, menopausal status, income, education, or ethnicity. The FDR was controlled by Benjamini–Hochberg procedure. The multivariate association analysis was conducted with minimum prevalence of 0.05, minimum abundance of 0.2%, and FDR-corrected p-value cutoff of 0.10.

Results
The healthy asymptomatic controls enrolled in the MAPP study who met study criteria and had microbiome data available included 110 females (mean age = 38; range 19-67) and 97 males (mean age =43; range 19-83). No bacterial species were identified in 30.9% (30/97) of male participants and 8.1% (9/110) of female participants. The total number of species detected was 66 with a mean number of species of 1.2 (sd 1.1) per male and 2.3 (sd 1.3) per female. The overlaps of detected species and genera are shown in the Venn diagrams (Figure 1). The taxa differences (present/absent) between male and female participants are shown in Table 1 (1a species; 1b genera and Figure 2 by species). Regarding species, Bifidobacterium subtile, Lactobacillus crispatus, and Lactobacillus johnsonii were more predominant in females. The genera Bifidobacterium, Staphylococcus, Lactobacillus, and Corynebacterium were more predominant in females. While for males the most prevalent organisms included Staphylococcus and Propionibacterium, only Propionibacterium approached a significant difference between genders. Species and genera diversity analyses by both Chao1 and Shannon’s index showed significantly greater richness and diversity in females (Figure 3).
Combining male and female participants at the species level, age was not associated with richness or the likelihood of a Shannon’s index value of 0 in the overall sample (p=0.26). For subjects with a Shannon’s index value >0, however, increasing age was associated with greater diversity (p=0.047). Sex-specific age trends were larger for females than males but did not reach statistical significance (Figure 4a and 4b). Results were largely similar at the genus level; however, the association between age and Shannon’s index reached statistical significance (p=0.043). At the genus level, older age was associated with increased Shannon’s Index in females (p=0.04 among those with Shannon > 0). Multivariate analysis revealed significant variation in overall composition by age in females (p=0.015 Bray-Curtis, p=0.002 Jaccard species) at the species level, but evidence was not as strong at the genus level (p=0.275 Jaccard, p=0.04 Bray-Curtis).

Comparing female participants by reported menopausal (pre- vs post-) status, there was no significant difference in the observed microbiome in terms of presence and/or diversity of micro-organisms at either the species (p=0.36 chao1, p=0.47 Shannon’s Index) or genus levels (p=0.39 chao1, p=0.46 Shannon’s Index) (Figure 5). A further species-level comparison of age trends (by decade) for male versus female cohorts showed no significant differences in diversity (both Chao1 and Shannon’s Indices).

As an adjunctive analysis, we also sought to understand if there were regional variations in urinary microbiota. Controlling for confounding variables of age, gender, and menopausal status, no significant differences in microbial composition could be seen for subjects recruited at different locations across the U.S. Even when income, education, and ethnicity were included as additional confounders, no regional differences in urinary microbiota could be identified.

**Discussion**
Although there is a paucity of studies of the urinary microbiome in the healthy asymptomatic human, we hypothesized that we would observe differences based on gender, age, and, for females, menopausal status. We did in fact observe a significant difference in both the species detected and diversity between males and females with *Bifidobacterium subtilis* and two *Lactobacilli* species (*Lactobacillus crispatus, Lactobacillus johnsonii*) more predominant in females and *Propionibacterium sp.* showing a marginally increased prevalence in males. Female midstream urine specimens showed significantly greater richness and diversity than in males. Surprisingly, while sex-specific age trends were larger for females than males, they did not reach statistical significance at the species level, only differing significantly by Shannon’s Index analysis at the genus level. We were also not able to confirm our hypothesis that menstrual status significantly impacted the microbiome in healthy women. Although there was no significant difference based on menstrual status in the observed microbiome in terms of presence and/or diversity of micro-organisms at either the species or genus levels, we did not capture hormonal use in the post-menopausal group.
The development of culture independent technologies has allowed us to explore the nature of the human urinary microbiome beyond simple culture-based approaches, but it can be difficult to keep up with the rapid evolution of these molecular techniques. The technology used in this study employed PCR-electrospray ionization mass spectrometry (PCR-ESI-MS), a molecular technique employed in other NIH MAPP studies\textsuperscript{3,8,18} to characterize microbial communities in urine specimens. This technique involves mass spectrometry of PCR amplicons such that the composition of nucleotides is deduced and compared against a database.\textsuperscript{17} While this technique appears to perform comparatively to 16S rRNA gene sequencing (but with shorter workflow times), it has not been widely implemented in the microbiome field. While our study may be limited by the technology employed, PCR-ESI-MS does have the ability to accurately identify microbiota\textsuperscript{17} and our interpretation of the results is simple, easily understood, and consistent with recently available, but much smaller, studies.

So how does this evaluation compare to other studies in the literature? One of the first studies to examine the healthy urinary microbiome using non-culture was published in 2013 and included 16 subjects (6 healthy males; 10 healthy females).\textsuperscript{21} The investigators concluded that the healthy human urinary microbiome included a heterogenous mix of microbial species and genera with females showing a slightly wider range of genera and greater diversity.\textsuperscript{21} A comprehensive review of studies\textsuperscript{9} evaluating the urinary tract microbiome in health and disease employing state of the art technology typically included control subjects without urinary disease. Most of these studies enrolled primarily females and most evaluated midstream urine specimens. The midstream urine of the healthy control subjects has been shown to contain various combinations of urinary and genital tract micro-organisms without a general concordance of observations (except for Lactobacilli in the urine of healthy female subjects).

Our findings are very similar to those from a recent study performed at the Helmholtz Centre for Infection Research in Braunschweig, Germany, in which midstream urine samples were collected from healthy employees as part of their routine examination at work, namely 31 healthy men (median age 29 years) and 49 healthy women (median age 29).\textsuperscript{22} One urotype dominated in relative abundance by L. crispatus was present in healthy women; no urotype characteristic of the male urinary microbiota was found, with the male group looking like the female subjects. Our larger study documented and confirmed many of these previous findings with respect to differences between species, genera, and diversity between healthy male and female subjects. In respect to the female urobiome, Curtiss et al.\textsuperscript{11} recently showed that few significant differences in the microbiota of midstream urine related to age could be identified except for Lactobacillus being more common in pre-menopausal women and Mobiluncus more common in post-menopausal women. In contrast, Price et al.\textsuperscript{4} noted that Lactobacillus was the most common organism in catheterized urine specimens for all ages of women while Gardnerella and Escherichia were more common in younger and older women, respectively. We similarly showed that Lactobacillus was the most common organism, and we further showed that
evaluating age as a continuous variable allowed us to confirm that there were microbiome changes related to age in females. However, we were not able to correlate that to menopausal status.

This MAPP study was not powered to show differences in the microbiome of healthy control subjects, but rather phenotypic differences between healthy control subjects and patients diagnosed with urologic chronic pelvic pain syndromes. Another consideration that must be taken into account, was that, as per MAPP protocol, only midstream clean catch urine specimens were available for evaluation. Recent reports indicate that the bladder microbiota of urine collected by catheter differs from that collected by midstream collection in both men and women. Midstream urine specimens represent a survey of the entire genitourinary microbiome which includes the bladder (perhaps even the kidney), urethra, the prostate and prepuce in men and the vagina/introitus in women. Further differences in the microbiome associated with the urethra and periurethral areas complicate the picture even more. One could argue however that catheter samples provide a very narrow window into the bladder lumen and only address hypotheses of bladder-lumen-specific influences while this purposeful, broad-based survey may provide a more comprehensive assessment of possible differences in the genitourinary microbiota. We could postulate that many of the gender differences we showed may be related to organisms residing in the female urethra and/or vagina, whereas male midstream urine would possibly show different organisms contributed by the prostate. This contamination might confound our findings, and the microbial composition of the two genders might be closer than seen here, similar to the findings of the previously described Gottschick study. We also excluded subjects with positive urine cultures for accepted uropathogenic organisms, so we cannot comment on the impact of asymptomatic bacteriuria.

While alternative collection methods (catheterization, bladder aspiration) would provide a more accurate representation of the bladder-specific microbiome, urinary microbial analysis can have multiple goals. While a detailed examination of the bladder-resident microbiome might be helpful in examining the role of bacteria in bladder physiology or confusing clinical presentations, for clinical purposes, primarily diagnostic and prognostic testing, non-invasive urine sampling is highly preferred. Knowledge and characterization of the healthy urinary microbiome in typical clinical specimens from males and females is essential to be able to develop clinically useful testing protocols and parameters. Bladder aspiration, and even catheterization, for urine sampling is unlikely to gain traction in the real-world clinical setting, except for complicated and/or confusing cases. We believe our findings will help further the ability to develop and interpret molecular diagnostics reports evaluating urinary bacteria collected as midstream specimens from patients with urinary symptoms.

**Conclusions**
The urinary microbiome of healthy asymptomatic subjects was found to differ between genders and differ by age in females but not menopausal status. The differences in gender might be attributable to the detection of urethral/vaginal organisms in females and prostate organisms in males. The urinary microbiome of healthy individuals may be more similar than different and this has important ramifications when interpreting the results of microbiome reports in the midstream urine specimens of patients with urinary symptoms.
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Figures and Tables

**Figure 1.** Overlap between species and genera between genders. Venn diagrams accompany the list of all taxa detected in males (yellow) or females (blue) alone or both genders (green) by (A) species and (B) genus.

![Venn diagram showing overlap between species and genera between genders.](image-url)
**Figure 2.** Heatmap of species by abundance for male vs. female healthy controls. More abundant species are indicated according to the color key at the upper left.
**Figure 3.** Diversity analysis by species using Chao1 and Shannon’s indices. The distributions of (A) Chao1 and (B) Shannon’s indices for healthy control males (blue) were lower than the Chao1 (C) and Shannon’s (D) indices seen for healthy control females (gold).
Figure 4. Sex-specific trends in diversity measures. (A) Chao1 and (B) Shannon’s indices diversity measures change differently with age in male (blue) and female (gold) healthy control subjects.
Figure 5. Heatmap of species abundance by menopausal status. More abundant species are indicated according to the color key at the upper left.
Table 1A. Species prevalence in asymptomatic male and female midstream urine specimens

| Species                        | Males_nt | Males_N (%) | Females_nt | Females_N (%) | p.pres  | p.wilc  |
|--------------------------------|----------|-------------|------------|---------------|---------|---------|
| Bifidobacterium subtile*       | 97       | 6 (6.2%)    | 110        | 20 (18.2%)    | 0.0129  | 0.0075  |
| Burkholderia cenocepacia       | 97       | 3 (3.1%)    | 110        | 7 (6.4%)      | 0.2834  | 0.2945  |
| Finegoldia magna               | 97       | 3 (3.1%)    | 110        | 7 (6.4%)      | 0.2834  | 0.2613  |
| Lactobacillus acidophilus      | 97       | 4 (4.1%)    | 110        | 13 (11.8%)    | 0.054   | 0.0377  |
| Lactobacillus crispatus*       | 97       | 1 (1%)      | 110        | 32 (29.1%)    | 0.0003  | 0       |
| Lactobacillus johnsonii*       | 97       | 0 (0%)      | 110        | 27 (24.5%)    | 0.9865  | 0       |
| Propionibacterium acnes        | 97       | 13 (13.4%)  | 110        | 6 (5.5%)      | 0.0553  | 0.0517  |
| Staphylococcus epidermidis/haemolyticus | 97 | 24 (24.7%)  | 110        | 37 (33.6%)    | 0.1626  | 0.1867  |
| Staphylococcus haemolyticus    | 97       | 5 (5.2%)    | 110        | 7 (6.4%)      | 0.7108  | 0.6793  |
| Staphylococcus hominis         | 97       | 9 (9.3%)    | 110        | 10 (9.1%)     | 0.9628  | 0.9076  |

Table 1B. Genera prevalence in asymptomatic male and female midstream urine specimens

| Genera                        | Males_nt | Males_N (%) | Females_nt | Females_N (%) | p.pres  | p.wilc  |
|-------------------------------|----------|-------------|------------|---------------|---------|---------|
| Bifidobacterium               | 97       | 7 (7.2%)    | 110        | 21 (19.1%)    | 0.0161  | 0.009   |
| Burkholderia                  | 97       | 3 (3.1%)    | 110        | 7 (6.4%)      | 0.2834  | 0.2945  |
| Corynebacterium               | 97       | 0 (0%)      | 110        | 14 (12.7%)    | 0.9917  | 3.00E-04 |
| Finegoldia                    | 97       | 3 (3.1%)    | 110        | 7 (6.4%)      | 0.2834  | 0.2613  |
| Lactobacillus                 | 97       | 7 (7.2%)    | 110        | 61 (55.5%)    | 0       | 0       |
| Propionibacterium             | 97       | 13 (13.4%)  | 110        | 6 (5.5%)      | 0.0553  | 0.0517  |
| Staphylococcus                | 97       | 40 (41.2%)  | 110        | 62 (56.4%)    | 0.0305  | 0.0479  |
| Streptococcus                 | 97       | 8 (8.2%)    | 110        | 12 (10.9%)    | 0.519   | 0.4528  |