Author's Accepted Manuscript

Redefining Healthy Urine: A Cross-Sectional Exploratory Metagenomic Study of People With and Without Bladder Dysfunction

Suzanne L. Groah , Marcos Pérez-Losada , Ljubica Caldovic , Inger H. Ljungberg , Bruce M. Sprague , Eduardo Castro-Nallar , Neel J. Chandel , Michael H. Hsieh , Hans G. Pohl

PII: S0022-5347(16)00127-0
DOI: 10.1016/j.juro.2016.01.088
Reference: JURO 13278

To appear in: *The Journal of Urology*
Accepted Date: 8 January 2016

Please cite this article as: Groah SL, Pérez-Losada M, Caldovic L, Ljungberg IH, Sprague BM, Castro-Nallar E, Chandel NJ, Hsieh MH, Pohl HG, Redefining Healthy Urine: A Cross-Sectional Exploratory Metagenomic Study of People With and Without Bladder Dysfunction, *The Journal of Urology*® (2016), doi: 10.1016/j.juro.2016.01.088.

DISCLAIMER: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our subscribers we are providing this early version of the article. The paper will be copy edited and typeset, and proof will be reviewed before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to The Journal pertain.

Embargo Policy

All article content is under embargo until uncorrected proof of the article becomes available online.

We will provide journalists and editors with full-text copies of the articles in question prior to the embargo date so that stories can be adequately researched and written. The standard embargo time is 12:01 AM ET on that date. Questions regarding embargo should be directed to jumedia@elsevier.com.

ACCEPTED MANUSCRIPT

TITLE: Redefining Healthy Urine: A Cross-Sectional Exploratory Metagenomic Study of People With and Without Bladder Dysfunction

RUNNING TITLE: Metagenomics Redefines Healthy Urine

By:

Suzanne L. Groah, MD, MSPH[1,2*]

Marcos Pérez-Losada, PhD*[3,4,5]

Ljubica Caldovic, PhD[4]

Inger H. Ljungberg, MPH[1]

Bruce M. Sprague, BS[6]

Eduardo Castro-Nallar[3, 7]

Neel J. Chandel, MD[1]

Michael H. Hsieh, MD, PhD[6]

Hans G. Pohl, MD[6]

From:

[1]MedStar National Rehabilitation Hospital, 102 Irving St., NW, Washington, DC, 20010;

[2]Georgetown University Hospital, Department of Rehabilitation Medicine, Washington, DC;

[3]Computational Biology Institute, The George Washington University, Ashburn, VA;

ACCEPTED MANUSCRIPT

[4]Department of Integrative Systems Biology, Children's National Health System, Washington, DC;

[5]CIBIO-InBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Universidade do Porto, 4485-661 Vairão, Portugal;

[6]Division of Urology, Children's National Health System, Washington, DC;

[7]Universidad Andres Bello, Center for Bioinformatics and Integrative Biology, Facultad de Ciencias Biológicas, Av. República 239, Santiago, Chile 8370146

*Co-1st Authors

ABSTRACT WORD COUNT: 256 (including headers)

TEXT WORD COUNT: 2,992

KEYWORDS: Neuropathic bladder, microbiome, metagenomics

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

FOOTNOTE PAGE

CONFLICT OF INTEREST: Dr.'s Groah, Pohl and Caldovic hold a provisional patent related to this data. Dr.'s Groah, Pohl, Caldovic and Hsieh are currently receiving funding by the Patient Centered Outcomes Research Institute (PCORI) to conduct research related to this data. None of the remaining authors have a commercial or other association that might pose a conflict of interest (e.g., pharmaceutical stock ownership, consultancy, advisory board membership, relevant patents, or research funding)

FUNDING: This work was supported by NCMRR/NINDS 2R24HD050846-06 (NCMRR-DC Core for Molecular and Functional Outcome Measures in Rehabilitation Medicine, PI Hoffman)

MEETINGS WHERE THE INFORMATION HAS BEEN PREVIOUSLY PRESENTED: Portions of this data have been presented at the 2013 American Spinal Injury Association Annual Meeting (Chicago, Ill), the 2014 American Spinal Injury Association Annual Meeting (San Antonio, TX), the 2015 combined meeting of the American Spinal Injury Association and the International Spinal Cord Society (Montreal, Quebec) and the 2015 American Urological Association Annual Meeting (New Orleans, LA).

CORRESPONDING AUTHOR CONTACT INFORMATION:

Suzanne L. Groah, MD, MSPH

MedStar National Rehabilitation Hospital

102 Irving St, NW

Washington, DC 20010

Phone: 202.877.1196

Email: Suzanne.L.Groah@Medstar.net

ACCEPTED MANUSCRIPT

ABSTRACT

Purpose: To utilize the PathoScope platform to conduct species-level analyses of publicly available, 16S rRNA pyrosequenced asymptomatic urine data to determine relationships between microbiomes and clinical and functional phenotypes.

Materials and Methods: Reanalysis of previously reported cross-sectionally acquired urine samples from 47 asymptomatic subjects (23 controls and 24 subjects with neuropathic bladder (NB)). Urine was originally collected by the usual method of bladder drainage and analyzed with urinalysis, culture, and pyrosequencing. Urinalysis and culture values were stratified as follows: leukocyte esterase (LE) 0 or ≥1, nitrite (+, -), pyuria <5 or ≥5 white blood cells/high power field (WBC/hpf), cloudy urine (+, -), and bacterial growth <50,000 or ≥50,000 colony forming units (cfu). PathoScope was used for next-generation sequencing alignment, bacterial classification, and characterization of microbial diversity.

Results: NB subjects were significantly more likely to have LE+, pyuria+, cloudy urine and bacterial growth. 23/47 samples had bacterial growth on culture while all samples had bacteria identified by pyrosequencing. The non-NB urine microbiomes had greater proportions of *Lactobacillus crispatus* (females) and *Staphylococcus haemolyticus* (males). The *Lactobacillus* community differed significantly amongst females depending on bladder function. Irrespective of gender, NB subjects had greater proportions of *Enterococcus faecalis*, *Proteus mirabilis*, and *Klebsiella pneumonia*. In 4 NB subjects, *Actinobaculum* was detected by sequencing+PathoScope but not by cultivation, and in all cases was associated with pyuria.

ACCEPTED MANUSCRIPT

Conclusions: Utilizing PathoScope plus 16S pyrosequencing, we were able to identify unique phenotype-dependent species-level microbes. Novel findings included an absence of *Lactobacillus crispatus* in female NB urine, and the presence of Actinobaculum in NB subjects only.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

INTRODUCTION

Explosive growth in microbiome research is driving discoveries in therapeutic microbiology, from our understanding of gastrointestinal and metabolic disorders to the role of probiotics to prevent and treat disease[1]. In contrast, only a handful of *urine* microbiome studies have been performed. This dearth of literature is striking when considering the many infections and infection-induced forms of inflammation that afflict the urinary tract, including prostatitis, urethritis, cystitis, pyelonephritis, and less well-characterized disorders such as painful bladder syndrome. A deeper understanding of how the urine microbiome interacts with the human host will facilitate discoveries likely to improve diagnostics and therapeutics for a number of urologic disorders.

Utilizing 16S rRNA and next generation sequencing (NGS) to identify bacteria at the genus level, our group was one of the first to show that healthy urine is not sterile and that a healthy urine microbiome exists, suggesting that the composition of healthy urine is much more diverse than previously thought.[2] These findings of bladder function, gender and catheterization-dependent microbiomes to the genus level have profound implications for our understanding of bladder health and disease. However, like many microbiome studies using operational taxonomic units (OTUs) and analytical pipelines not originally developed to identify pathogenic species or strains, our prior work was only able to grossly characterize the urine microbiome. Therefore, the role individual bacterial species may play in the maintenance or induction of health and disease was poorly explored due to these technologic and analytic limitations. Since our original analysis, bioinformatics tools have become more sophisticated.

Herein, we use PathoScope as part of a new microbiome analytical pipeline designed to more consistently characterize the urine microbiome at the species level. PathoScope utilizes a Bayesian statistical framework that accommodates information on sequence quality, mapping quality, and provides posterior probabilities of matches to a known database, considering ambiguous read reassignment and the possibility that the sample species is not in the reference database. Moreover, this is more efficiently accomplished, without the need for multiple alignment steps, extensive homology searches, or genome assembly.[3] In other work, we have successfully used PathoScope to generate robust and accurate estimates of bacterial diversity of airway and skin microbiomes in the analysis of PCR-amplified 16S ribosomal DNA.[4,5] We have also *in silico* validated PathoScope[8] using known bacteria for which 16S was sequenced. We found that PathoScope correctly identified 5 of 6 known bacteria with high confidence, with minor ambiguities in discriminating *E. coli* from *Shigella* and *P. aeruginosa* from *P. otitidis*, which have nearly identical 16S sequences.

The purpose of this new study is to utilize PathoScope to conduct species-level microbiome analyses of publicly available, 16S rRNA pyrosequenced asymptomatic urine data to determine relationships between specific microbes, clinical phenotype (defined by urinalysis and urine culture), and functional phenotype.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

MATERIALS AND METHODS

This is a re-analysis of previously reported 16S microbiome data collected cross-sectionally from asymptomatic volunteers with and without bladder dysfunction due to neuropathic bladder (NB). Complete data was available on 47 subjects (23 controls without known bladder dysfunction (non-NB) and 24 subjects with NB due to spinal cord injury (SCI)). Patient populations, sample collection, isolation of DNA from bacteria, and pyrosequencing of 16S rRNA genes have been described previously.[2] Urine was collected by usual method of emptying (clean catch in those who void, directly from an unused catheter in those who use intermittent catheterization, and directly from the catheter in those with suprapubic catheters). Clinical phenotype is described in terms of urinalysis and urine culture results. As no clear evidence-based guidelines exist for "positive" urinalysis and urine culture values, for the purposes of this study values were stratified a priori as follows: leukocyte esterase (LE) 0 or ≥1, nitrite (+ or -), pyuria <5 or ≥5 white blood cells/high power field (WBC/hpf), cloudy urine (+ or -), and urine culture growth <50,000 or ≥50,000 colony forming units (CFU/ml).

For PathoScope analysis, the raw 16S rDNA data were obtained from the NCBI under BioProject ID 97505.[2] Cutadapt[6] and PRINSEQ-lite[7] were used to filter out reads of <220 bp, trim primer sequences, and eliminate low-complexity or poor-quality reads. Potential chimeras were also eliminated using UCHIME.[8] Duplicates were retained for downstream analyses. Microbial diversity was characterized in PathoScope[9,10] by mapping reads against two bacterial 16S rRNA reference datasets, "The All-Species Living Tree" Project LTP115; [11] and a curated version of the Silva 119 Ref NR 99 (all unclassified and marine microbiome sequences purged).[12] Bowtie2[13]

was used to map reads according to the PathoMap module. An average of 7,541 reads per sample aligned to the target libraries.

Statistical analysis. Exploratory analysis and differences in taxon relative abundances or proportions were assessed in R and Bioconductor[14] using packages xlsx, gtools, CHNOSZ, plyr, ggplot2, reshape2, gplots, Phyloseq, and DESeq2, and in STAMP.[15] Alpha diversity indexes of Shannon, Simpson, InvSimpson and Fisher were estimated. Abundance differences among multiple groups of samples were compared using ANOVA and Kruskal-Wallis' tests.[16] If significant ($P < 0.05$), the Games-Howell's test[16] was used to determine significantly different means between group pairs. Group abundance differences between any taxonomic category were compared using Welch's[16] or White's non-parametric t-test (proposed for the analysis of clinical metagenomic data).[17] Confidence intervals were estimated by inverting Welch's t-test and using a percentile bootstrapping method (10,000 replications), respectively. False discovery rate (FDR) in multiple testing was controlled in each analysis by using the Benjamini-Hochberg FDR[18] or Storey's FDR[19] methods.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

RESULTS

Clinical and functional phenotype data are shown in Table 1. When urine sample characteristics of 23 non-NB subjects (mean age 35.3 years) and 24 NB subjects (mean age 40.3 years) were compared, the NB group were significantly more likely to have urinalyses positive for LE ($p<.001$), nitrite ($p<.001$), pyuria ($p=.001$), cloudy urine ($p<.001$), and positive culture ($p< .001$).

<<<INSERT TABLE 1 APPROXIMATELY HERE>>>

Microbiomes by Clinical Phenotype: All subjects (47/47) had bacteriuria based on 16S pyrosequencing, while only 23 had positive urine cultures. Among the 23 positive urine cultures, *Escherichia coli* was the most frequently identified species, identified in nine by standard cultivation methods, four of which as the single species, and in the remainder as part of a polymicrobial culture (see Table 2). One culture-positive *E. coli* sample was not confirmed by sequence analysis by either database (although PathoScope detected *Shigella,* which has a nearly identical 16S gene). In two additional cases, bacteria (*Enterococcus faecalis* and *Pseudomonas aeruginosa*) were identified by cultivation, as part of polymicrobial cultures, whose presence could not be confirmed by sequence analysis (although PathoScope detected other species from the same genera). Overall, there was high correlation in the bacterial species identified by both databases with the exception of *E. coli*. None of the *E. coli* strains were identified using the LTP115 database, while 8 of 9 *E. coli* culture positive samples were confirmed to have *E. coli* rRNA using the Silva database. This difference between the databases is attributable to the LTP115 database including only one *E. coli* reference, while the curated

SILVA database includes 1256 *E. coli* (some redundant) references. These results also confirm the good performance of PathoScope at assessing bacterial composition using 16S sequences.

<<<INSERT TABLE 2 HERE>>>

The non-NB female urine microbiome was characterized by Lactobacillaceae, Aerocacaeae, and Enterobacteriacea, with only Lactobacillaceae being significantly more abundant when compared with non-NB males (75% greater abundance, p=.002), NB males (60% greater abundance, p=.01), and NB females (55% greater abundance, p=.02). There was no gender difference in proportional representation of Lactobacillaceae within the NB group (p≥.1). The non-NB female *Lactobacillus* community was characterized by *L. crispatus* and *L. iners*, whereas the *Lactobacillus* community of NB females was characterized by *L. iners*. *L. crispatus* was not identified in the microbiome of any subject with NB.

The non-NB male urine microbiome was characterized by a significantly greater proportion of Streptococcaceae than non-NB females (40-45% greater abundance, p=.014) (Figure 1a), and NB males and females (both p<.05). These trends were similar at the genus level (Figure 1b), but did not persist to the species level. *Staphylococcus haemolyticus* was the only bacterial species present to a significantly greater degree when compared with non-NB females (p=.023) (see Figure 1c). Supplemental Figure 1 confirms these differences using the SILVA reference database.

<<<INSERT FIGURE 1 APPROXIMATELY HERE>>>

191 At the genus level, NB females had a significantly greater proportion of *Lactobacillus* (20%
192 greater, p=.018), *Gardnerella* (8% greater, p=.02), and *Enterobacter* (6% greater, p=.04) than NB
193 males. At the species level, *G. vaginalis* (8% greater, p=.009) and *L. iners* (17% greater, p=.01)
194 were significantly more predominant in NB females.

195 To determine urine microbiome differences by bladder function, non-NB males and females
196 were combined and compared with NB males and females. Figure 2c shows that NB group
197 microbiomes had significantly greater representation from *Enterococcus faecalis* (p=.006),
198 *Pseudomonas aeruginosa* (p=.023) and *Klebsiella pneumonia* (p=.023). Using the SILVA
199 reference database, significant abundance of *E. faecalis* (p=.005) and *L. crispatus* (p=.02) were
200 confirmed for the NB and non-NB groups, respectively, while *E. coli* was also shown to be
201 present in the NB group to a greater extent (p=.028, see Supplemental Figure 2).

202 <<<INSERT FIGURE 2 APPROXIMATELY HERE>>>

203 When the NB group was further stratified by catheterization status, NB subjects using
204 suprapubic catheters (SP; p<.05) and intermittent catheterization (IC; p<.01), but not those who
205 void (p≥.1) had significantly greater abundance of the family Enterobacteriaceae than the non-
206 NB group (see Figure 3a). No differences were observed in intra-NB group comparisons. The
207 non-NB group had significantly greater proportions of Lactobacillaceae than subjects with NB
208 using SP catheters (p<.001) and IC (p<.01), but not those with NB who void (p>.1; see Figure
209 3b).

210 <<<INSERT FIGURE 3 APPROXIMATELY HERE>>>

211 Microbial diversity was assessed using the Shannon, Simpson, Inverse Simpson and Fisher
212 diversity indices. While there was no significant difference in diversity between the non-NB, NB-
213 void, NB-IC, or NB-SP groups, the NB-void group trended toward less diversity. Similarly, there
214 was no difference in diversity by gender between the non-NB and NB groups. All study groups
215 independent of gender and bladder status had a median of two to 18 phylotypes, underscoring
216 the concept that polymicrobial urine is a ubiquitous condition.

217 When the relationship between microbial diversity and pyuria was assessed, there was no
218 difference in diversity between the NB group in those with and without pyuria. Further analysis
219 by white blood cell (WBC) count demonstrated no association (positive or negative) between
220 the presence of *Lactobacillus* (*L. iners, L. crispatus, L. fornicalis,* and *L. gasseri*), *Streptococcus*,
221 *Klebsiella*, or *Shigella* and pyuria between the non-NB and NB groups and within the NB group
222 when data were analyzed using both the LTP115 (see Fig 4) or SILVA (Supplemental Fig 3)
223 reference databases.

224 <<<INSERT FIGURE 4 APPROXIMATELY HERE>>>

225 When we analyzed the microbiomes of NB subjects by the presence or absence of pyuria we
226 found that the Genus *Actinobaculum* was strongly associated with the presence of pyuria
227 (p=.009). Further analysis of this genus revealed that none of the four *Actinobaculum* species
228 were present in any non-NB subject or NB subjects with WBC<5, while *Actinobaculum sp*.
229 (*A.schaalii* and *A.massiliense*) were present in 36% (4 of 11) NB subjects with pyuria.

ACCEPTED MANUSCRIPT

DISCUSSION

In this paper we aimed to build upon our previous work disputing clinical dogma that healthy urine is sterile, by describing unique asymptomatic urine microbiomes by clinical and functional phenotype. We extend our prior work by correlating clinical assessments (urinalysis and urine culture) with urine microbiome findings, and demonstrating that the asymptomatic urine microbiome varies by gender and function. *Lactobacillus sp*. and *S. haemolyticus* characterize non-NB females and males, respectively, while the urine microbiome of those with NB dysfunction is characterized by known uropathogens, *E. coli*, *E. faecalis*, *P. aeruginosa* and *K. pneumoniae*. Lastly, we identified emerging uropathogens of the genus *Actinobaculum* in healthy NB subjects, all of whom had pyuria.

Our demonstration of the discordance between urinalysis findings and urine culture bacterial growth between the non-NB and NB groups support clinical observations. Further, there was the suggestion of increasingly abnormal findings with increased exposure to a urinary catheter. Because these patients were asymptomatic, these findings loosely support disregard of WBC (at least at the WBC≥5 level) for catheter-associated UTI diagnosis endorsed by the IDSA.[20]

Our findings of significant differences in urine microbiome composition by gender, regardless of NB or catheterization status, are not surprising. The vaginal microbiome is rich in Lactobacilli during health, and characterized by reduced Lactobacilli and heightened bacterial diversity during disease states.[21] Related, *L. crispatus* vaginal microbiomes are considered the 'healthiest' and less likely to be associated with disease states than *L. iners* vaginal microbiomes. If the urine microbiome follows vaginal microbiome physiology then this finding

leads us to hypothesize that absence of *L. crispatus* in favor of *L. iners* in NB subjects may be indicative of a microbiome more prone to disease.

The preponderance of Enterococcaceae in the urine microbiome of people with NB is consistent with clinical observations. Our NB participants were all affected by SCI, which results in near universal presence of neuropathic bladder and bowel. Fecal incontinence or bowel care regimes may alter colonization of the perineum by fecal flora. Alternatively, shifts in the gut microbiome may influence the ability of specific bacteria to colonize the urinary tract independent of mechanical delivery.

Lastly, we were surprised that we did not find any differences in diversity amongst the groups. The evidence suggests that microbiome diversity is not consistently associated with health or disease across body systems. Whereas increased bacterial diversity is associated with disease states in the female vagina,[21] decreased gut microbiome diversity is implicated in obesity and allergic/immunologic conditions.[22] Our data provide preliminary evidence about urine microbiome diversity during the asymptomatic state.

These findings are highly clinically relevant to the NB population, who face a disproportionately high risk of genitourinary complications. UTIs were historically the most common cause of death for people with SCI,[23] and while early mortality due to UTI and subsequent kidney failure has declined with improved prevention and management, UTIs remain the most common cause of emergency department visits and rehospitalization among people with neuropathic bladder.[24,25]

Our results demonstrate that the Genus *Actinobaculum*, comprised of *A. massiliense*, *A. schaalii*, *A. suis* and *A. urinale,* was present in four NB microbiomes, none of which were detected by cultivation, and the presence was strongly associated with pyuria. Characterized by 16S rRNA sequencing between 1997 (*A. schaalii*) and 2003 (*A. urinale*), the four *Actinobaculum* species have been identified as emerging uropathogens in adults and children with underlying pathophysiology, including cases of a child with neuropathic bladder due to meningomyelocele;[26] urosepsis;[27] and UTI in the elderly population with chronic cystitis.[28] In a study of 10 Danish patients infected with *A. schaalii*, 3 of these were similar to our NB population in that they either utilized a urinary catheter for bladder drainage and/or had neuropathic bladder due to syringomyelia or chronic paraplegia.[29]

A. schaalii is a facultative anaerobic gram-positive rod that resembles normal skin or mucosal flora. It is slowly growing and is often overgrown in culture media by faster growing or commensal species. Because of these features and because traditional urine samples are incubated for 24-48 hours in ambient air, *A. schaalii* growth is impeded, making it challenging to isolate.[29] In a retrospective series of 20 cases of *A. schaalii* infection, leukocytes were present in all culture positive cases (10/10) while nitrite was negative in all cases.[30] *A. schaalii* has diminished sensitivity to first line antibiotics used to treat readily cultured uropathogens (i.e. ciprofloxacin and trimethoprim/sulfamethoxazole),[30] while being susceptible to amoxicillin, ceftriaxone, gentamicin, and nitrofurantoin. This is clinically relevant as people with NB due to spinal cord injury frequently have pyuria and bacterial growth on urine cultures despite a lack of symptoms. Also, they frequently experience “recurrent” infections that do not respond as well as anticipated to antimicrobials. Thus, we speculate that in people with NB due to spinal

293 injury, the presence of *Actinobaculum* species may influence abnormal urinary findings and that
294 standard urine culture data might sometimes mislead antibiotic choice.

295 The major limitation of this study was the small sample size, which limited the robustness of
296 analyses when multiple stratifications were performed. While we found unique microbiomes by
297 gender and bladder function, our sample was not large enough to stratify by both variables
298 simultaneously. Our ability to identify E. coli using 16S rRNA gene sequencing depended on the
299 reference database used. Because this was a cross-sectional study of the urine microbiome
300 during the healthy state, we are unable to make any correlations to UTI or to fluctuations in the
301 microbiome over time. Lastly, we cannot fully exclude contamination of bladder urine with
302 urethral and vaginal bacteria. Despite these limitations, given that all 47 patients had bacterial
303 16s rRNA detected we suspect that bacteria are always present in the urine. Prospective studies
304 of people during asymptomatic, symptomatic and post-antimicrobial therapy will be helpful in
305 better understanding any relationships between these states.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

CONCLUSIONS

Several findings from this study suggest a change in our clinical and research approaches to urinary health and disease. Asymptomatic bacteriuria is often considered an 'unhealthy' state or possibly a precursor to disease. Our data suggest that asymptomatic bacteriuria is universal as opposed to being a rare and episodic event. Metagenomics allows greater specificity and perhaps will allow us to identify urine microbes that are associated with more or less healthy urologic states, as has been done in other body systems.

Defining the healthy urine microbiome provides yet undiscovered insights into novel diagnostic and therapeutic approaches worthy of future scientific pursuit. Our findings call into question our current approach to cultivation and perhaps the diagnostic utility of identifying the most prevalent bacterial species as the etiology of infection. Instead of a goal being eradication of bacterial load, perhaps future goals might involve identification of and subsequent manipulation of the urine microbiome toward a healthier state. For example, *L. crispatus,* which we found in non-NB females, is currently being explored as a probiotic in clinical trials. Different microbiomes (such as those of the gut, vagina, and bladder), and changes within these microbiomes, may be found to influence each other such that deviations toward or away from health in one may affect the others. Understanding the behavior of *bacteria* within and between microbiomes offers great potential for clinical advancement and benefit to the patient.

ACCEPTED MANUSCRIPT

FUNDING: This work was supported by NCMRR/NINDS [Grant number: 2R24HD050846-06] (NCMRR-DC Core for Molecular and Functional Outcome Measures in Rehabilitation Medicine, PI Hoffman)

ACKNOWLEDGEMENT

We would like to acknowledge the support provided by the Eric Hoffman and Keith Crandall laboratories at Children's National Medical Center, Center for Genetic Medicine Research and the George Washington University Computational Biology Institute, respectively.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

REFERENCES

1. Hur KY, Lee MS. Gut Microbiota and Metabolic Disorders. *Diabetes Metab J.* 2015;3(39):198-203.
2. Fouts D, Pieper R, Szpakowski S, et al. Integrated next-generation sequencing of 16S rDNA and metaproteomics differentiate the healthy urine microbiome from asymptomatic bacteriuria in neuropathic bladder associated with spinal cord injury. *J Transl Med.* 2012/08/28 2012;10(1):1-17.
3. Francis OE, Bendall M, Manimaran S, et al. Pathoscope: species identification and strain attribution with unassembled sequencing data. *Genome research.* Oct 2013;23(10):1721-1729.
4. Toma I, Siegel MO, Keiser J, et al. Single-molecule long-read 16S sequencing to characterize the lung microbiome from mechanically ventilated patients with suspected pneumonia. *Journal of clinical microbiology.* Nov 2014;52(11):3913-3921.
5. Carney BC, Moffatt LT, Randad PR, et al. Microbiome analysis of burn wounds reveals the presence of species not currently known to be involved in burn. *Journal of Burn Care & Research.* In Press.
6. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal.* 2011;17:10-12.
7. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. *Bioinformatics.* Mar 15 2011;27(6):863-864.
8. Edgar RC, Haas BJ, Clemente JC, et al. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* Aug 15 2011;27(16):2194-2200.
9. Byrd AL, Perez-Rogers JF, Manimaran S, et al. Clinical PathoScope: rapid alignment and filtration for accurate pathogen identification in clinical samples using unassembled sequencing data. *Bmc Bioinformatics.* Aug 4 2014;15.
10. Francis OE, Bendall M, Manimaran S, et al. Pathoscope: Species identification and strain attribution with unassembled sequencing data. *Genome research.* Oct 2013;23(10):1721-1729.
11. Munoz R, Yarza P, Ludwig W, et al. Release LTPs104 of the All-Species Living Tree. *Syst Appl Microbiol.* May 2011;34(3):169-170.
12. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research.* Jan 2013;41(Database issue):D590-596.
13. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* Apr 2012;9(4):357-U354.
14. Gentleman RC, Carey VJ, Bates DM, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* 2004;5(10).
15. Parks DH, Tyson GW, Hugenholtz P, et al. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics.* 2014;30(21):3123-3124.
16. Bluman AG. *Elementary statistics: A step by step approach.* 6th edition ed. New York: McGraw Hill Higher Education, New York; 2007.
17. White JR, Nagarajan N, Pop M. Statistical Methods for Detecting Differentially Abundant Features in Clinical Metagenomic Samples. *Plos Comput Biol.* Apr 2009;5(4).
18. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B.* 1995;57(1):289-300.
19. Storey JD, Taylor JE, Siegmund D. Strong control, conservative point estimation and simultaneous conservative consistency of false discovery rates: a unified approach. *J Roy Stat Soc B.* 2004;66:187-205.

ACCEPTED MANUSCRIPT

20. IDSA. Infectious Diseases Society of America (IDSA). Diagnosis, Prevention, and Treatment of Catheter-Associated Urinary Tract Infection in Adults: 2009 International Clinical Practice Guidelines from the Infectious Diseases Society of America. 2009; http://www.idsociety.org/IDSA/Site_Map/Guidelines/Patient_Care/IDSA_Practice_Guidelines/Infections_by_Organ_System/Genitourinary/Complicated_Urinary_Tract_Infections_%28UTI%29.aspx. Accessed May 4, 2014, 2014.

21. van de Wijgert JH, Borgdorff H, Verhelst R, et al. The vaginal microbiota: what have we learned after a decade of molecular characterization? *PloS one.* 2014;9(8):e105998.

22. Le Chatelier E, Nielsen T, Qin J, et al. Richness of human gut microbiome correlates with metabolic markers. *Nature.* Aug 29 2013;500(7464):541-546.

23. Ahmad I, Granitsiotis P. Urological follow-up of adult spina bifida patients. *Neurourology and urodynamics.* 2007;26(7):978-980.

24. Caterino JM, Scheatzle MD, D'Antonio JA. Descriptive analysis of 258 emergency department visits by spina bifida patients. *The Journal of emergency medicine.* Jul 2006;31(1):17-22.

25. Haisma JA, van der Woude LH, Stam HJ, et al. Complications following spinal cord injury: occurrence and risk factors in a longitudinal study during and after inpatient rehabilitation. *J Rehabil Med. .* 2007;39:393-398.

26. Zimmermann P, Berlinger L, Liniger B, et al. Actinobaculum schaalii an emerging pediatric pathogen? *BMC infectious diseases.* 2012;12:201.

27. Sturm PD, Van Eijk J, Veltman S, et al. Urosepsis with Actinobaculum schaalii and Aerococcus urinae. *Journal of clinical microbiology.* Feb 2006;44(2):652-654.

28. Bank S, Jensen A, Hansen TM, et al. Actinobaculum schaalii, a common uropathogen in elderly patients, Denmark. *Emerging infectious diseases.* Jan 2010;16(1):76-80.

29. Reinhard M, Prag J, Kemp M, et al. Ten cases of Actinobaculum schaalii infection: clinical relevance, bacterial identification, and antibiotic susceptibility. *Journal of clinical microbiology.* Oct 2005;43(10):5305-5308.

30. Beguelin C, Genne D, Varca A, et al. Actinobaculum schaalii: clinical observation of 20 cases. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases.* Jul 2011;17(7):1027-1031.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 1. Clinical and functional bladder phenotype summary.

	Age *(years, range)*	**Urinalysis**				**Urine Culture**
		+LeukEst (%)	*+Nitrite (%)*	*+Pyuria (WBC>5/hpf) (%)*	*Urine Cloudy (%)*	*Positive (>50,000-100,000 CFU/ml) (%)*
Non-NB (N=23)	35.3 (22-57)	2 (8.69%)	0	1 (4.35%)	0	3 (13.04%)
Males (N=9)	34.4 (24-50)	1 (11.11%)	0	0	0	0
Females (N=14)	35.8 (22-57)	1 (7.14%)	0	1 (7.14%)	0	3 (21.42%)
NB (N=24)	40.33 (19-61)	13 (54.17%)	11 (45.80%)	11 (45.80%)	19 (79.10%)	17 (70.80%)
Males (N=12)	32.8 (19-48)	8 (66.66%)	6 (50%)	6 (50%)	11 (91.66%)	10 (83.33%)
Void (N=3)	32.6 (19-48)	1 (33.33%)	1 (33.33%)	1 (33.33%)	2 (66.66%)	3 (100%)
Intermittent Catheter (N=4)	39.25 (21-48)	2 (50%)	3 (75%)	1 (25%)	4 (100%)	3 (75%)
Suprapubic Catheter (N=5)	27.8 (20-48)	5 (100%)	2 (40%)	4 (80%)	5 (100%)	4 (80%)
Females (N=12)	47.8 (36-61)	5 (41.66%)	5 (41.66%)	5 (41.66%)	8 (66.66%)	7 (58.33%)
Void (N=1)	41.0	0	0	0	0	1 (100%)
Intermittent Catheter (N=6)	50.8 (36-55)	2 (33.33%)	2 (33.33%)	2 (33.33%)	4 (66.66%)	2 (33.33%)
Suprapubic Catheter (N=5)	45.6 (40-61)	3 (60%)	3 (60%)	3 (60%)	4 (80%)	4 (80%)

ACCEPTED MANUSCRIPT

Table 2. Comparison of Urine Culture and Sequencing Findings (using two databases)				
	Patient	*Culture*	*LTP115*	*silva119refNRclean*
E. coli as sole organism	GU008	*Escherichia coli*	-	+
	GU021	*Escherichia coli*	-	+
	GU026	*Escherichia coli*	-	-
	GU032	*Escherichia coli*	-	+
	GU034	*Escherichia coli*	-	+
E. coli as part of polymicrobial culture	GU014	*Enterococcus faecalis*	+	-
		Escherichia coli	-	+
		Pseudamonas aeruginosa	+	+
	GU015	*Enterococcus faecalis*	+	+
		Escherichia coli	-	+
		Klebsiella pneumoniae	+	-
		Providencia stuartii	+	+
		Pseudomonas aeruginosa	+	+
	GU029	*Citrobacter koseri (diversus)*	+	+
		Enterococcus faecalis	-	-
		Escherichia coli	-	+
	GU056	*Enterococcus faecalis*	+	-
		Escherichia coli	-	+
non E. coli bacteria as monobacterial culture	GU001	*Proteus mirabilis*	+	+
	GU005	*Klebsiella pneumoniae*	+	+
	GU037	*Klebsiella pneumoniae*	+	+

ACCEPTED MANUSCRIPT

	GU057	*Klebsiella oxytoca*	-	+
	GU006	*Enterococcus faecalis*	+	+
	GU028	*Enterococcus faecalis*	+	+
	GU018	*Pseudomonas aeruginosa*	+	+
	GU025	*Staphylococcus*	+	+
	GU046	*Lactobacillus species*	+	+
	GU047	*Lactobacillus species*	+	+
	GU049	*Streptococcus beta-hemolytic*	+	+
	GU053	*Streptococcus beta-hemolytic*	+	+
Samples with non E. coli bacteria as polymicrobial culture	GU031	*Diphtheroids (Corynebacterium)*	+	-
		Lactobacillus	+	+
		Pseudomonas aeruginosa	-	-
	GU040	*Enterococcus faecalis*	+	+
		Pseudomonas aeruginosa	+	-

ACCEPTED MANUSCRIPT

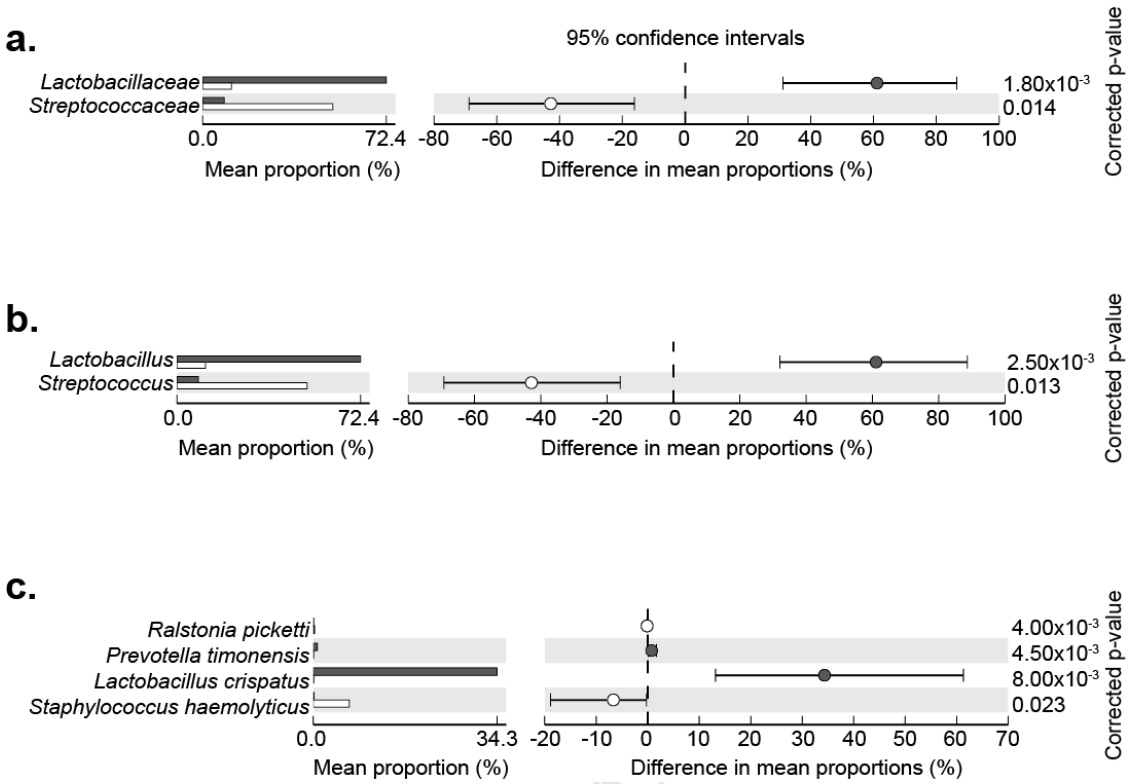


Figure 1. Significant differences in the urine microbiomes of all females (shaded) versus all males (not shaded) at the (a.) family, (b.) genus, and (c.) species levels.

ACCEPTED MANUSCRIPT

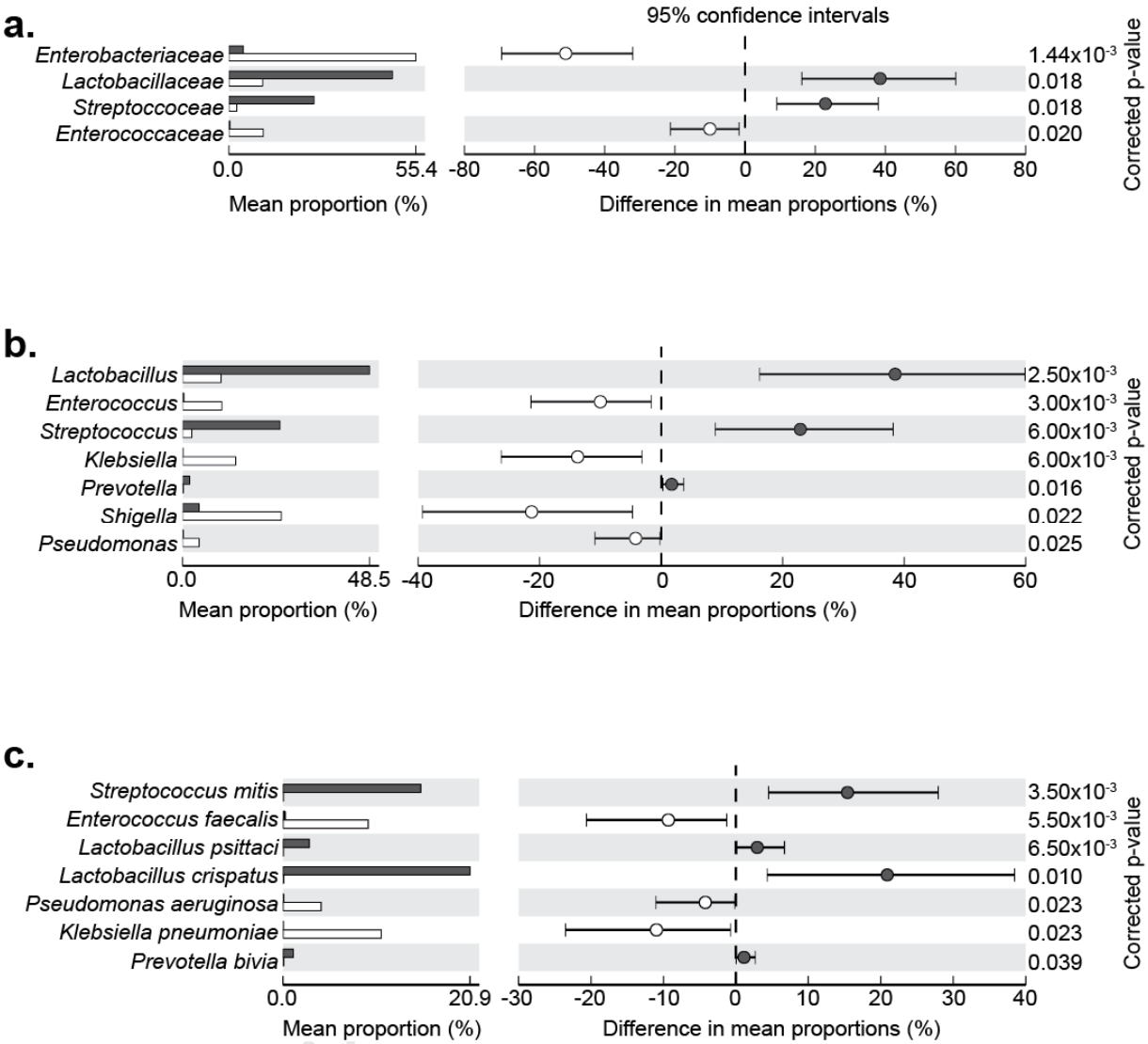


Figure 2. Significant differences in group urine microbiome analyses of non-NB subjects (shaded) versus NB subjects (not shaded) at the (a.) family, (b.) genus, and (c.) species levels.

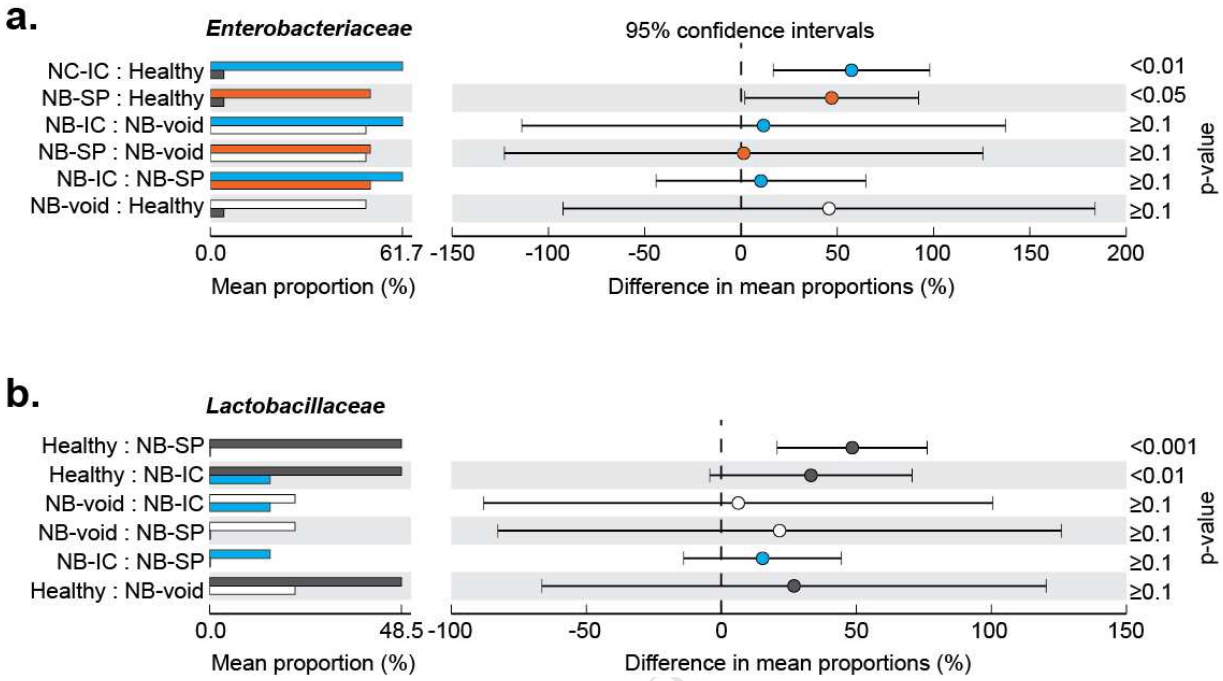


Figure 3. Significant differences in Enterobacteriaceae (a) and Lactobacillaceae (b) by bladder function and bladder management method (cyan = NB subjects using IC; orange = NB subject using SP; shaded = non-NB; no shading = NB subject who void).

ACCEPTED MANUSCRIPT

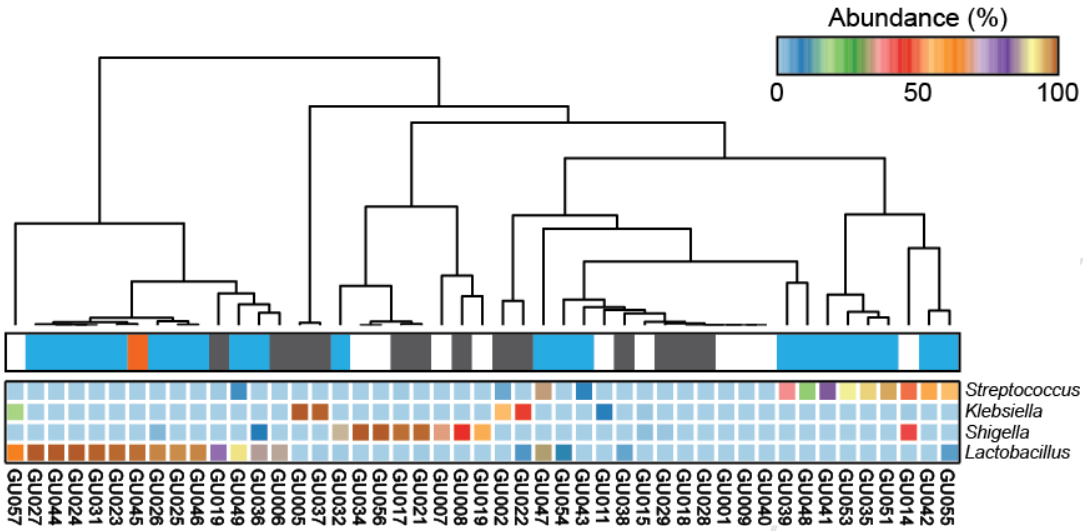


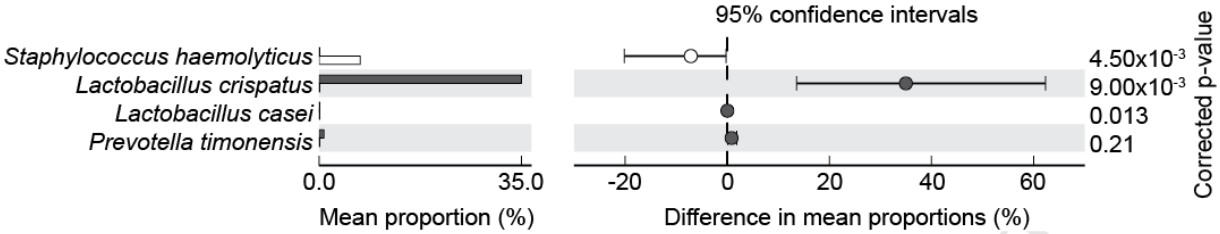
Figure 4. Heatmap and dendrogram of predominant genera by patient group and pyuria status (cyan = non-NB subjects without pyuria; orange = non-NB subjects with pyuria; shaded = NB subjects without pyuria; no shading = NB subjects with pyuria).

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

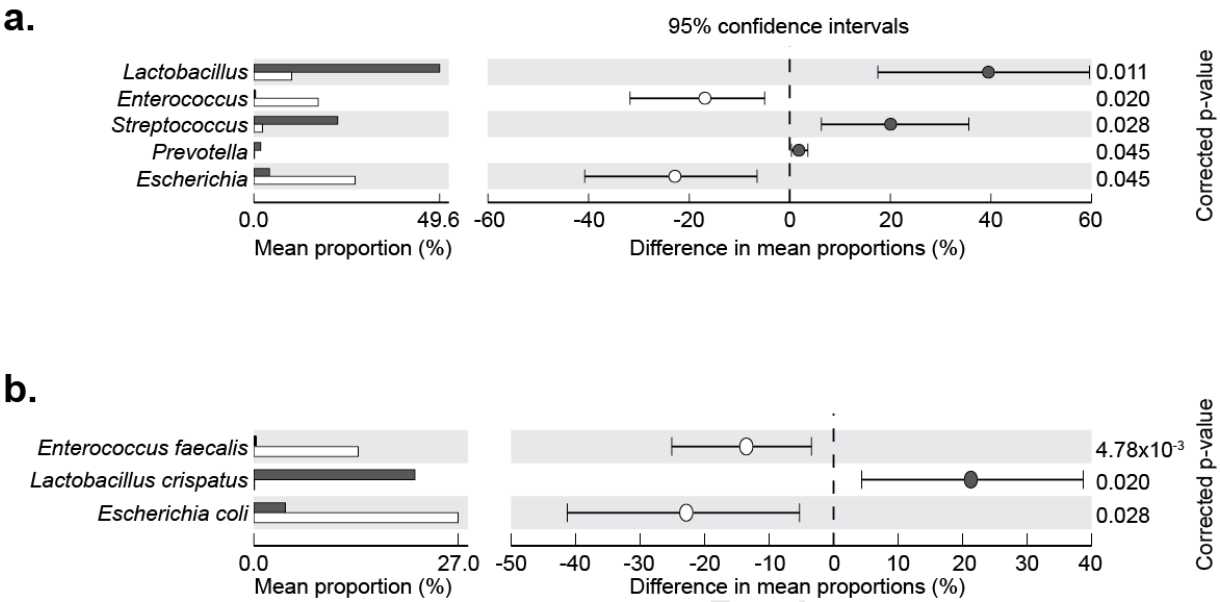
ABBREVIATION	WORD/PHRASE
NB	Neuropathic bladder
SCI	Spinal cord injury
UTI	Urinary tract infection
ASB	Asymptomatic bacteriuria

ACCEPTED MANUSCRIPT



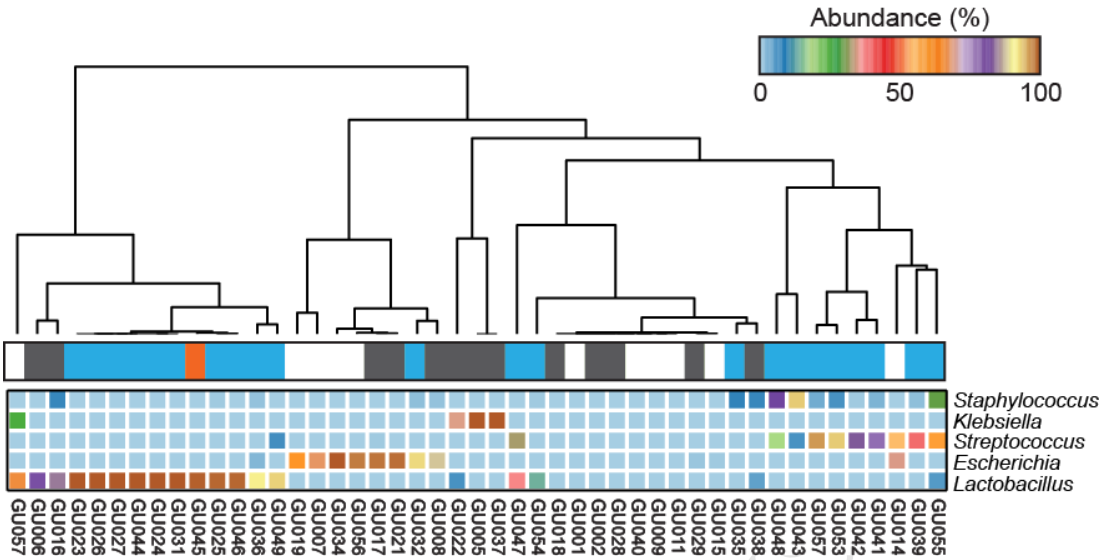
Supplementary Figure 1. Confirmation of significant differences in the urine microbiomes of all females (shaded) versus all males (not shaded) at the species level using the SILVA database.

ACCEPTED MANUSCRIPT



Supplemental Figure 2. Significant differences in group urine microbiome analyses of non-NB subjects (shaded) versus NB subjects (not shaded) at the genus and species levels.

ACCEPTED MANUSCRIPT



Supplemental Figure 3. Heatmap and dendrogram of predominant genera by patient group and pyuria (cyan = non-NB subjects without pyuria; orange = non-NB subjects with pyuria; shaded = NB subjects without pyuria; no shading = NB subjects with pyuria).

ACCEPTED MANUSCRIPT