Recurrent urinary tract infection and estrogen shape the taxonomic ecology and functional potential of the postmenopausal urobiome

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Abstract

Community-acquired urinary tract infection (UTI) is among the most common bacterial infections observed in humans. Postmenopausal women are a rapidly growing and underserved demographic group who are severely affected by rUTI with a >50% recurrence rate. In this population, rUTI can persist for years, reducing quality of life and imposing a significant healthcare burden. rUTI is most often treated by long-term antibiotic therapy, but development of antibiotic resistance and allergy leave physicians with fewer treatment options. The female urobiome has been identified as a key component of the urogenital environment. However, structural and functional changes in the urobiome underlying rUTI susceptibility in postmenopausal women are not well understood. Here, we used strictly curated, controlled cross-sectional human cohorts of postmenopausal women, urobiome whole genome (shotgun) metagenomic sequencing (WGMS), advanced urine culturing techniques, extensive biobanking of >900 patient-derived urinary bacterial and fungal isolates, and mass spectrometry-based estrogen profiling to survey the urobiome in rUTI patients during infection relapse and remission as well as healthy comparators with no lifetime history of UTI. Our results suggest that a history of rUTI strongly shapes the taxonomic and functional ecology of the urobiome. We also find a putative protective commensal population, consisting of species known to convey protection against bacterial vaginosis such as *Lactobacillus crispatus*, within the urobiome of women who do not experience UTI. Integration of clinical metadata detected an almost exclusive enrichment of putative protective species belonging to the genus, *Lactobacillus*, in women taking estrogen hormone therapy (EHT). We further show that the urobiome taxonomic ecology is shaped by EHT, with strong enrichments of putatively protective lactobacilli, such as *L. crispatu*s and *L. vaginalis*. Integrating quantitative metabolite profiling of urinary estrogens with WGMS, we observed robust associations between urobiome taxa, such as *Bifidobacterium breve* and *L. crispatus*, and urinary estrogen conjugate concentrations, suggesting that EHT strongly alters the taxonomic composition of the female urobiome. We have further used functional metagenomic profiling and patient-derived isolate phenotyping to identify microbial metabolic pathways, antimicrobial resistance genes (ARGs), and clinically relevant antimicrobial resistance phenotypes enriched between disease-states. Our data suggest distinct metabolic and ARG signatures of the urobiome associated with current rUTI status and history. Taken together, our data suggests that rUTI history and estrogen use strongly shape the functional and taxonomic composition of the urobiome in postmenopausal women.

Introduction

Urinary tract infection (UTI) is among the most common adult bacterial infections and imparts particularly significant medical burden on women, with more than 50% of women suffering UTI in their lifetime (Gaitonde et al., 2019; Jhang and Kuo, 2017). Historically, UTI has largely been underprioritized in medical research due to low mortality rates and the effectiveness of available antibiotics for most UTI episodes. However, UTI is a disease of disproportionate burden as age is one of the strongest associated risk factors for UTI and the development of recurrent UTI (rUTI) (Flores-Mireles et al., 2015). Indeed, it is estimated that approximately 50% of UTIs in postmenopausal women develop into rUTI, which is clinically defined as ≥ 3 symptomatic UTIs in 12 months (Gaitonde et al., 2019; Malik et al., 2018b). rUTI can last for years, dramatically decreasing quality of life and, if treatment is unsuccessful, can develop into life-threatening urosepsis. Current therapeutic strategies mostly rely on the chronic use of antibiotics to achieve urinary tract sterility (Flores-Mireles et al., 2015; Malik et al., 2018a; Neugent et al., 2020). However, increasing rates of antibiotic refractory rUTI make this strategy unsustainable and ultimately ineffective. For example, a recent prospective study of 86 postmenopausal women observed resistance or allergy incidence rates of 76%, 56%, and 35% to the frontline antibiotics trimethoprim-sulfamethoxazole (TMP-SMX), fluoroquinolones, and nitrofurantoin, respectively (Malik et al., 2018a). These observations highlight the need for alternative therapeutic strategies to combat the growing antibiotic resistance rates in the treatment of rUTI.

A promising source of therapeutic strategies for rUTI lies in modulating or restoring the urinary microbiome, termed here the "urobiome" (Flores-Mireles et al., 2015; Stamm and Norrby, 2001). Decades of medical dogma have largely assumed sterility of urine and the urinary tract. However, a large body of work has robustly established the existence of a resident microbiome within the human urinary tract (Brubaker and Wolfe, 2017; Hilt et al., 2014; Lewis et al., 2013; Price et al., 2019; Siddiqui et al., 2011; Wolfe et al., 2012). Initial taxonomic analyses have associated dysbiosis of the urobiome taxonomic composition to urinary incontinence, overactive bladder, and bladder cancer (Bucevic Popovic et al., 2018; Karstens et al., 2016; Pearce et al., 2014). However, fundamental knowledge of urobiome composition and function in postmenopausal women is lacking. Studies surveying the microbial ecologies of the urobiome associated with UTI have almost exclusively focused on premenopausal women or cohorts of mixed age (Barraud et al., 2019; Brubaker and Wolfe, 2017; Hilt et al., 2014; Neugent et al., 2020; Price et al., 2019; Siddiqui et al., 2011; Wolfe et al., 2012). As a result, the relationship between the urobiome and rUTI susceptibility or treatment outcome is poorly understood in postmenopausal women. Interestingly a 2021 report showed that pre and postmenopausal women displayed different core urinary

102   microbiota, providing strong rationale to characterize the postmenopausal urobiome in urogenital
103   disease (Ammitzboll et al., 2021).

104   Recently, the female urobiome has been reported to exhibit interconnection with the vaginal
105   microbiome (Thomas-White et al., 2018). For example, vaginal D(-)Lactate-producing lactobacilli,
106   known to protect the vagina from colonization by bacterial and fungal pathogens, have been
107   consistently observed in the female urobiome in multiple independent studies (Edwards et al.,
108   2019; Pearce et al., 2014). These observations beg the question of whether these known
109   protective vaginal species serve a similar protective role in the urobiome. Interestingly, a 2011
110   randomized clinical trial found a moderate reduction of rUTI incidence among women receiving
111   an intravaginal *L. crispatus* probiotic (Stapleton et al., 2011). While this study was performed in
112   premenopausal women and has yet to be validated in a larger clinical study, it does suggest that
113   lactobacilli may support urinary tract health. However, the niche-specific colonization and
114   metabolic needs of lactobacilli have not been assessed for this understudied ecosystem.

115   While numerous studies have used 16S rRNA amplicon sequencing to taxonomically profile the
116   microbial ecology of the human urobiome, no whole genome metagenomic sequencing (WGMS)
117   datasets have been generated to profile the genomic ecology of the urobiome in postmenopausal
118   women. Given the genomic diversity observed within and among taxonomic clades, metagenomic
119   information beyond 16S rRNA sequence enrichment is needed to assess the functional potential
120   of microbial communities (Quince et al., 2017). Whole-metagenome analysis of the urobiome is
121   required to identify the genes and metabolic pathways associated with urinary tract health, a
122   critical step towards the development of rationally designed probiotic therapies for rUTI. Here, we
123   present a WGMS survey of the urobiome of a strictly curated, cross-sectional cohort of
124   postmenopausal women separated into three groups defined by rUTI history and current UTI
125   status. Two of the cohort groups were curated to have recent rUTI history, differing only by current
126   infection status. The third cohort group consisted of postmenopausal women with no known
127   lifetime history of UTI. Our analysis has defined the urobiome ecology among the three cohorts
128   and has determined that the large-scale structure of the urobiome is not altered by rUTI history.
129   However, our taxonomic biomarker analysis using a Bayesian differential abundance model
130   identified significant species-level enrichments of gut bacteria and known uropathogens within the
131   urobiomes of women with rUTI history but no current UTI, suggesting that an imprint of past UTI
132   remains in the urobiome. We also observed a striking association between the use of estrogen
133   hormone therapy (EHT) and the presence of *L. crispatus* in the urobiome. Interestingly, we
134   observed differential enrichment of *Lactobacillus* spp. between patients using different EHT
135   modalities with strong *Lactobacillus* spp. enrichment among women using oral and patch EHT,

136   an observation which was not observed in patients using vaginal EHT. These findings mirror the
137   detected levels of excreted urinary estrogen conjugates in EHT(+) women. Functional analysis
138   revealed that the encoded metabolic potential is different between uropathogen-dominated and
139   commensal-dominated communities and that a history of rUTI dramatically alters the resistome
140   of the urinary microbiota with significant enrichments of beta-lactam, sulfonamide, and
141   aminoglycoside antimicrobial resistance genes associated with rUTI history. Taken together,
142   these results suggest that rUTI history and EHT shape the taxonomic and functional ecology of
143   the urobiome in postmenopausal women.
144
145   **Results**
146   **Cohort curation, metagenomic DNA preparation, and whole genome metagenomic dataset**
147   **generation**
148   Microbiome studies often observe high inter-sample variability, which can make interpretation
149   of ecological and associative analyses difficult (Fouts et al., 2012). With this in mind, we
150   implemented a strict set of exclusion criteria to guide patient recruitment and enrollment into the
151   study. Patients were excluded if they were premenopausal, seeking medical intervention for
152   complicated rUTI, diabetic, currently receiving chemotherapy, exhibiting renal insufficiency
153   (creatinine >1.5 mg/dL), using indwelling or intermittent catheters, had neurogenic bladder, or had
154   recent exposure to antibiotics within the last 4 weeks, unless an active infection was detected by
155   initial culture screening. rUTI often follows a cyclic pattern of infection relapse interrupted by
156   periods of remission (Figure 1A). To model this pattern of relapse and remission, the cohort of
157   postmenopausal women was striated into three groups based on rUTI history. Group 1 served as
158   a healthy comparator and consisted of postmenopausal women with no lifetime history of
159   symptomatic UTI (No UTI History), group 2 consisted of postmenopausal women with a recent
160   history of rUTI but no active infection at the time of urine donation (rUTI Remission), and group 3
161   consisted of postmenopausal women with a history of rUTI and an active UTI at the time of urine
162   donation (rUTI Relapse) (Figure 1B). In total, 258 patients were recruited and screened for
163   enrollment candidacy through interview, clinical assessment, and electronic medical records.
164   29.8% of recruited patients passed the exclusion criteria and were enrolled. We determined that
165   25 patients per group was sufficient to balance a-priori sample size estimation (Figure S 1A, B)
166   with clinical feasibility and enrollment rates. Cohort demographic information is reported in Table
167   1.
168   Urine was collected via the clean catch midstream method after patients were educated about
169   the procedure for sample collection. It should be noted the clean-catch midstream urine samples

170   are representative of the urogenital microbiome (Karstens et al., 2018), which is inclusive of the

171   bladder, urethral, and, in some cases, vaginal microbiomes. Metagenomic DNA yields following

172   purification from cohort urine reflected the anticipated biomass of the urobiome in each group with

173   the highest DNA yields observed in women with active rUTI (rUTI Relapse). We collected a

174   median (± interquartile range) DNA mass of 62.1 ± 345.5 ng from No UTI History samples, 46.7

175   ± 198.2 ng from rUTI Remission samples, and 1822 ± 544 ng from rUTI Relapse samples (Figure

176   S 1C).

177   WGMS sequencing of human samples can suffer from a high degree of host contamination.

178   Previous reports of human contamination in WGMS sequencing of the urobiome range from 1-

179   99% of obtained reads (Moustafa et al., 2018; Quince et al., 2017). We therefore targeted 50

180   million reads per sample, balancing the higher cost of deep sequencing with the added advantage

181   of increased microbial sampling power. To remove host contamination, we mapped all reads

182   against the human genome reference (hg38) and collected unmapped reads, which represent all

183   non-human DNA sequenced. We observed and average of 67.57% host contamination within the

184   metagenomic sequencing data (Figure S 1E). After host removal, we generated a dataset with an

185   average of $2.6\text{x}10^7$ non-human reads per sample.

186   To observe and measure potential contaminating background and environmental taxonomic

187   signals, a pure water sample was randomly inserted into the metagenomic DNA isolation

188   workflow, subjected to identical DNA isolation procedures, NGS sequencing, and taxonomically

189   analyzed with MetaPhlan2 (Segata et al., 2012). The majority of microbial reads observed in the

190   water control mapped to common nucleic acid purification kit and environmental contaminants

191   (Figure S 2A) (Salter et al., 2014). Except for known members of the human microbiome and

192   urobiome, these background taxa were censored from sample data when observed. We observed

193   a small relative abundance of taxa salient to the human urobiome in the water control, such as

194   *Pseudomonas*, *Escherichia*, *Klebsiella*, *Enterococcus*, *Staphylococcus*, and *Corynebacterium.*

195   These signals ranged from 0.0510%-11.5% of approximately 3 million mappable reads observed

196   in the water control.

197

198   **Validation of viable urobiome species through advanced urinary culture and WGMS hybrid**

199   **taxonomic profiling.**

200   To observe and validate the presence of living microbiota within the urobiomes sampled, we

201   coupled WGMS with advanced urine culture, a modification of the previously reported EQUC

202   protocol (Price et al., 2016). Advanced culture of cohort urine both generated a detailed, species-

203   level taxonomic profile and validated the presence of living taxa detected within the urobiomes

204 sampled. Taxonomic profiling by WGMS revealed that the aggregate of the urobiomes sampled
205 was dominated by the kingdom, Bacteria, which represented 99.4% of the detected non-viral,
206 microbial taxa (Figure 1C). Consistent with what is known about the taxonomic composition of
207 urobiomes studied to date (Brubaker and Wolfe, 2017; Siddiqui et al., 2011), the detected
208 bacterial taxa across the entire cohort belong to 4 major phyla: Firmicutes (44.7%), Actinobacteria
209 (22.3%), Proteobacteria (20.6%), and Bacteroidetes (12%) (Figure 1C). Advanced urine culture
210 captured 93.9% of bacterial genera detected in WGMS with observed aggregate relative
211 abundance ≥5% in any sample (Figure 1D). At the patient-level, advanced urine culturing was
212 able to validate the viability of an average of 74.5% of genera detected by WGMS (Figure S 2A).
213 The most frequent cultivable genera detected across all samples were *Lactobacillus*, *Escherichia*,
214 *Streptococcus*, *Bifidobacterium*, *Gardnerella*, *Klebsiella*, *Staphylococcus*, *Finegoldia*,
215 *Enterococcus*, and *Facklamia*. We cryo-preserved pure isolates from every cultivable species
216 detected through advanced urine culture and generated a large isolate biobank of 904 speciated
217 bacterial and fungal isolates, a first of its kind resource for the field.
218
Analysis of urobiome ecology reveals similar ecological structure between women with
No lifetime UTI history and rUTI Remission.
221 We next analyzed the genus and species-level taxonomic profiles and ecological structure
222 within the No UTI History, rUTI Remission and rUTI Relapse groups (Figure 2A, B, Figure S 3A).
223 Inspection of the rUTI Relapse group revealed urobiomes mainly dominated by single bacterial
224 uropathogens with little detected abundance of *Eukarya* and *Archaea* (Figure 2B, Figure S 3A).
225 The most prevalent uropathogen detected in our analysis was *Escherichia coli* (15/25, 60%), a
226 species known to be the major uropathogen among most types of UTI (Flores-Mireles et al.,
227 2015). Along with *E. coli*, our analysis also detected known uropathogens, *Klebsiella pneumoniae*
228 (2/25, 8%), *Enterococcus faecalis* (1/25, 4%), and *Streptococcus agalactiae* (1/25, 4%). We also
229 observed a low relative abundance of fungal species within the rUTI Relapse urobiomes including
230 *Candida glabrata* and *Malassenzia globosa*. Similarly low amounts of Archaeal taxa were
231 detected, such as, *Methanobrevibacter spp* (Figure S 3A). Interestingly, we observed viral taxa
232 present within the urobiomes of the rUTI Relapse cohort. The most observed virus was JC
233 polyomavirus (4/25 16%) but Human herpes virus 4 (1/25 4%) and Enterobacteria phage lke (1/25
234 4%) were also detected (Figure S 3B).
235 The most frequently observed bacterial species in urobiomes of women without active infection
236 (No UTI History and rUTI Remission) belonged to the genera *Lactobacillus*, *Bifidobacterium*,
237 *Gardnerella*, *Streptococcus*, *Staphylococcus*, and *Actinobaculum* (Figure 2 A, B). A subset of

238 these samples (54%) were dominated by one taxon while others were diverse and exhibited no

239 single dominant taxon. We observed 13 patients (24%) in the No UTI History and rUTI Remission

240 groups with a >50% relative abundance of various *Lactobacillus spp.*, including *L. crispatus, L.*

241 *iners*, and *L. gasseri* (Figure 2B). Interestingly, we also observed a subset of urobiomes in the No

242 UTI History and rUTI Remission groups dominated by *Bifidobacterium spp.*, such as *B. breve, B.*

243 *dentium*, and *B. longum*, as well as *Gardnerella vaginalis* (Figure 2B). Fungal and archaeal

244 species were also observed in low abundance (0-8.8% relative abundance) in the No UTI History

245 and rUTI Remission urobiomes (Figure S 3A). Three species of *Candida*, including *C. albicans*,

246 *C. glabrata*, and *C. dubliniensis* were detected. Other fungal taxa observed include *Malassezia*

247 *globosa*, *Naumovozyma spp.*, and *Eremothecium spp.* Observed archaeal species within the No

248 UTI History and rUTI Remission urobiomes included *Methanosphaera stadtmanae* and

249 *Methanobrevibacter spp.* Viral taxa were more frequently observed in the No UTI history and rUTI

250 Remission groups as compared to the rUTI Relapse group (Figure S 3B). The most observed

251 viral taxa in the No UTI history and rUTI Remission groups were JC polyomavirus, BK

252 polyomavirus, and Merkel cell polyomavirus (Figure S 3B).

253 To model the urobiome ecological structure within the three groups, we calculated alpha-

254 diversity indices including the observed taxa count, Shannon, Simpson, Chao 1, and ACE indices

255 (Thomas-White et al., 2017). Our analysis found that women in the No UTI History and rUTI

256 Remission groups had similarly diverse urobiomes across different alpha diversity indices (Figure

257 2C, D, Figure S 4 A, B, C). These data suggest that if there are ecological differences in the

258 urobiomes of postmenopausal women who are susceptible to rUTI (rUTI Remission) versus those

259 who are not (No UTI History), they are not reflected in alpha diversity metrics. The rUTI Relapse

260 group exhibited significantly lower alpha diversity indices as compared to the rUTI Remission

261 cohort, an observation that is likely attributed to uropathogen niche dominance during active

262 infection (Figure 2C, D, Figure S 4 A, B, C).

263 To assess large-scale taxonomic signatures associated with rUTI history and infection status,

264 we used double principal coordinate analysis (DPCoA), a beta-diversity ordination method which

265 allows for dimensionality reduction through relative abundance weighted phylogenetic distance

266 calculation between samples (Pavoine et al., 2004). Visualization of the first two PCoAs applying

267 weighted DPCoA to the aggregate dataset of detected species in the cohort revealed clustering

268 of the urobiomes of the rUTI Relapse group along a vector defined by the enrichment of *E. coli*

269 and were ecologically distinct from the urobiomes of the No UTI History and rUTI Remission

270 groups. The No UTI History and rUTI Remission groups exhibited relatively similar clustering

271 patterns in the first two PCoAs (Figure 2E) and many of these women clustered along opposing

272 vectors defined by the enrichment of either *L. crispatus* or *G. vaginalis*, which are associated with

273 a healthy vaginal microbiome or bacterial vaginosis, respectively (Ravel and Brotman, 2016;

274 Ravel et al., 2011). This similar clustering pattern between the No UTI history and rUTI Remission

275 cohorts suggests that a history of rUTI does not significantly alter the large-scale taxonomic

276 structure of the urobiome in postmenopausal women. However, the clustering of the No UTI

277 History and rUTI Remission urobiomes along the vectors defined by *L. crispatus* or *G. vaginalis*

278 supports an association between the urinary and vaginal microbiomes as previously reported

279 (Thomas-White et al., 2018).

280

281 **Taxonomic co-occurrence analysis identifies three non-interacting microbial clusters**

282 **within the urobiome.**

283 Microbial communities can harbor intricate interactions between member taxa (Alteri et al.,

284 2015; Keogh et al., 2016). Exceedingly little is known about interactions and co-occurrence of

285 bacterial species within the urobiome. We used the CCREPE pipeline to compute pairwise

286 associations and a compositionally corrected statistical association assessment between genera

287 detected within the cohort urobiomes (https://huttenhower.sph.harvard.edu/ccrepe/) (Figure 2F).

288 This analysis identified 2025 non-zero taxa correlations that were further filtered to 87 statistically

289 significant associations ($P<0.05$) (Figure 2F). After multiple hypothesis testing correction, a total

290 of 17 unique microbial associations exhibited robust statistical significance ($Q<0.05$) (Figure 2F).

291 Network visualization of significant associations ($P<0.05$) revealed three non-interacting microbial

292 clusters (Figure 2G). Cluster 1 member taxa were all known members of the human gut

293 microbiome (Human Microbiome Project, 2012). Cluster 2 exhibited the largest member set and

294 diversity and captured associations between genera known to inhabit the urobiome but whose

295 association has not yet been reported. Cluster 2 member genera, such as *Peptoniphilus* and

296 *Finegoldia*, have been reproducibly observed in the human urobiome in multiple, independent

297 studies (Anglim et al., 2021; Thomas-White et al., 2017). Interestingly, Cluster 2 grouped strongly

298 around the genus *Peptoniphilus*, a member of the vaginal microbiome that is known to be

299 associated with dysbiosis (Diop et al., 2019). Cluster 3 was identified as a pairwise interaction

300 between the genera *Gardnerella* and *Atopobium*, two taxa that have been previously associated

301 in vaginal dysbiosis and bacterial vaginosis (Bradshaw et al., 2006; Hardy et al., 2016; Hardy et

302 al., 2015). Taken together, these data suggest that taxonomic signatures within the urobiome of

303 postmenopausal women may follow patterns of co-occurrence, an observation which merits

304 further mechanistic study.

305

Taxonomic biomarker analysis reveals that rUTI history alters the species-level taxonomic signature of the urobiome.

rUTI follows a cyclic pattern of active infection followed by periods of no infection or remission (Figure 1A). We and others have observed that the taxonomic profiles of the urobiomes of individuals with UTI are significantly different than healthy individuals (Figure 2 A,B) (Flores-Mireles et al., 2015). Taxonomic remodeling associated with infection has also been shown to significantly alter the microbiome in other niches throughout the human body. Interestingly, taxonomic remodeling of the gut microbiome associated with antibiotic use is the strongest risk factor associated with the development of *Clostridium difficile* infection (Theriot et al., 2014). To test the hypothesis that rUTI history alters the underlying urobiome, we performed genus and species-level differential taxonomic enrichment analysis between the urobiomes of the No UTI History and the rUTI Remission patients. We employed the linear discriminant analysis of effect size (LEfSe) to incorporate an extensively employed non-parametric assessment of differential taxonomic abundance (Segata et al., 2011). We also employed the Bayesian microbial differential abundance (BMDA) model that can account for common characteristics that complicate differential analysis of microbiome data, such as data sparsity (zero inflation), over-dispersion, and uneven sampling depth (Li et al., 2019). Differentially enriched taxa detected by these analyses may serve as candidate taxonomic biomarkers for urobiome dysbiosis and possibly rUTI susceptibility. LEfSe detected no differentially abundant taxa between the No UTI History and rUTI Remission groups. However, using the BMDA model, we detected multiple differentially abundant taxa between No UTI History and rUTI Remission groups. BMDA detected two genera, *Aerococcus* ($\log_{10}$(Posterior Effect Size) = 0.70, PPI = 0.97) and *Lactobacillus* ($\log_{10}$(Posterior Effect Size) = 0.52, PPI = 0.96), as well as two species of lactobacilli, *L. vaginalis* ($\log_{10}$(Posterior Effect Size) = 7.66, PPI = 1) and *L. crispatus* ($\log_{10}$(Posterior Effect Size) = 1.36, PPI = 1), as significantly enriched in the No UTI History group compared to the rUTI Remission group (Figure 3A, B). At the genus-level, *Klebsiella*, *Gemella*, *Bacteroides*, *Clostridiales Family XIII Incertae Sedis unclassified*, *Eggerthella*, and *Escherichia* were among the most significantly enriched in the rUTI Remission group compared to the No UTI History group. Fifteen species were identified as significantly enriched in the rUTI Remission group, including *Ureaplasma parvum*, *Bacteroides uniformis*, *E. faecalis*, *Staphylococcus hominis*, and *Staphylococcus epidermidis* (Figure 3B). Many taxa enriched in the rUTI Remission group are primarily known to be native to the human gut microbiome and not the female urobiome or vaginal microbiome (Figure 3 A, B). Taken together, these results suggest that rUTI history may leave an imprint on urobiome composition that may be missed by common ecological indices (alpha or beta diversity) and traditional

340   differential abundance pipelines that do not consider the sparsity, over-dispersion and uneven

341   sampling that is common in low biomass microbiomes like the urobiome (Karstens et al., 2018).

342

343   **Estrogen hormone therapy and urinary estrogen concentration is associated with**

344   ***Lactobacillus* abundance in the urobiome of postmenopausal women**

345   Given that many of the urobiomes of women without active rUTI were dominated by species of

346   lactobacilli (26%, 13/50) (Figure 2 A, B), we sought to further characterize this taxonomic

347   enrichment in the No UTI History and rUTI Remission groups. We screened the cohort-associated

348   clinical metadata for any variables associated with *Lactobacillus* abundance. Interestingly, we

349   found that estrogen hormone therapy (EHT) was strongly associated with the presence of

350   *Lactobacillus* in the urobiome (Figure 4 A, B, C). Ecological modeling revealed that the urobiomes

351   of EHT(+) women were significantly less diverse than those of EHT(-) women and tended to be

352   dominated by a single species belonging to the genus, *Lactobacillus* (Figure 4 D, E, F). To

353   determine how EHT shapes the taxonomic profile of the urobiome, we performed differential

354   taxonomic enrichment analysis using LEfSe and BMDA. The LEfSe identified the enrichment of

355   the species *L. crispatus* in patients using EHT and the enrichment of the genus, *Streptococcus*,

356   in patients not using EHT (*L. crispatus* LDA=5.1, *P*=0.0185; *Streptococcus* LDA=4.8, *P*=0.0081)

357   (Figure 4 G). Differential enrichment analysis using BMDA captured a similar result but was able

358   to further resolve species-level differential enrichment (Figure 4 H). *L. crispatus* and *L. vaginalis*,

359   were significantly enriched in the urobiomes of EHT(+) women (*L. crispatus* $\log_{10}$(Posterior Effect

360   Size) = 13.5, PPI=1; *L. vaginalis* $\log_{10}$(Posterior Effect Size) = 8.5, PPI=1). Interestingly, BMDA

361   detected species-level enrichment of the *Streptococcus mitis/oralis/pneumoniae* (*S. m/o/p*) group,

362   *Streptococcus infantis*, and *Atopobium vaginae* within the urobiomes of EHT(-) women (*S. m/o/p*

363    $\log_{10}$(Posterior Effect Size)= 8.8, PPI=1; *S. infantis* $\log_{10}$(Posterior Effect Size)= 9.2, PPI=1; *A.*

364   *vaginae* $\log_{10}$(Posterior Effect Size)= 13.9, PPI=1) (Figure 4H). Taken together, these data

365   suggest a strong association between EHT use and urobiome dominance by *Lactobacillus*

366   species known to convey protection against bacterial pathogens (Edwards et al., 2019).

367   EHT can be administered via multiple modalities including oral supplementation, transdermal

368   patch, and topical vaginal cream (Lobo, 2017). Using the cohort-associated clinical metadata, we

369   striated patients based on EHT modality. Interestingly, we observed that women using oral and

370   patch EHT exhibited significant urobiome enrichment of *Lactobacillus*. However, urobiome

371   *Lactobacillus* enrichment varied widely in women using vaginal EHT (vEHT) and was not

372   significantly different from EHT(-) women (Figure 5A). We hypothesized that the vEHT may differ

373   from oral and transdermal patch in composition, metabolism, or dosage. We therefore used a

374 modified version of previously published targeted liquid-chromatography mass spectrometry (LC-

375 MS) methods for the measurement of urinary estrogens to quantify excreted urinary estrogen

376 conjugates of women in the No UTI History and rUTI Remission groups (van der Berg et al.,

377 2020). Limiting our analysis to the known major excreted sulfate and glucuronide conjugates of

378 estrone (E1) and 17β–estradiol (E2), we observed significantly higher urinary E1 and E2

379 conjugate abundance, both sulfates and glucuronides, in women using oral EHT, an observation

380 concordant with observed urinary *Lactobacillus* abundance (Figure 5 B, C, D Figure S 6 A, B, C,

381 D). Women using patch EHT also exhibited high urinary *Lactobacillus* abundance and we

382 observed a significant enrichment of E1-sulfate in patch EHT(+) women compared to EHT(-)

383 women (Figure S 5C). Consistent with urinary *Lactobacillus* abundance, we observed no

384 statistically significant difference in urinary estrogen conjugate concentrations between vEHT(+)

385 women and EHT(-) women (Figure 4 A, B, C, D Figure S 5 A, B, C, D).

386 We further sought to determine if rUTI history differentially affected the EHT-associated

387 taxonomic signature by dichotomizing patients by No UTI History and rUTI Remission group

388 membership. We then performed exploratory correlation analysis of creatinine-normalized

389 estrogen metabolite concentrations and the species-level taxonomic profile. Multiple studies have

390 assessed association between urinary and vaginal *Lactobacillus* abundance and host estrogen

391 status with varying conclusions (Anglim et al., 2021; Thomas-White et al., 2020). However, it

392 remains unknown if urinary *Lactobacillus* abundance is directly correlated with host systemic or

393 excreted estrogen concentration. Interestingly, we observed a striking difference in the estrogen-

394 associated taxonomic profile between the No UTI History and rUTI Remission groups (Figure 5

395 E, F). We observed strong correlations between urinary E1 and E2 conjugates and three species

396 of *Lactobacillus, L. crispatus, L. iners, and L. gasseri,* in the No UTI History group, correlations

397 which were not detected in the rUTI Remission group (Figure 5 E, F Figure S 5 E, F). Interestingly,

398 *Bifidobacterium breve*, an *Actinobacterium* often used in probiotics and associated with colon

399 health, exhibited the strongest positive association across estrogen conjugates in the No UTI

400 History group (Figure 5 E, G). This observation was again absent in the urobiomes of the rUTI

401 Remission group. We observed one species, *Anaerococcus prevotii*, which was consistently and

402 significantly negatively associated with urinary estrogens in the No UTI History group (Figure 5 E,

403 Figure S 5 E). Interestingly, fewer and distinct taxa correlated with estrogen conjugates in the

404 rUTI Remission group (Figure 5 F, Figure S 5F). It should be noted that most EHT(+) women in

405 the rUTI Remission group used vEHT, which was not found to be associated with urobiome

406 *Lactobacillus* abundance (Figure 5 A). Taken together, these data demonstrate a direct

407 correlation between excreted urinary estrogen conjugate concentration and urobiome abundance

408   of known protective urogenital lactobacilli, such as *L. crispatus*. The data indicate that the

409   correlation between specific estrogen metabolites and urobiome is dependent on previous rUTI

410   history, suggesting that both rUTI history and estrogen shape the taxonomic ecology of the

411   urobiome.

412

413   **Functional profiling reveals significant differences in the metabolic potential of cohort**

414   **urobiomes.**

415   The genes encoded within a microbial community define the phenotypic capabilities of its

416   members. We used the HUMAnN (v2.0) pipeline to profile the metabolic potential encoded within

417   the cohort urobiomes (Franzosa et al., 2018). We sought to determine if rUTI history leaves a

418   detectable imprint on the functional metabolic potential of the urobiome. We further sought to

419   define the metabolic potential of the urobiomes of women who do not experience UTIs compared

420   to the rUTI Relapse group. Principal component analysis (PCA) performed on the relative

421   abundance of detected encoded metabolic pathways in the three groups identified discriminating

422   clusters that separated many of the rUTI Relapse urobiomes from the rUTI Remission and No

423   UTI History urobiomes in the first two principal components (PCs) (35.39% cumulative variance

424   explained in PC1 and 2) (Figure 6A). These results were consistent with those observed in the

425   taxonomic beta-diversity analysis (Figure 2 E). Analysis of the PCA loadings within the first two

426   PCs found that the rUTI Relapse group ordinated along vectors defined by the enrichment of

427   lipopolysaccharide (LPS) biosynthesis (*n*=4 pathways), demethylmenaquinol-8 biosynthesis,

428   Fucose and Mannose degradation, D-galacturonate degradation, Sucrose degradation, and the

429   TCA cycle (Figure 6B). Interestingly, rUTI Remission and No UTI History groups, which were not

430   discriminated in the first two PCs, tended to ordinate along vectors defined by the genetic

431   enrichment of nucleotide biosynthesis pathways (*n*=8 pathways), L-lysine biosynthesis II, S-

432   adenosyl methionine (SAM) biosynthesis, and UDP-N-acetyl-glucosamine biosynthesis (Figure

433   6B). These data suggest that the large-scale genetic potential of the urobiome is relatively similar

434   between rUTI Remission and No UTI History groups but is dramatically altered during active rUTI.

435   Because dimensional reduction techniques tend to lose fine-scale discriminating features, we

436   next sought to identify unique metabolic enrichments using pairwise comparisons. We used the

437   LEfSe microbial biomarker identification pipeline to identify discriminatory metabolic pathway

438   enrichments between the No UTI History group and the rUTI Remission or rUTI Relapse groups

439   (Segata et al., 2011). First, we tested the hypothesis that rUTI history imparts functional changes

440   on the urobiomes of PM women by comparing the No UTI History and rUTI Remission groups.

441   The analysis identified 49 metabolic pathways differentially enriched between the No UTI History

442   and rUTI Remission groups with an FDR-corrected $P<0.05$ and LDA >2 (Figure 6C). Forty-five
443   discriminatory metabolic pathways were significantly enriched in the rUTI Remission urobiomes
444   and 4 metabolic pathways significantly enriched in the No UTI History urobiomes. The top 40
445   differentially enriched pathways discriminating No UTI History from rUTI Remission urobiomes
446   primarily belonged to carbohydrate metabolism (n=14), electron carrier biosynthesis (n=8), amino
447   acid metabolism (n=5), cell envelope building block biosynthesis (n=4), vitamin and cofactor
448   biosynthesis (n=4), and polysaccharide degradation (n=3) (Figure 6C). While most of the
449   discriminatory carbohydrate metabolic pathways were enriched in the rUTI Remission urobiomes
450   (13/14, 92.9%), we observed an enrichment of D-galactose degradation (Leloir pathway) in the
451   urobiomes of the No UTI History group (LDA=3.06, P=0.028). Interestingly, we observed a strong
452   enrichment of electron carrier biosynthesis pathways in the rUTI Remission urobiomes.
453   Biosynthetic pathways for ubiquinol 7, 8, 9, and 10 as well as menaquinol 6, 9 and 10 and
454   demethylmenaquinol 9 were strongly enriched in the rUTI Remission urobiomes (Figure 6C). L-
455   lysine biosynthesis, L-threonine biosynthesis, and L-tryptophan degradation were observed to be
456   enriched in the No UTI History urobiomes, while L-ornithine biosynthesis and L-arginine
457   degradation were found to be enriched in the rUTI Remission urobiomes. The remaining
458   discriminating metabolic pathways were enriched in the rUTI Remission urobiomes and included
459   cell envelope biosynthesis (e.g. LPS biosynthesis), vitamin metabolism (e.g. coenzyme A
460   biosynthesis), polysaccharide degradation (e.g. 4-deoxy L-threo hex-4-enopyranuronate
461   degradation), cinnamate and hydroxy cinnamate degradation, and ppGpp biosynthesis (Figure
462   6C). These data suggest that the functional metabolic landscape of the urobiome may be
463   significantly altered by rUTI history.
464   We next performed pairwise differential pathway enrichment analysis between the No UTI
465   History and rUTI Relapse groups to test the hypothesis that the functional potential of the
466   urobiome is altering during rUTI. This analysis identified 183 discriminatory metabolic pathways
467   differentially enriched between the No UTI History and rUTI Relapse groups with a P-value less
468   than 0.05 and LDA >2 (Figure 6D). We observed 50 and 133 metabolic pathways significantly
469   enriched in No UTI History and Relapsed rUTI groups, respectively (Figure 6D). In line with the
470   taxonomic enrichment of Gram-negative species, we observed a strong enrichment of
471   biosynthetic pathways for lipopolysaccharide (LPS) within the rUTI Relapse cohort. The top 40
472   most discriminating pathways mainly represent carbohydrate (n=13), nucleotide (n=9), and amino
473   acid metabolism (n=6), as well as cell envelope biosynthesis (n=5) (Figure 6D). We also observed
474   a striking enrichment of diverse carbohydrate degradation and central carbon metabolism
475   pathways, including rhamnose, fucose, glyoxylate, and fructuronate degradation in rUTI Relapse

476   urobiomes (Figure 3D). This was coupled with a significant enrichment of TCA cycle metabolism,

477   particularly 2-oxoglutarate decarboxylase and ferroreductase, within the urobiomes of the rUTI

478   relapse cohort (Figure 3D). Interestingly, only four metabolic pathways involved in carbohydrate

479   metabolism, including glycolysis from glucose and glucose 6-phosphate (G6P), pyruvate

480   fermentation, and N-acetyl glucosamine biosynthesis, were among the top 40 differentially

481   enriched within the No UTI History urobiomes (Figure 3D). There was a strong enrichment of

482   nucleic acid biosynthesis pathways among the urobiomes of the No UTI History group while the

483   Relapsed rUTI group was strongly enriched for nucleic acid degradation pathways (Figure 6D).

484   Differentially enriched amino acid metabolism pathways included strong enrichments for L-lysine,

485   L-threonine, and L-isoleucine biosynthesis in the No UTI History group as well as L-phenylalanine

486   biosynthesis in the rUTI Relapse group (Figure 3D). These results suggest that the metabolic

487   potential of the urobiome is significantly altered during active rUTI with the urobiomes of the rUTI

488   Relapse group displaying the ability to utilize a more diverse nutrient set than the urobiome of

489   women who do not experience UTI.

490

491   **History of rUTI alters the resistance genotype and phenotype of the urobiome**

492   Antibiotic therapy is currently the most prescribed treatment for the management of rUTI.

493   However, resistance to front-line antibiotic regimens, such as TMP-SMX, fluoroquinolones, and

494   nitrofurantoin, is becoming a significant barrier to the successful treatment of rUTI (Malik et al.,

495   2018a). We used the GROOT resistome analysis pipeline to generate a detailed profile of the

496   antimicrobial resistance genes (ARGs) encoded within the urobiomes of the women in each study

497   group (Rowe and Winn, 2018). This analysis detected an aggregated total of 55 distinguishable

498   ARGs distributed among all three patient groups. We observed significantly more ARG's present

499   in the urobiomes of the rUTI Remission (*P*=0.0455) and rUTI Relapse (*P*=0.0302) groups as

500   compared to the No UTI History urobiomes (Figure 7A). Interestingly, there was no significant

501   difference in ARG count between rUTI Relapse and rUTI Remission urobiomes, suggesting that

502   the urobiomes of women with rUTI history, independent of current rUTI status, harbor more ARGs

503   than the urobiomes of women with No UTI History. These results suggest that a history of rUTI

504   leaves an imprint on the underlying resistome of the urinary microbiota in postmenopausal

505   women.

506   To directly assess ARG enrichments associated with rUTI history, we performed differential

507   enrichment analysis using a Bayesian model of proportional enrichment with an objective Jeffrey's

508   prior (Jeffreys, 1946). This analysis identified the TEM β-lactamase family, the sulfonamide

509   resistance genes *sul1* and *sul2*, and the *straA* aminoglycoside 3'-phosphotransferase as

510   significantly differentially enriched in the rUTI Relapse group. We further observed significant

511   enrichments of the aminoglycoside 3'-phosphotransferase genes, *aph(3')-III* and *aph(3')-Ia*, the

512   macrolide resistance gene, *ermB*, the β-lactam resistance gene *mecA*, and the aminoglycoside

513   O-nucleotidyltransferase gene, *ant(6)-Ia*, in the urobiomes of the rUTI Remission group (Figure

514   7B). Interestingly, no ARGs were found to be statistically enriched in the No UTI History group,

515   further suggesting that rUTI history is associated with an accumulation of ARGs within the

516   urobiome. Taken together, these data suggest that urobiomes of women with active rUTI

517   frequently harbor ARGs conferring resistance to the commonly used antibiotic classes: β-lactams,

518   sulfanomides, and aminoglycosides. Of note, the urobiomes of the rUTI Relapse group were

519   found to be significantly enriched for *sul1* and *sul2*, which confer resistance to sulfanomides such

520   as sulfamethoxazole, one of the most common prescribed antibiotic therapies for rUTI (Kõljalg et

521   al., 2009).

522   Genotypic assessments of ARG presence in metagenomes can only predict the phenotype of

523   a microbe or set of microbes belonging to the sampled population (Quince et al., 2017). Given

524   the observed enrichment of ARGs within rUTI Relapse urobiomes, we sought to phenotypically

525   assess the resistance characteristics, by disc diffusion, of 22 unique bacterial uropathogens

526   isolated from 22 women of the actively infected rUTI Relapse group. The bacterial isolates

527   generated from these women represented many known uropathogens including *E. coli* (UPEC)

528   (*n*=15), *K. pneumoniae* (*n*=2), *Klebsiella oxytoca* (*n*=1), *Streptococcus anginosus* (*n*=2), *S.*

529   *agalactiae* (*n*=1), *E. faecalis* (*n*=1), and *S, epidermidis* (*n*=1). We observed 15 isolates exhibiting

530   either complete or intermediate resistance to the penicillin β-lactam, Ampicillin (Amp), and 4

531   isolates exhibiting either complete or intermediate resistance to the cephalosporin β-lactam

532   Cefixime (Cfx) (Figure 7C). Of the isolates exhibiting β-lactam resistance phenotypes, 80%

533   (12/15) and 50% (2/4) were isolated from urobiomes for which WGMS resistome profiling detected

534   ARGs conferring resistance to Amp and CFX, respectively (Figure 7C). We observed that 50% of

535   isolates exhibiting resistance to Trimethoprim/Sulfamethoxazole (TMP/SMX) were isolated from

536   urobiomes for which WGMS resistome profiling detected the presence of the ARGs Sul I/II and

537   DrfA1 (Figure 7C). Aminoglycoside resistance to Gentamycin (Gen), Kanamycin (Kan), Amikacin

538   (Amk), and Streptomycin (Str) was also assayed. We observed 50% (1/2), 60% (5/5), 100% (1/1),

539   87.5% (7/8) of the isolates exhibiting complete or intermediate resistance to Gen, Kan, Amk, and

540   Str, respectively, were isolated from urobiomes for which WGMS resistome profiling detected

541   ARGs conferring resistance (Figure 7C). We observed that 27.3% (3/11) and 33.3% (2/6) of the

542   isolates with complete or intermediate resistance to the fluoroquinolones, Ciprofloxacin and

543   Levofloxacin, were isolated from urobiomes for which WGMS resistome profiling detected ARGs

544   conferring resistance (Figure 7C). However, because GROOT does not detect single nucleotide
545   polymorphisms (SNPs) and fluoroquinolone resistance is often conferred by SNPs in the
546   quinolone resistance-determining regions (QRDR) of the genes encoding gyrase and
547   topoisomerase I, fluoroquinolone ARG identification was less sensitive (Correia et al., 2017).
548   Relatively few urobiomes harbored resistance factors to macrolides, which are not clinically used
549   in the treatment of Gram-negative infections (Arsic et al., 2018). However, while 60% (3/5) of the
550   urobiomes dominated by Gram-positive uropathogens harbored macrolide ARGs, such as *mphA*
551   and *erm* genes, we observed that all Gram-positive isolates were resistant to erythromycin.
552   Tetracycline and phenicol ARGs were less common in the WGMS data and we observed little
553   resistance to these antibiotics among tested isolates (Figure 7C). Taken together, these data
554   suggest an overall fair accuracy of prediction of antibiotic resistance phenotype by WGMS ARG
555   analysis that varies depending on drug class.

556

557   **Discussion**

558   This study provides a robust survey of the urobiome in health and rUTI using a cross-sectional,
559   controlled human cohort of postmenopausal women. This age group is particularly burdened by
560   UTI and rUTI with more than 50% of UTIs progressing into rUTI (Gaitonde et al., 2019). Given
561   that many postmenopausal women experience UTIs, the frequency of progression into rUTI
562   imparts a significant impact on quality of life and if treatment is unsuccessful can lead to life
563   threatening urosepsis. However, few postmenopausal women experiencing rUTI find rapid relief
564   from the cycles of infection and millions of patients suffer the significant medical burden of a
565   chronic disease. A decade of research has identified and characterized the urobiome; the
566   microbial communities inhabiting the urinary tract (Brubaker and Wolfe, 2017; Hilt et al., 2014;
567   Lewis et al., 2013; Price et al., 2019; Siddiqui et al., 2011; Wolfe et al., 2012). Through these
568   research endeavors, it has become evident that the urobiome is involved or affected by urinary
569   tract disease. Given the interconnection between host health and microbiome composition, the
570   urobiome has drawn significant attention in further understanding the pathobiology of UTI and
571   rUTI (Thomas-White et al., 2018). Currently, most metagenomic studies of the female urobiome
572   are performed in mostly younger or mixed age cohorts with little focused representation of
573   postmenopausal women (Vaughan et al., 2021). This lack of focus on postmenopausal women
574   leaves critical gaps in our knowledge of a demographic that is heavily impacted by UTI, rUTI, and
575   other urological diseases. In a focused effort to understand the postmenopausal urobiome
576   ecology and function in rUTI, we used a comprehensive approach, coupling whole genome
577   metagenomic sequencing (WGMS) with advanced urine culture, mass spectrometry-based

578   metabolite profiling, and a combination of traditional and Bayesian statistical methodologies to
579   provide an unbiased, robust survey of the postmenopausal urobiome during rUTI pathology. We
580   generated a unique, controlled human cohort which models both the active infection (rUTI
581   Relapse) and intervening stages of rUTI (rUTI Remission) in postmenopausal women, a
582   demographic which is underrepresented in infection biology, microbiome, and clinical research.

583   While the urobiome has been importantly and robustly characterized using bacterial 16S
584   amplicon sequencing, few WGMS studies have been reported (Ammitzboll et al., 2021; Moustafa
585   et al., 2018; Siddiqui et al., 2011; Thomas-White et al., 2020; Vaughan et al., 2021). This unbiased
586   approach allows for the profiling of prokaryotic, eukaryotic, archaeal, and viral taxa present in the
587   sample. Beyond taxonomic profiling, WGMS also allows the characterization of the functional,
588   genetic content of the urobiome (Neugent et al., 2020; Quince et al., 2017). By looking beyond
589   the species-level taxonomic composition, we can define metabolic pathways, virulence factors,
590   and genes critical in urobiome health and urinary tract pathology using WGMS.

591   Taxonomic profiling by WGMS is a very sensitive technique to profile the ecology of a microbial
592   niche. However, in silico analysis of metagenomic sequencing data can suffer from database bias
593   and does not have the ability to distinguish living from dead microbial community members
594   (Quince et al., 2017). To address these issues, we combined a bioinformatics analysis of deeply
595   sequenced WGMS dataset with advanced urine culture based on a modified version of the
596   previously reported EQUC protocols (Hilt et al., 2014). This hybrid approach was able to confirm
597   the presence of 93.9% of the genera observed at >5% relative abundance in the aggregate
598   metagenomic data. Our culturing efforts also led to the generation of an extensive biobank of 904
599   speciated and cryopreserved pure isolates, a unique resource for the field.

600   Our taxonomic analysis of the three groups determined that bacteria make up the majority of
601   the non-human, non-viral metagenome of the postmenopausal urobiome. The detected bacterial
602   taxa across the three cohort groups were mainly members of the Phyla *Firmicutes*, *Actinobacteria*,
603   and *Proteobacteria*. The main uropathogen detected in the rUTI Relapse group was UPEC, an
604   observation consistent with the fact that UPEC is responsible for 75% of all UTIs (Flores-Mireles
605   et al., 2015). The urobiomes of the No UTI History and rUTI Remission groups were either
606   dominated by a single bacterial species or were relatively diverse. Both the No UTI History and
607   rUTI Remission group exhibited similar subsets of patients dominated by species of vaginal
608   *Lactobacillus*, *Gardnerella*, and *Bifidobacterium*, which mainly consisted of the species *L.*
609   *crispatus, L. gasseri*, *L. iners*, *B. breve*, *B. dentium*, *B. longum*, and *G. vaginalis* (Ravel et al.,
610   2011). These data support the observations by Thomas-White et al. of an interconnected
611   urogenital microbiome (Thomas-White et al., 2018). Many of the diverse urobiomes exhibited the

612   presence of streptococci, such as, *S. agalactiae* and *S. anginosis*. These species of streptococci

613   are not only known uropathogens but have also been linked to vaginal dysbiosis (Gilbert et al.,

614   2021). We detected relatively small abundances of fungal and archaeal taxa. Currently, little is

615   known about the impact or function of fungal and archaeal taxa in the urobiome, particularly of

616   postmenopausal women. This represents an area of significant interest for future research.

617   Ecological modeling with alpha- and beta-diversity metrics found that the urobiomes of the rUTI

618   Relapse group exhibited a less diverse and taxonomically distinct signature than those of the No

619   UTI History and rUTI Remission groups. This can be attributed to uropathogen domination of the

620   urobiome during infection. A critical question for future research will be to determine the

621   longitudinal impact of infection on the underlying urobiome. Interestingly, the No UTI History and

622   rUTI Remission groups were virtually indistinguishable in alpha- and beta-diversity measures.

623   This observation indicates that the large-scale taxonomic structure of the urobiome is relatively

624   similar between women with and without rUTI history. This analysis does not measure fine-scale

625   taxonomic changes as information is lost during the dimensionality reduction techniques used for

626   beta-diversity assessment. Further, this analysis does not consider possible changes in the

627   functional potential of the urobiome, such as metabolic enrichment or antimicrobial resistance.

628   Within microbial communities, co-occurrence is often observed between different taxa that

629   presumably mutually benefit one another. The opposite, *e.g.,* mutual exclusivity is also observed

630   among microbial communities and is attributed to inter-taxa competition during the colonization

631   of a given niche (Faust et al., 2012). Little is currently known of taxonomic associations within the

632   urobiome of postmenopausal women. Interestingly, we observed three non-interacting bacterial

633   co-occurrence networks in the urobiomes of the No UTI history and rUTI Remission cohort

634   groups. These networks include a cluster of genera known to be members of the human gut

635   microbiome: *Bacteroides, Collinsella, Lachanospiracea, Eggerthella, Eubacterium, Blautia, and*

636   *Subdoligranulum*. The largest co-occurring bacterial cluster consisted mainly of known members

637   of the female urogenital microbiome. Interestingly, these taxa clustered strongly around the

638   genus, *Peptoniphilus*, a member of the vaginal microbiome believed to be associated with

639   bacterial vaginosis (Marrazzo et al., 2008; Onderdonk et al., 2016). The third cluster detected was

640   a pairwise association between the genera *Gardnerella* and *Atopobium*, two taxa again known to

641   be associated with bacterial vaginosis (Bradshaw et al., 2006). Of note, we did not detect any co-

642   occurrence networks that included *Lactobacillus*. We hypothesize that this is due to the tendency

643   for lactobacilli to dominate the microbial community and will require further research to

644   mechanistically understand. A body of work has characterized the ability of vaginal lactobacilli to

645   protect against bacterial vaginosis by the secretion of D-lactate among other mechanisms (Atassi

646   et al., 2019; Daniel S. C. Butler, 2016; Edwards et al., 2019; Jespers et al., 2015). In the vaginal

647   environment, the major *Lactobacillus spp.*, *L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*, are

648   critical for maintaining vaginal pH and resisting invasive pathogens associated with bacterial

649   vaginosis (Ravel et al., 2011). It is still largely unknown if these same *Lactobacillus* species play

650   a similar protective role in the urobiome.

651   We hypothesized that rUTI would leave a detectable taxonomic imprint of the underlying

652   urobiome. Our initial alpha- and beta- diversity analysis did not detect any significant differences

653   between the No UTI History and rUTI Remission groups. However, this analysis is not sensitive

654   to individual taxa-level enrichments. Differential taxonomic enrichment detected genus- and

655   species-level taxonomic biomarkers enriched in women with rUTI history. We observed genus-

656   level enrichments for many of the known uropathogenic species in the rUTI Remission group as

657   compared to the No UTI History group. These enriched uropathogenic genera included *Klebsiella*,

658   *Escherichia*, and *Enterococcus*. It is unclear whether these uropathogen enrichments are

659   representative of persisting populations of previous infections. The enrichment of the genus

660   *Lactobacillus* in the No UTI History group was also expected given the role many vaginal

661   lactobacilli play in protection from bacterial vaginosis (Jespers et al., 2015). At the species-level,

662   we observed that *L. vaginalis* and *L. crispatus,* two species associated with vaginal health, were

663   strongly enriched among the women of the No UTI History group (Jespers et al., 2015).

664   Conversely, in the rUTI Remission group we observed strong species-level enrichments of

665   *Ureaplasma parvum*, *Anaerococcus hydrogenalis, E. faecalis, S. hominis, Peptoniphilus*

666   *lacrimalis*, and *Anaerococcus prevotii,* suggesting that a history of rUTI changes the underlying

667   taxonomic structure of the urobiome. Interestingly a 2021 report by Vaughan et al. studying the

668   urobiome of postmenopausal women with rUTI identified multiple taxonomic differences

669   associated with rUTI compared to controls, including differences in the order, *Bacteroidales*, and

670   the family, *Prevotellaceae* (Vaughan et al., 2021).

671   Postmenopausal women are disproportionately affected by rUTI (Glover et al., 2014). Evidence

672   suggests that approximately 50% of UTIs experienced by postmenopausal women will progress

673   into rUTI (Glover et al., 2014; Ikaheimo et al., 1996). These associative observations beg

674   questions about menopausal changes which may make PM women more susceptible to rUTI.

675   One major physiological change during menopause is a dramatic decrease in circulating levels of

676   the sex hormones in the estrogen family. Menopause-associated decreases in estrogen levels

677   are associated with many changes in physiology, including vasomotor symptoms, sleeping

678   disorders, sexual health, urogenital discomfort, and quality of life (Fait, 2019). Estrogen hormone

679   therapy (EHT) is a common medical intervention many PM women use to treat discomfort

680 associated with menopause (Fait, 2019). Our analysis of cohort-associated metadata found a
681 strong link between EHT use and the presence of *Lactobacillus* in the urobiome of non-infected
682 patients. These observations support those made by Thomas-White et al. and are of particular
683 interest to research endeavors seeking to identify possible UTI-protective members of the urinary
684 and urogenital tract microbial communities (Thomas-White et al., 2020). Lactobacilli control the
685 local chemical environment of the vagina and are responsible for not only maintaining an acidic
686 pH, but also secrete large amounts of the antimicrobial D (-) lactate isomer (Amabebe and
687 Anumba, 2018). A 2019 report by Edwards et. al showed that vaginal *L.crispatus* directly confers
688 protection against *Chlamydia trachomatis* infection through the secretion of large amounts of D (-
689) lactate (Edwards et al., 2019). Given the prevalence of vaginal species of lactobacilli (*i.e. L.*
690 *crispatus, L.* gasseri) in the urobiome, it is possible that these species play a similar role in
691 preventing uropathogen colonization the urinary tract, and that disruptions in urogenital lactobacilli
692 populations may increase susceptibility to UTI and rUTI (Thomas-White et al., 2018). In the
693 present study, we found that the two largest sources of variances in taxonomic ecology between
694 groups were the genera *Escherichia* and *Lactobacillus*. The variance explained by *Escherichia*
695 abundance can be attributed to UPEC infection whereas we the presence of *Lactobacillus spp.*
696 was strongly predicted by EHT use. We further identify *L. crispatus and L. vaginalis* as uniquely
697 associated with EHT use. Interestingly, EHT(-) women exhibited urobiome enrichment of
698 streptococci, such as *S. infantis* and *S. mitis/oralis/pneumoniae*. We also observed that EHT(-)
699 women were enriched for *A. vaginae*, a Gram-positive species associated with *G. vaginalis* in
700 bacterial vaginosis. These differential taxonomic enrichment signatures may suggest a level of
701 urobiome dysbiosis among the postmenopausal women not using EHT. Future work is needed to
702 understand the taxonomic perturbations of the urobiome in rUTI pathology.
703   The cohort studied here exhibited women using three different modalities of EHT (oral,
704 transdermal patch, and vaginal cream). Our findings suggest that oral and patch EHT modalities
705 are associated with significant enrichment of *Lactobacillus* in the urobiome. However, we
706 observed a large amount of variance in the abundance of *Lactobacillus* among patients taking the
707 vaginal modality of EHT. We hypothesized that EHT modalities may differ in dosage, composition,
708 patient compliance, or primary metabolism. Oral and patch EHT modalities used by women in the
709 cohort contained 17β-estradiol as the active ingredient. However, vaginal EHT modalities include
710 two estrogen compositions. 64.7% of vaginal EHT(+) women used a 17β-estradiol cream while
711 35.3% of vaginal EHT(+) women used conjugated estrogens creams, which contain mainly equilin
712 sulfate and estrone sulfate as the active ingredients (Whittaker et al., 1980). Circulating estrogens
713 are metabolized in the liver and conjugated with polar chemical groups, such as sulfates or

714   glucuronides, for urinary excretion (Raftogianis et al., 2000). Our data suggests that both oral and

715   patch EHT modalities are associated with elevated excreted urinary estrogen conjugates, while

716   vaginal EHT modalities were not. In a 2021 report, Anglim et al. found no difference in urinary

717   lactobacilli among postmenopausal women with and without rUTI taking vaginal EHT compared

718   to baseline controls (Anglim et al., 2021). However, Thomas-White et al. reported a significant

719   enrichment of lactobacilli in the bladders of postmenopausal women with overactive bladder

720   symptoms using vaginal EHT (Thomas-White et al., 2020). These results along with our report

721   suggest nuances in the association between urinary lactobacilli and EHT that merit future

722   mechanistic research.

723   We further performed an exploratory correlation analysis to globally determine the taxa

724   associated with excreted urinary estrogen conjugates. Interestingly, we found that disease state

725   affected the results of this analysis with the No UTI History and rUTI Remission groups exhibiting

726   different patterns of taxonomic association with excreted urinary estrogen. The species *B. breve,*

727   *L. iners*, *L. crispatus*, and *L. gasseri*, correlated with urinary estrogen conjugates in the No UTI

728   history group only. We did not observe strong taxonomic associations with urinary estrogens in

729   the rUTI Remission group. We hypothesize that this may be due to the fact the rUTI Remission

730   EHT(+) women were predominantly taking vaginal EHT, which we observe to not be strongly

731   associated with urinary lactobacilli. This observation may also be attributable to perturbations of

732   the underlying urobiome by the history of infection and treatment in the rUTI Remission patients.

733   The use of WGMS allowed for the assessment of the total genetic potential of the urobiome

734   between disease states (Quince et al., 2017). Our analysis found that cohort urobiomes cluster

735   similarly by function as they do by taxonomy. Of note, the metabolic pathways facilitating this

736   distinct clustering of the rUTI relapse group away from the No UTI history and rUTI Remission

737   groups were mainly LPS biosynthesis, the TCA cycle, sugar utilization, and biosynthesis of

738   electron carriers, such as demethylmenaquinol-8. This pattern of discrimination may represent a

739   metabolic signature of carbon sources and central metabolism shared among uropathogen-

740   dominated microbial communities. Pairwise differential enrichment analysis revealed that

741   compared to the urobiomes of the No UTI history group, the urobiomes of the rUTI Remission

742   group exhibited greater metabolic potential for the degradation of carbohydrates, biosynthesis of

743   electron carriers, production of cell envelope, and polysaccharide metabolism among others.

744   These data suggest that a history of rUTI, even in the absence of an active infection, alters

745   urobiome functional potential. Future work may focus on understanding whether these alterations

746   are signatures of dysbiosis or sensitization factors for future rUTI Relapse events. Interestingly,

747   we observed that L-lysine biosynthesis II (acetylase variant pathway observed among *Firmicutes*

748 (Bartlett and White, 1985) was very strongly enriched in the No UTI History urobiomes, a
749 discriminatory feature that was also found in comparison to the active infections of the rUTI
750 Relapse urobiomes. Finally, the No UTI History urobiomes exhibited strong enrichments for
751 nucleotide biosynthesis pathways while the rUTI Relapse cohort were enriched for nucleotide
752 degradation pathways. These data may suggest critical differences in the metabolic phenotypes
753 of commensal and uropathogenic members of the urobiome.

754 A critical functional analysis enabled by WGMS is resistome profiling. This is essential for rUTI,
755 which is most often managed by antibiotic therapy (Waller et al., 2018). The cyclic nature of rUTI
756 treated by frequent antibiotic regimens is thought to facilitate the evolution of antibiotic resistance
757 within the urobiome and among uropathogens. Consequences of acquired antibiotic resistance
758 include treatment failure, a need for urgent escalation of therapeutic strategies, and if
759 unsuccessful, cystectomy or life-threatening urosepsis. Currently, there is no single dataset
760 profiling the rUTI-associated antimicrobial resistance genes (ARGs) encoded in the urobiomes of
761 a controlled cohort of postmenopausal women. As expected, we found that the resistome of the
762 infected urobiomes of the rUTI Relapse contained significantly more ARGs than the No UTI
763 History group. Interestingly, we also found that the rUTI Remission urobiome also harbored a
764 significantly larger resistome than the No UTI History urobiomes. This suggests that even in the
765 absence of infection, a history of rUTI is associated with significantly more urobiome ARGs than
766 found in healthy comparators with no lifetime history of UTI. rUTI Relapse urobiomes exhibited
767 significant enrichments for the TEM β-lactamase alleles. It should be noted that the analytical
768 pipeline used was not able to distinguish between TEM alleles. Interestingly, rUTI Relapse
769 urobiomes also exhibited significant enrichments for ARGs which confer resistance to
770 sulfonamide antibiotics, such as the frontline antibiotic, sulfamethoxazole. Phenotypic analysis
771 showed that bacterial isolates generated from the rUTI Relapse cohort exhibited similar antibiotic
772 resistance phenotypes as what WGMS predicted. These data suggest that, at least among
773 uropathogenic bacteria, antibiotic resistance predicted by WGMS resistome profiling was
774 reasonably consistent with observed phenotypes. One major limitation of currently available
775 resistome profiling pipelines for metagenomic datasets; however, is that they are limited to
776 detecting ARGs and do not detect antibiotic resistances conferred by point mutations. This
777 limitation makes detection of quinolone resistance by this method incomplete. Further study is
778 needed to understand the evolution of the resistome associated with rUTI

779 This work aimed to survey the taxonomic and functional ecology of the urobiome associated
780 with rUTI and rUTI history through WGMS analysis of a clinically relevant group of
781 postmenopausal women. These robust sequencing and analytical datasets, associated clinical

782 metadata, and extensive microbial isolate collection represent an important resource for the field.

783 Our analysis has identified taxonomic and functional biomarkers associated with rUTI history, as

784 well as a set of putatively protective taxa correlated with particular modalities of EHT. We also

785 find that a history of rUTI significantly alters the number of and composition of resistance genes

786 encoded in the urobiome, an observation with implications towards future and current treatment

787 paradigms rUTI. Taken together, this work provides robust foundation for further mechanistic

788 studies of how the urobiome is associated with rUTI pathobiology and perhaps may serve as a

789 resource for the development of urobiome-aware alternative therapies for a disease that affects

790 millions of women worldwide.

791

792 **Methods**

793 **<u>Patient Recruitment and Cohort Curation</u>**

794 The current study is approved under IRBs STU032016-006 (University of Texas Southwestern

795 Medical Center) and 19MR0011 (University of Texas at Dallas). To estimate the number of

796 patients needed to enroll into each cohort group and predict statistical power, we performed a

797 series of power analyses using the 'pwr' package (https://github.com/heliosdrm/pwr) in the R-

798 statistical language based on ranging effect sizes from small to large for both multivariate analysis

799 of 3 groups with equal sample size and pairwise-based comparisons of two groups. Balancing

800 cost and clinical feasibility with predicted statistical power, we chose a sample size of 25 for each

801 cohort group. Patients were recruited from the Urology Clinic at University of Texas Southwestern

802 Medical Center between April 2018 and October 2019. Written informed consent was obtained

803 from each patient prior to recruitment into the study cohorts. All patients were postmenopausal

804 females. The following set of exclusion criteria were used to initially screen patient's candidacy

805 for enrollment into the cohort: pre- or perimenopausal status; antibiotic exposure within the 4

806 weeks prior to urine sample donation unless an active infection was detected by culture; pelvic

807 malignancy or history of pelvic radiation within 3 years before urine sample donation; most recent

808 post void residual (PVR) greater than 100 mL; greater than stage 2 prolapse; pelvic procedure for

809 incontinence within 6 months prior to urine sample donation; use of intermittent catheterization;

810 neurogenic bladder; any upper urinary tract abnormality which may explain rUTI; and Diabetes

811 Mellitus (DM) type 1 or 2. All urine samples were obtained by clean-catch midstream urine

812 collection and therefore were representative of the urogenital microbiome, rather than specifically

813 just the bladder microbiome. Patients were educated about the cleaning and urine collection

814 needs for this sampling technique prior to urine collection. Urine samples were stored at 4°C for

815 no more than 4 hours before sample processing, aliquoting, and biobanking at -80°C.

816 Patients were further vetted by self-reporting of UTI history, mining clinical history from
817 electronic patient records, clinical (standard) urine culture, and urine culture on Chromogenic agar
818 (BBL CHROMagar Orientation, BD). Clinical urine culture was performed on samples from all
819 patients with active UTI symptoms by the Clinical Microbiology Laboratory at UT Southwestern
820 Medical Center. Group assignment criteria were as follows. No UTI History: no self-reported or
821 clinical history of UTI, no UTI symptoms at the time of urine collection. rUTI Remission: recent
822 history of rUTI, no UTI symptoms at time of sample collection. rUTI Relapse: recent history of
823 rUTI, active UTI symptoms at time of urine collection, positive clinical urine culture. Positive urine
824 culture was defined as $>10^4$ bacterial CFU/mL.

825

826 **Advanced Urine Culture, Isolate Identification, and Isolate Biobanking**
827 Glycerol-stocked urine samples (stored at -80ºC) were thawed at room temperature, and then
828 diluted 1:3 and 1:30 in sterile 1X Phosphate Buffered Saline to adjust plating density for high and
829 low biomass samples. 100 µl of urine from each dilution was plated onto blood agar plates (BAP),
830 CHROMagar Orientation, De Man, Rogosa, and Sharpe (MRS) agar, Rabbit BAP (R-BAP), BD
831 BBL CDC anaerobe blood agar (CDC AN-BAP), and Columbia Colistin Naladixic Acid Agar
832 (CNA). Following plating, BAP was incubated in ambient and 5% CO_2 atmospheres, CHROMagar
833 Orientation in 5% CO_2, MRS and R-BAP in microaerophilic conditions, BD BBL CDC anaerobe
834 blood agar (CDC AN-BAP) in microaerophilic and anaerobic conditions and CNA in all four
835 atmospheric conditions. Plates were incubated at 35°C for 4 days in the respective atmosphere.
836 It should be noted that we were unable to culture *Gardnerella spp.* using these methods. However,
837 WGMS profiling frequently detected *G. vaginalis* in the sampled urobiomes. For targeted isolation
838 of *Gardnerella spp.*,100 µl urine was plated onto Human polysorbate-80 (HBT) bilayer medium in
839 microaerophilic atmosphere for 3 days. To isolate fungal species, 100 µl urine was plated onto
840 Brain Heart Infusion Agar supplemented with 20 g/L glucose and 50 mg/µl of chloramphenicol
841 (BHIg-Cam) and incubated at 5% CO2 for 3 days.
842 Bacterial identification was performed by PCR amplification and Sanger sequencing of the 16S
843 rRNA gene from well-isolated colonies as described previously (De Nisco et al., 2019). Briefly,
844 16S rRNA gene was amplified using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and
845 1492R (5'-GGTTACCTTGTTACGACTT-3') by colony PCR (Vaishnava et al., 2011) using
846 DreamTaq Master Mix (ThermoFisher Scientific) and 0.2µM primers. Amplicon size was
847 confirmed on 1% agarose gel, followed by gel purification (Bio basic) and Sanger Sequencing
848 (Genewiz) using the 8F primer. Sequences were analyzed using BLASTn against the NCBI 16S
849 ribosomal RNA (Bacteria and Archaea) database.

850   For fungal identification, ITS1 and ITS2 regions were amplified using the primer sequences
851   ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS2: 5'-GCTGCGTTCTTCATCGATGC-3' from
852   well-isolated colonies and Sanger sequenced (Genewiz). Sequences were analyzed using
853   BLASTn against the NCBI ITS from Fungi type and reference material database.

854   All the isolated and taxonomically identified isolates (n=904) were assigned a distinct ID and
855   biobanked at -80ºC in glycerol. The isolates were grown in Brain Heart Infusion broth, Tryptic Soy
856   Broth (BD 211825), MRS broth or NYCIII according to their growth preferences and stocked in
857   16% sterile glycerol for long-term storage at -80C.

858

859   **Metagenomic DNA Isolation, Library Construction, and Sequencing**

860   Prior to WGMS, we assessed the quality and reproducibility of 3 metagenomic DNA extraction
861   techniques: a modified genomic DNA (gDNA) isolation based on the Qiagen blood and tissue
862   DNAeasy Kit, the Zymo Research DNA/RNA microbiome miniprep, and a modified
863   phenol/chloroform/isoamyl alcohol extraction as demonstrated by Moustafa et al. (Moustafa et al.,
864   2018) After assessing the quality and yield of metagenomic DNA isolated using the three
865   methods, we chose the Zymo Research method. Urine samples were allowed to thaw on ice at
866   4°C overnight. 10-20 mL of urine was centrifuged for 15 minutes at 4000 x g at 4°C. Urine pellets
867   were resuspended in 750 µL of DNA/RNA Shield (Zymo Research), transferred to a bead beating
868   tube, and subjected to ten 30 sec cycles of mechanical bead beating, with 5 min cooling between
869   each cycle. After mechanical lysis, the maximum volume of sample was collected and transferred
870   to a new microcentrifuge tube with DNA/RNA lysis buffer (Zymo). Nucleic acids were purified via
871   the Zymo Research DNA/RNA microbiome miniprep kit per the manufacturer's instruction. Elution
872   of DNA from the column was performed in nuclease-free water and each column was eluted twice
873   to maximize DNA recovery. As a control to internally assess gDNA extraction efficiency and
874   WGMS limit of detection (LOD), gDNA was concurrently extracted from commercially available
875   community standards (ZymoBiomics) using the same methods. gDNA was also extracted from
876   nuclease-free water to account for kit and environmental contamination. All DNA samples were
877   subjected to 16S rRNA gene amplification by PCR and visualized by agarose gel electrophoresis
878   to ensure microbial DNA was present before proceeding with WGMS. DNA yield and purity for all
879   samples were assessed by agarose gel electrophoresis, and by fluorescence-based Qubit
880   quantitation of DNA, RNA, and protein. Prior to library preparation the DNA concentration of each
881   sample was normalized and 20pg of spike-in gDNA was added (Zymo Research High Bacterial
882   Load Spike-in), which contains gDNA from the bacterial species *Imchella halotolerans* and
883   *Allobacillus halotolerans*, which are known to not be associated with humans.

884   WGMS was performed at the University of Texas at Dallas Genome Center using 2x150 bp

885   paired-end reads on a Illumina NextSeq 500. Library preparation was performed using Nextera

886   DNA Flex kit. Library preparation of the entire cohort and community standard and water controls

887   was distributed over 2 batches with overlapping samples. All samples were sequenced using

888   2x150 base pair paired-end sequencing in high output mode with a target of ≥50 million paired

889   end reads per sample.

890

891   **Bioinformatic Analyses**

892   All taxonomic, functional, and resistome bioinformatic analyses were performed on an in-house

893   Dell PowerEdge T630 server tower with 256GB RAM, 12 core Intel Xenon processor with 16TB

894   storage capacity or at the Texas Advanced Computing Center (TACC).

895   **Data Preprocessing**

896   The fastq files were checked for read quality, adapter content, GC contents, species

897   contamination using fastqc (v0.11.2) and fastq_screen (v0.4.4) (Andrews, 2015; Wingett and

898   Andrews, 2018). Low-quality reads (a quality score of less than Q20) and adapter were removed

899   using Trim galore (v 0.4.4) (Krueger). Human DNA sequences were removed using KneadData

900   (Huttenhower).

901   **Taxonomic Profiling, Ecological Modeling, and Co-Occurrence Analysis**

902   The taxonomic assignment and estimation of composition of microbial species present in each

903   sample was performed using MetaPhlAn2 (Segata et al., 2012). MetaPhlAn2 estimates the

904   relative abundance of species by mapping the metagenomic reads against a clade specific marker

905   gene database. The database consists of bacterial, archaeal, viral and eukaryotic genomes. We

906   further used merge_metaphlan_tables module of MetaPhlAn2 to combine the relative abundance

907   estimates of samples in a cohort into one table.

908   To identify kit, environmental, and background contaminating taxonomic signals, we sequenced

909   a water sample which was randomly inserted into the metagenomic DNA preparation protocol.

910   Sequencing and taxonomic analysis of this sample revealed known kit and environmental

911   contaminants, such as *Delftia*, *Stenotrophomonas*, *Ralstonia*, *Bradyrizobium*, and others (Salter

912   et al., 2014). Unless a known member of the human microbiome, these taxa were censored from

913   the entire dataset. We further assessed the WGMS limit of reliable detection using a commercially

914   available log community standard (ZymoBiomics), which is composed of multiple Gram-positive

915   and Gram-negative bacterial and fungi. We observed a strong linear correlation between the

916   theoretical and observed relative abundance above 0.001%. We therefore set a relative

917   abundance threshold of 0.001% for a taxon to be considered as detected within a sample.

918 Species-level MetaPhlAn 2 taxonomic assignments were not included in analysis if they were
919 "unclassified".

920 Alpha-diversity analysis was performed at the species-level using phyloseq (version 1.16.2)
921 (McMurdie and Holmes, 2013). Beta-diversity analysis was performed using DPCoA on the
922 species-level taxonomic relative abundance dataset using phyloseq (version 1.16.2)(McMurdie
923 and Holmes, 2013). Taxonomic co-occurrence was performed with CCREPE pipeline using the
924 Pearson correlation and compositionally corrected P-values
925 (https://github.com/biobakery/biobakery/wiki/ccrepe#22-ccrepe-function). Network analysis of
926 taxonomic co-occurrences was performed using CytoScape (Version 3.8.2) with edges defined
927 by the correlation coefficients between taxa nodes.

928 **Functional Metabolic Profiling**

929 Functional metabolic profiling was performed using HUMAnN 2.0 (Franzosa et al., 2018).
930 HUMAnN2 uses a tiered approach to identify the functional profile of microbial communities.
931 Firstly, it maps the sample reads to clade specific markers and creates a database of pangenomes
932 for each sample. In the second tier, it performs the nucleotide level mapping of samples reads
933 against pangenome database. Lastly, a translated search against Uniref90 is performed for
934 unaligned reads in each sample (Suzek et al., 2015). The output result is the mapping of reads to
935 gene sequences with known taxonomy. The reads are normalized to gene sequence length to
936 give an estimate of per-organism and community total gene family abundance. Next, gene
937 families are analyzed to reconstruct and quantify metabolic pathways using MetaCyc (Caspi et
938 al., 2018). Different modules of HUMAnN such as humann2_join_table and
939 humann2_renorm_table were used to merge the pathway abundance of all the samples in a
940 cohort and normalize the abundance to cpm respectively. We filtered the results to only include
941 pathways whose taxonomic range included bacteria. We further censored pathways which were
942 specifically associated with a particular taxon due to database bias toward commonly isolated
943 and studied species. PCA of functional pathways was performed on the pathway level relative
944 abundance dataset using factroextra (https://cran.r-
945 project.org/web/packages/factoextra/readme/README.html). Pathway differential abundance
946 analysis was performed using LEfSe (Segata et al., 2011) on the pathway-level relative
947 abundance dataset. LEfSe uses Kruskal Wallis and Wilcoxon tests to find the differential
948 pathways between microbial communities. Finally, it uses LDA model to rank the pathways.

949 **Resistome Profiling and ARG Enrichment**

950 We used the GROOT (Graphing Resistance Out Of meTagenomes) to generate a profile of
951 antimicrobial resistance genes within the urobiomes of the present study (Rowe and Winn, 2018).

952　The default database ARG-ANNOT was used for alignment of the metagenomics reads.

953　Subsequently GROOT report command was used to generate a profile of antibiotic resistance

954　genes at a read coverage of 90%. Filtering of the GROOT results was performed to insure high

955　confidence in ARG presence within the urobiomes. We used a conservative cutoff of a sufficient

956　amount of reads to generate 10x coverage of an ARG to qualify its detection within a urobiome.

957　We further collapsed alleles of the β-lactamase genes TEM, CTX, OXA, OXY2, SHV, and *cfxA* as

958　well as the aminoglycoside ARG Aac3-IIa and Aac3-IIe alleles into single gene-level features to

959　account for multiple-mapping reads.

960　Bayesian modeling of the resistome data was performed as follows. Resistome data for the

961　three cohorts (Never = 1, Remission = 2, Relapse = 3) consisted of 186 antimicrobial resistance

962　genes (ARG) which were collapsed into family-level genes ($G = 55$). Each cell in the data set

963　contained a binary indicator of no detection (0) or detection (1) of the resistance family-level gene

964　within each patient sample such that $x_{gik} = \{0,1\}, g = 1, \cdots, 55, i = 1, \cdots, 25, k = 1,2,3$ indicates no

965　detection or detection of resistance family-level gene g respectively for sample $i = 1, \dots, 25$ in

966　cohort k.

967　A Bayesian Beta-Bernoulli model with Jeffreys prior was used to model the posterior

968　distributions of group proportions and pairwise differences for the G family-level genes $G =$

969　$1, \dots, 55$. Three posterior inferences were performed. First, we removed any family-level genes

970　that had no significant pairwise contrasts using 95% credible intervals as criteria. We determined

971　that a significant family level-gene does not have zero contained in a 95% credible interval for at

972　least one pairwise contrast. Second, we computed the posterior probability and Bayes Factor (BF)

973　to make inferences on each pairwise contrast of cohort proportions of only the significant family-

974　level genes. The BF computed for each contrast represented the odds of H_1: "at least one cohort's

975　proportion for gene g is different" in favor of H_0: $\omega_{g1} = \omega_{g2} = \omega_{g3}$.

976　**Taxonomic Biomarker Analysis**

977　We applied two methods of taxonomic differential abundance analysis employing the robust and

978　widely used LEfSe pipeline as well as BMDA, a recently described Bayesian model of differential

979　abundance (Li et al., 2019). LEfSe analysis was performed as previously described (Segata et

980　al., 2011). For the BMDA model we first applied the quality control step (detailed in the supplement

981　of Li et al., 2019) to the raw count data. We then fitted the BMDA model, which is a novel Bayesian

982　hierarchical framework that uses a zero-inflated binomial model to model the raw count data and

983　a Gaussian mixture model with feature selection to identify differentially abundant taxa. The

984　BMDA can fully account for zero-inflation, over-dispersion, and varying sequencing depth. We

985　chose weakly informative priors on all parameters of the model to avoid biased results. For model

986 fitting and posterior inference, BMDA implements the Metropolis-Hastings algorithm within a
987 Gibbs sampler. The marginal posterior probability of inclusion (PPI) was used to identify the set
988 of discriminating taxa between the control and disease groups. Marginal PPI is the proportion of
989 MCMC samples in which a taxon is selected to be discriminatory if it is greater than a pre-specified
990 value. We chose a threshold such that the expected Bayesian false discovery rate (FDR) was
991 less than 0.05.

992

993 **Antibiotic susceptibility testing**

994   Assessment of antibiotic (abx) susceptibility was performed via the Kirby-Bauer disk diffusion
995 susceptibility test (Hudzicki, 2009). Antibiotic disks were prepared by aliquoting 10uL of antibiotic
996 stock (GEN 1mg/ml, AMP 1mg/ml, CIP 0.5mg/ml, LVX 0.5mg/ml, ERM 1.5mg/ml, CHL 3mg/ml,
997 TMP/SMX 1.25/23.75mg/ml, NIT 30mg/ml, DOX 3mg/ml) onto the disk in a sterile petri dish and
998 drying at room temperature in the dark. Vehicle control disks were prepared similarly using the
999 diluents of each antibiotic. Strains were streaked from frozen glycerol stocks onto CHROMagar
1000 or Blood Agar (species dependent) and incubated overnight at 37ºC in ambient conditions or 35ºC
1001 in 5% CO2. Single, well-isolated colonies were inoculated into 3 mL Brain-Heart-Infusion broth
1002 and incubated at the respective atmospheric conditions for 16 – 18 hours. After incubation,
1003 cultures were normalized to 0.5 McFarland standard, washed, and resuspended in sterile 1X
1004 Phosphate-Buffered Saline (PBS). 150 μL of standardized culture were pipetted onto 150 mm
1005 Mueller-Hinton Agar plates and spread using sterile glass beads. Plates were dried in sterile
1006 conditions before abx-impregnated disks were placed on the surface of the agar. *E. coli* strain
1007 ATCC25922 was used for quality and vehicle controls. Sterile 1X PBS was plated as sterility
1008 control. Plates were incubated inverted per the recommendations of Clinical and Laboratory
1009 Standards Institute (CLSI) M100-ED30: 2020 Performance Standards for Antimicrobial
1010 Susceptibility Testing, 30th Edition
1011 (https://clsi.org/standards/products/microbiology/documents/m100/). After incubation,
1012 antimicrobial susceptibility was evaluated by measurement of the zone of inhibition and using
1013 CLSI established zone diameter breakpoints.

1014

1015 **Liquid Chromatography Mass Spectrometry Measurement of Estrogen Metabolites**

1016   Direct measurement of urinary estrogen metabolites was performed via a modification of
1017 previously reported methods(van der Berg et al., 2020). Briefly, urine (500 μL) was diluted and
1018 spiked with 100 ng stable isotope-labeled internal standards of d3-Estrone 3-Glucuronide and d4-
1019 Estradiol 3-Sulfate. Diluted and spiked samples were loaded onto an equilibrated Phenomenex

1020 C18 cartridge for solid phase extraction to separate conjugated estrogens. Following aqueous
1021 methanolic extraction of estrogen conjugates and non-polar extraction of free estrogens with
1022 methanolic acetone, fractions were dried by vacuum centrifugation and prepared for LC-MS/MS
1023 analysis. Estrogen conjugates (sulfates and glucuronides) were directly assayed using a curated
1024 and optimized MRM library by LC-MS/MS.

1025 High sensitivity quantitative LC-MS/MS was performed on a Waters Xevo TQ tandem
1026 quadrupole MS lined to an ACQUITY UPLC with a Selectra C8 RP column (100x2.1 mm 1.8μm,
1027 UCT). MRM libraries of estrogen conjugates have been curated to include both analytical and
1028 confirmatory transitions for each analyte at optimal retention times to maximize separation. Briefly,
1029 data analysis was performed by integrating the peak area of the analytical transition for each
1030 analyte. Peak areas were normalized to molecular class-matched internal spike-in standards and
1031 mapped to a standard curve to accurately estimate analyte concentration. Urine estrogen
1032 metabolite concentrations were then normalized to urinary creatinine, which was measured by
1033 colorimetric assay (Sigma).

1034

1035 **Statistical Analysis**
1036 Statistical analysis was performed using R statistical programing, GraphPad Prism 9, and
1037 Microsoft Excel. For hypothesis testing, non-parametric Mann-Whitney U-test was used for
1038 pairwise comparisons and the Kruskal-Wallis non-parametric ANOVA with multiple comparison
1039 post-hoc was used to for non-paired and unmatched comparisons of 3 or more groups. Multiple
1040 comparison adjustment was performed using false discover rate (FDR) when appropriate. An
1041 alpha of 0.05 was considered significant to control type I error.

1042

1043 **Acknowledgements**

1053

Author Contributions

Supplemental materials

Figure S1. Power analysis and metagenomic dataset characteristics.

Figure S2. Power analysis and metagenomic dataset characteristics.

Figure S3. Taxonomic profiles of detected *Archea, Eukaryota*, and Vial species.

Figure S4. Ecological modeling indices among the cohort groups.

Figure S5. Urinary estrogen conjugate concentrations and taxonomic associations.

Figure legends

Figure 1. Study design and summary of genera detected by WGMS and advanced urine culture. (A) Schematic diagram of rUTI pathobiology cycle depicting periods of active, symptomatic UTI defined by positive urine culture followed by periods of remission defined by negative clinical urine culture. (B) Schematic diagram of clinical cohort structure and datasets generated for the study. (C) Taxonomic cladogram of taxa detected in all sequenced metagenomes (n=75) by Metaphlan2. Node size indicates relative abundance and branch length is arbitrary. The top 20 genera by average relative abundance are displayed. (D) Venn diagram depicting the coverage of WGMS metagenomes by advanced urine culture. Coverage is calculated at the genus level considering all bacterial genera exhibiting relative abundance >5% by WGMS in at least one patient.

Figure 2. The bacterial taxonomic profile of rUTI in postmenopausal women. (A) Genus-level taxonomic profile of the top 15 bacterial genera among cohort groups (No UTI History (*n*=25), rUTI Remission (*n*=25), rUTI Relapse (*n*=25)). All genera not within the top 15 are combined into "Other". (B) Species-level taxonomic profile represented within the top 15 bacterial genera among cohort groups (No UTI History (*n*=25), rUTI Remission (*n*=25), rUTI Relapse (*n*=25)). All species

1088   not represented within the top 15 genera are combined into "Other". Alpha-diversity comparison

1089   of the observed species counts (C) and Shannon index (D) between cohort groups (1 = No UTI

1090   History (*n*=25), 2 = rUTI Remission (*n*=25), 3 = rUTI Relapse (*n*=25)). Solid lines represent

1091   medians while dotted lines represent the interquartile range. *P*-value was generated by Kruskal-

1092   Wallis test with uncorrected Dunn's multiple correction post hoc. (E) Beta-diversity sample

1093   ordination of the WGMS dataset by Double Principal Coordinate Analysis (DPCoA) in the first two

1094   PCoAs. Each dot represents an individual sample color-coded by group (No UTI history in blue,

1095   rUTI remission in purple, and rUTI relapse in salmon). Vectors (grey) are drawn to represent the

1096   major discriminating loadings (*i.e.* species). (F) Volcano plot depicting co-occurrence of genera

1097   within the WGMS dataset by Pearson correlation. *P*-value generated by permutation. Red dots

1098   represent associations which were considered significant after FDR correction of the *P*-value.

1099   Blue dots represent associations which exhibited a nominal *P*-value less than 0.05, but an FDR-

1100   corrected *P*-value greater than 0.05. (G) Network analysis of all genera co-occurrence

1101   associations with *P*-value less than 0.05. Nodes represent genera edges are defined by Pearson

1102   correlation. Node size is proportional to the degree of the node.

1103

1104   **Figure 3. Bayesian modeling detects the taxonomic imprint of rUTI history on the urobiome**

1105   **of postmenopausal women.** (A) Schematic representation of the BMDA model. (B) Bayesian

1106   differential abundance model comparing taxonomic enrichment between the No UTI history

1107   (*n*=25) and rUTI Remission (*n*=25) cohort groups. Dots, indicating the $\log_{10}$(posterior effect size),

1108   are color-coded by their enrichment in a particular cohort group with taxa enriched in No UTI

1109   history in blue and taxa enriched in rUTI remission. Lines indicate the minima and maxima of the

1110   95% credible interval and PPI denotes posterior probability index.

1111

1112   **Figure 4. Estrogen hormone therapy shapes the urobiome of postmenopausal women.**

1113   (A) Genus-level taxonomic profile of the relative abundance of the top 22 bacterial genera among

1114   women reporting no use of estrogen hormone therapy (EHT(-), *n*=21) and women reporting use

1115   of EHT (EHT(+), *n*=29) among the No UTI History and rUTI Remission groups. All genera not

1116   within the top 22 are combined into "Other". (B) Taxonomic profile of the relative abundance of

1117   species represented within the top 22 bacterial genera among EHT(-) (*n*=21) and EHT(+) (*n*=29)

1118   non-infected women from the No UTI History and rUTI Remission groups. All species not

1119   represented within the top 22 genera are combined into "Other". (C) Comparison of the relative

1120   abundance of the genus *Lactobacillus* between EHT(-) (grey) and EHT(+) (pink) women in the No

1121   UTI history and rUTI remission groups. Violin plot depicts the smoothed distribution of the data.

1122   Solid lines represent the median. Dotted lines represent the interquartile range. P-value generated

1123   by Wilcoxon rank-sum test. Alpha-diversity comparison of the observed species count (D),

1124   Shannon index (E), and Simpson index (F) between EHT(-) (grey) and EHT(+) (pink) women in

1125   the No UTI history and rUTI remission cohorts. Violin plot depicts the smoothed distribution of the

1126   data. Solid lines represent medians while dotted lines represent the interquartile range. P-value

1127   generated by Wilcoxon rank-sum. (G) Two significantly differentially enriched taxa detected by

1128   LEfSe biomarker analysis performed between EHT(-) (grey) and EHT(+) (pink) women in the No

1129   UTI history and rUTI remission groups. LDA denotes the $\log_{10}$(linear discriminant analysis score)

1130   and the P-value was generated by Wilcoxon Rank Sum Test. (H) Differentially enriched taxa

1131   between EHT(-) (grey) and EHT(+) (pink) women in the No UTI history and rUTI remission cohorts

1132   detected by the Bayesian differential abundance model. Dots indicate $\log_{10}$(posterior effect size)

1133   and PPI denotes posterior probability index. S. m/o/p denotes *Steptococcus*

1134   *mitus/oralis/pneumoniae.*

1135

1136   **Figure 5. Distinct taxa-urinary estrogen metabolite associations between postmenopausal**

1137   **women with and without rUTI history.** (A) Comparison of the relative abundance of the genus

1138   *Lactobacillus* between EHT(-) (grey, *n*=21) and EHT(+) (pink, *n*=29) women from the No UTI

1139   History and rUTI Remission groups striated by EHT modality (Oral, Patch, Vaginal). Error bars

1140   are drawn from minimum to maximum of the data distribution. Boxes represent the interquartile

1141   range. Solid lines denote the median. P-value generated by Kruskal-Wallis test with uncorrected

1142   Dunn's multiple correction post hoc. Summed creatinine (Cr)-normalized urinary E1 and E2

1143   conjugates (B), E1 conjugates (C) and E2 conjugates (D) measured in the urine of EHT(-) and

1144   EHT(+) women from the No UTI History and rUTI Remission groups striated by EHT modality

1145   (Oral (*n*=6), Patch (*n*=6), Vaginal (*n*=17)). Error bars are drawn from minimum to maximum of the

1146   data distribution. Boxes represent the interquartile range. Solid lines denote the median. P-value

1147   generated by Kruskal-Wallis test with uncorrected Dunn's multiple correction post hoc. Volcano

1148   plots depicting correlation of bacterial species with summed Cr-normalized urinary E1 and E2

1149   conjugates by Spearman correlation in No UTI History (E) and rUTI remission (F) groups. P-value

1150   generated by permutation. Red dots represent significant ($p<0.05$) positive associations. Blue

1151   dots represent significant negative associations. (G) Correlation scatter plots of the association

1152   between *B. breve*, *L. iners*, *L. crispatus*, and *A. prevotti,* and summed creatinine-normalized

1153   urinary E1 and E2 conjugates among No UTI History women (*n*=25) (blue) and rUTI Remission

1154   women (*n*=23) (purple). Linear regression trend line (solid line) is shown with 95% confidence

1155   intervals (dashed lines).

1156

1157 **Figure 6. rUTI history and active infection shape the metabolic potential of the urobiome.**

1158 (A, B) Principal component analysis (PCA) of cohort samples by metagenome-encoded pathway-

1159 level metabolic potential. Depiction of sample ordination and clustering in the first two PCAs in

1160 (A) and vectors (grey) defining major discriminatory loadings (*i.e.* metabolic pathways) in (B). Top

1161 40 differentially enriched pathways detected by LEfSe analysis performed between the No UTI

1162 History (blue, n=25) and rUTI Remission (purple, *n*=25) cohort groups (C) and the No UTI History

1163 (n=25) and rUTI Relapse (red, *n*=25) cohort groups (D). Pathways presented exhibit an FDR-

1164 corrected *P*-values less than 0.05. LDA score indicates $\log_{10}$(linear discriminant analysis score).

1165

1166 **Figure 7. rUTI history and active infection shape the resistome of the postmenopausal**

1167 **urobiome.** (A) Comparison of the observed Antibiotic Resistance Genes (ARGs) detected within

1168 the urobiomes of the No UTI History (*n*=25), rUTI Remission (*n*=25), and rUTI Relapse (*n*=25)

1169 cohort groups. Violin plot depicts the smoothed distribution of the data. Solid lines represent

1170 median while dotted lines represent the interquartile range. *P*-value was generated by Kruskal-

1171 Wallis test with uncorrected Dunn's multiple correction post hoc. (B) Bayesian differential

1172 enrichment analysis of ARG presence within the urobiomes of the No UTI History (*n*=25), rUTI

1173 Remission (*n*=25), and rUTI Relapse (*n*=25) cohort groups. Group comparisons are were

1174 determined by the difference in ARG(+) proportion within a pairwise group comparison. 95%

1175 credible intervals, Bayes factor, and posterior probability are presented for interpretation of

1176 Bayesian analysis. P-value generated by the Fisher Exact test is also provided. (C) Phenotypic

1177 assessment of the agreement between urobiome ARG detection and antibiotic resistance of

1178 isolates of the most abundant uropathogen species present in each patient in the rUTI Relapse

1179 cohort group (*n*=23, *E. coli* (n=15), *Klebsiella* (n=3), *Streptococcus* (n=3), *E. faecali*s (n=1), *S.*

1180 *epidermidis* (n=1)). Upper diagonal colors represent WGMS GROOT profiling results (blue = ARG

1181 (+), white = ARG(-)). Lower diagonal color represents Isolate Phenotype (red = resistant, yellow

1182 = intermediate resistance, white = sensitive, grey = not tested). Amp, Ampicillin; Cfx, Cefixime;

1183 Cex, Cephalexin; TMP/SMX, Trimethoprim/sulfamethoxazole; Gen, Gentamicin; Kan,

1184 Kanamycin; Amk, Amikacin; Str, Streptomycin; Cip, Ciprofloxcain; Lvx, Levofloxacin; Erm,

1185 Erythromycin; Dox, Doxycycline; Chlor, Chloramphenicol.

1186

1187 **References**

1188 Alteri, C.J., Himpsl, S.D., and Mobley, H.L. (2015). Preferential use of central metabolism in vivo
1189 reveals a nutritional basis for polymicrobial infection. PLoS Pathog *11*, e1004601.

1190   Amabebe, E., and Anumba, D.O.C. (2018). The Vaginal Microenvironment: The Physiologic Role
1191   of Lactobacilli. Front Med (Lausanne) *5*, 181.

1192   Ammitzboll, N., Bau, B.P.J., Bundgaard-Nielsen, C., Villadsen, A.B., Jensen, A.M., Leutscher,
1193   P.D.C., Glavind, K., Hagstrom, S., Arenholt, L.T.S., and Sorensen, S. (2021). Pre- and
1194   postmenopausal women have different core urinary microbiota. Sci Rep *11*, 2212.

1195   Andrews, S. (2015). FastQC: a quality control tool for high throughput sequence data.

1196   Anglim, B., Phillips, C., Shynlova, O., and Alarab, M. (2021). The effect of local estrogen therapy
1197   on the urinary microbiome composition of postmenopausal women with and without recurrent
1198   urinary tract infections. Int Urogynecol J.

1199   Arsic, B., Barber, J., Cikos, A., Mladenovic, M., Stankovic, N., and Novak, P. (2018). 16-
1200   membered macrolide antibiotics: a review. Int J Antimicrob Agents *51*, 283-298.

1201   Atassi, F., Pho Viet Ahn, D.L., and Lievin-Le Moal, V. (2019). Diverse Expression of Antimicrobial
1202   Activities Against Bacterial Vaginosis and Urinary Tract Infection Pathogens by Cervicovaginal
1203   Microbiota Strains of Lactobacillus gasseri and Lactobacillus crispatus. Front Microbiol *10*, 2900.

1204   Barraud, O., Ravry, C., Francois, B., Daix, T., Ploy, M.C., and Vignon, P. (2019). Shotgun
1205   metagenomics for microbiome and resistome detection in septic patients with urinary tract
1206   infections. Int J Antimicrob Agents.

1207   Bartlett, A.T.M., and White, P.J. (1985). Species of Bacillus That Make a Vegetative
1208   Peptidoglycan Containing Lysine Lack Diaminopimelate Epimerase but Have Diaminopimelate
1209   Dehydrogenase. Microbiology *131*, 2145-2152.

1210   Bradshaw, C.S., Tabrizi, S.N., Fairley, C.K., Morton, A.N., Rudland, E., and Garland, S.M. (2006).
1211   The association of Atopobium vaginae and Gardnerella vaginalis with bacterial vaginosis and
1212   recurrence after oral metronidazole therapy. J Infect Dis *194*, 828-836.

1213   Brubaker, L., and Wolfe, A.J. (2017). The female urinary microbiota, urinary health and common
1214   urinary disorders. Ann Transl Med *5*, 34.

1215   Bucevic Popovic, V., Situm, M., Chow, C.T., Chan, L.S., Roje, B., and Terzic, J. (2018). The
1216   urinary microbiome associated with bladder cancer. Sci Rep *8*, 12157.

1217   Caspi, R., Billington, R., Fulcher, C.A., Keseler, I.M., Kothari, A., Krummenacker, M.,
1218   Latendresse, M., Midford, P.E., Ong, Q., Ong, W.K., *et al.* (2018). The MetaCyc database of
1219   metabolic pathways and enzymes. Nucleic Acids Res *46*, D633-D639.

1220   Correia, S., Poeta, P., Hebraud, M., Capelo, J.L., and Igrejas, G. (2017). Mechanisms of
1221   quinolone action and resistance: where do we stand? J Med Microbiol *66*, 551-559.

1222   Daniel S. C. Butler, A.S., Ann E. Stapleton (2016). Cytoprotective Effect of Lactobacillus crispatus
1223   CTV-05 against Uropathogenic E. coli. Pathogens *5(1)*.

1224   De Nisco, N.J., Neugent, M., Mull, J., Chen, L., Kuprasertkul, A., de Souza Santos, M., Palmer,
1225   K.L., Zimmern, P., and Orth, K. (2019). Direct Detection of Tissue-Resident Bacteria and Chronic

1226 Inflammation in the Bladder Wall of Postmenopausal Women with Recurrent Urinary Tract
1227 Infection. J Mol Biol *431*, 4368-4379.

1228 Diop, K., Diop, A., Michelle, C., Richez, M., Rathored, J., Bretelle, F., Fournier, P.E., and Fenollar,
1229 F. (2019). Description of three new Peptoniphilus species cultured in the vaginal fluid of a woman
1230 diagnosed with bacterial vaginosis: Peptoniphilus pacaensis sp. nov., Peptoniphilus raoultii sp.
1231 nov., and Peptoniphilus vaginalis sp. nov. Microbiologyopen *8*, e00661.

1232 Edwards, V.L., Smith, S.B., McComb, E.J., Tamarelle, J., Ma, B., Humphrys, M.S., Gajer, P.,
1233 Gwilliam, K., Schaefer, A.M., Lai, S.K*., et al.* (2019). The Cervicovaginal Microbiota-Host
1234 Interaction Modulates Chlamydia trachomatis Infection. MBio *10*.

1235 Fait, T. (2019). Menopause hormone therapy: latest developments and clinical practice. Drugs
1236 Context *8*, 212551.

1237 Faust, K., Sathirapongsasuti, J.F., Izard, J., Segata, N., Gevers, D., Raes, J., and Huttenhower,
1238 C. (2012). Microbial co-occurrence relationships in the human microbiome. PLoS Comput Biol *8*,
1239 e1002606.

1240 Flores-Mireles, A.L., Walker, J.N., Caparon, M., and Hultgren, S.J. (2015). Urinary tract infections:
1241 epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol *13*, 269-284.

1242 Fouts, D.E., Pieper, R., Szpakowski, S., Pohl, H., Knoblach, S., Suh, M.J., Huang, S.T.,
1243 Ljungberg, I., Sprague, B.M., Lucas, S.K*., et al.* (2012). Integrated next-generation sequencing of
1244 16S rDNA and metaproteomics differentiate the healthy urine microbiome from asymptomatic
1245 bacteriuria in neuropathic bladder associated with spinal cord injury. J Transl Med *10*, 174.

1246 Franzosa, E.A., McIver, L.J., Rahnavard, G., Thompson, L.R., Schirmer, M., Weingart, G., Lipson,
1247 K.S., Knight, R., Caporaso, J.G., Segata, N*., et al.* (2018). Species-level functional profiling of
1248 metagenomes and metatranscriptomes. Nat Methods *15*, 962-968.

1249 Gaitonde, S., Malik, R.D., and Zimmern, P.E. (2019). Financial Burden of Recurrent Urinary Tract
1250 Infections in Women: A Time-driven Activity-based Cost Analysis. Urology *128*, 47-54.

1251 Gilbert, N.M., Foster, L.R., Cao, B., Yin, Y., Mysorekar, I.U., and Lewis, A.L. (2021). Gardnerella
1252 vaginalis promotes group B Streptococcus vaginal colonization, enabling ascending
1253 uteroplacental infection in pregnant mice. Am J Obstet Gynecol *224*, 530 e531-530 e517.

1254 Glover, M., Moreira, C.G., Sperandio, V., and Zimmern, P. (2014). Recurrent urinary tract
1255 infections in healthy and nonpregnant women. Urol Sci *25*, 1-8.

1256 Hardy, L., Jespers, V., Abdellati, S., De Baetselier, I., Mwambarangwe, L., Musengamana, V.,
1257 van de Wijgert, J., Vaneechoutte, M., and Crucitti, T. (2016). A fruitful alliance: the synergy
1258 between Atopobium vaginae and Gardnerella vaginalis in bacterial vaginosis-associated biofilm.
1259 Sex Transm Infect *92*, 487-491.

1260 Hardy, L., Jespers, V., Dahchour, N., Mwambarangwe, L., Musengamana, V., Vaneechoutte, M.,
1261 and Crucitti, T. (2015). Unravelling the Bacterial Vaginosis-Associated Biofilm: A Multiplex
1262 Gardnerella vaginalis and Atopobium vaginae Fluorescence In Situ Hybridization Assay Using
1263 Peptide Nucleic Acid Probes. PLoS One *10*, e0136658.

1264 Hilt, E.E., McKinley, K., Pearce, M.M., Rosenfeld, A.B., Zilliox, M.J., Mueller, E.R., Brubaker, L.,
1265 Gai, X., Wolfe, A.J., and Schreckenberger, P.C. (2014). Urine is not sterile: use of enhanced urine
1266 culture techniques to detect resident bacterial flora in the adult female bladder. J Clin Microbiol
1267 *52*, 871-876.

1268 Hudzicki, J. (2009). Kirby-Bauer disk diffusion susceptibility test protocol.

1269 Human Microbiome Project, C. (2012). Structure, function and diversity of the healthy human
1270 microbiome. Nature *486*, 207-214.

1271 Huttenhower, C. KneadData.

1272 Ikaheimo, R., Siitonen, A., Heiskanen, T., Karkkainen, U., Kuosmanen, P., Lipponen, P., and
1273 Makela, P.H. (1996). Recurrence of urinary tract infection in a primary care setting: analysis of a
1274 1-year follow-up of 179 women. Clin Infect Dis *22*, 91-99.

1275 Jeffreys, H. (1946). An invariant form for the prior probability in estimation problems. Proc R Soc
1276 Lond A Math Phys Sci *186*, 453-461.

1277 Jespers, V., van de Wijgert, J., Cools, P., Verhelst, R., Verstraelen, H., Delany-Moretlwe, S.,
1278 Mwaura, M., Ndayisaba, G.F., Mandaliya, K., Menten, J.*, et al.* (2015). The significance of
1279 Lactobacillus crispatus and L. vaginalis for vaginal health and the negative effect of recent sex: a
1280 cross-sectional descriptive study across groups of African women. BMC Infect Dis *15*, 115.

1281 Jhang, J.F., and Kuo, H.C. (2017). Recent advances in recurrent urinary tract infection from
1282 pathogenesis and biomarkers to prevention. Ci Ji Yi Xue Za Zhi *29*, 131-137.

1283 Karstens, L., Asquith, M., Caruso, V., Rosenbaum, J.T., Fair, D.A., Braun, J., Gregory, W.T.,
1284 Nardos, R., and McWeeney, S.K. (2018). Community profiling of the urinary microbiota:
1285 considerations for low-biomass samples. Nat Rev Urol *15*, 735-749.

1286 Karstens, L., Asquith, M., Davin, S., Stauffer, P., Fair, D., Gregory, W.T., Rosenbaum, J.T.,
1287 McWeeney, S.K., and Nardos, R. (2016). Does the Urinary Microbiome Play a Role in Urgency
1288 Urinary Incontinence and Its Severity? Front Cell Infect Microbiol *6*, 78.

1289 Keogh, D., Tay, W.H., Ho, Y.Y., Dale, J.L., Chen, S., Umashankar, S., Williams, R.B.H., Chen,
1290 S.L., Dunny, G.M., and Kline, K.A. (2016). Enterococcal Metabolite Cues Facilitate Interspecies
1291 Niche Modulation and Polymicrobial Infection. Cell Host Microbe *20*, 493-503.

1292 Kõljalg, S., Truusalu, K., Vainumäe, I., Stsepetova, J., Sepp, E., and Mikelsaar, M. (2009).
1293 Persistence of *Escherichia coli* Clones and Phenotypic and Genotypic Antibiotic
1294 Resistance in Recurrent Urinary Tract Infections in Childhood. Journal of Clinical Microbiology *47*,
1295 99-105.

1296 Krueger, F. Trim Galore!

1297 Lewis, D.A., Brown, R., Williams, J., White, P., Jacobson, S.K., Marchesi, J.R., and Drake, M.J.
1298 (2013). The human urinary microbiome; bacterial DNA in voided urine of asymptomatic adults.
1299 Front Cell Infect Microbiol *3*, 41.

Li, Q., Jiang, S., Koh, A.Y., Xiao, G., and Zhan, X. (2019). Bayesian Modeling of Microbiome Data for Differential Abundance Analysis, pp. arXiv:1902.08741.

Lobo, R.A. (2017). Hormone-replacement therapy: current thinking. Nat Rev Endocrinol *13*, 220-231.

Malik, R.D., Wu, Y.R., Christie, A.L., Alhalabi, F., and Zimmern, P.E. (2018a). Impact of Allergy and Resistance on Antibiotic Selection for Recurrent Urinary Tract Infections in Older Women. Urology *113*, 26-33.

Malik, R.D., Wu, Y.R., and Zimmern, P.E. (2018b). Definition of Recurrent Urinary Tract Infections in Women: Which One to Adopt? Female Pelvic Med Reconstr Surg *24*, 424-429.

Marrazzo, J.M., Thomas, K.K., Fiedler, T.L., Ringwood, K., and Fredricks, D.N. (2008). Relationship of specific vaginal bacteria and bacterial vaginosis treatment failure in women who have sex with women. Ann Intern Med *149*, 20-28.

McMurdie, P.J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One *8*, e61217.

Moustafa, A., Li, W., Singh, H., Moncera, K.J., Torralba, M.G., Yu, Y., Manuel, O., Biggs, W., Venter, J.C., Nelson, K.E.*, et al.* (2018). Microbial metagenome of urinary tract infection. Sci Rep *8*, 4333.

Neugent, M.L., Hulyalkar, N.V., Nguyen, V.H., Zimmern, P.E., and De Nisco, N.J. (2020). Advances in Understanding the Human Urinary Microbiome and Its Potential Role in Urinary Tract Infection. mBio *11*.

Onderdonk, A.B., Delaney, M.L., and Fichorova, R.N. (2016). The Human Microbiome during Bacterial Vaginosis. Clin Microbiol Rev *29*, 223-238.

Pavoine, S., Dufour, A.B., and Chessel, D. (2004). From dissimilarities among species to dissimilarities among communities: a double principal coordinate analysis. J Theor Biol *228*, 523-537.

Pearce, M.M., Hilt, E.E., Rosenfeld, A.B., Zilliox, M.J., Thomas-White, K., Fok, C., Kliethermes, S., Schreckenberger, P.C., Brubaker, L., Gai, X.*, et al.* (2014). The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. MBio *5*, e01283-01214.

Price, T.K., Dune, T., Hilt, E.E., Thomas-White, K.J., Kliethermes, S., Brincat, C., Brubaker, L., Wolfe, A.J., Mueller, E.R., and Schreckenberger, P.C. (2016). The Clinical Urine Culture: Enhanced Techniques Improve Detection of Clinically Relevant Microorganisms. J Clin Microbiol *54*, 1216-1222.

Price, T.K., Hilt, E.E., Thomas-White, K., Mueller, E.R., Wolfe, A.J., and Brubaker, L. (2019). The urobiome of continent adult women: a cross-sectional study. BJOG.

Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J., and Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. Nat Biotechnol *35*, 833-844.

1336 Raftogianis, R., Creveling, C., Weinshilboum, R., and Weisz, J. (2000). Estrogen metabolism by
1337 conjugation. J Natl Cancer Inst Monogr, 113-124.

1338 Ravel, J., and Brotman, R.M. (2016). Translating the vaginal microbiome: gaps and challenges.
1339 Genome Med *8*, 35.

1340 Ravel, J., Gajer, P., Abdo, Z., Schneider, G.M., Koenig, S.S., McCulle, S.L., Karlebach, S., Gorle,
1341 R., Russell, J., Tacket, C.O., *et al.* (2011). Vaginal microbiome of reproductive-age women. Proc
1342 Natl Acad Sci U S A *108 Suppl 1*, 4680-4687.

1343 Rowe, W.P.M., and Winn, M.D. (2018). Indexed variation graphs for efficient and accurate
1344 resistome profiling. Bioinformatics *34*, 3601-3608.

1345 Salter, S.J., Cox, M.J., Turek, E.M., Calus, S.T., Cookson, W.O., Moffatt, M.F., Turner, P., Parkhill,
1346 J., Loman, N.J., and Walker, A.W. (2014). Reagent and laboratory contamination can critically
1347 impact sequence-based microbiome analyses. BMC Biol *12*, 87.

1348 Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., and Huttenhower, C.
1349 (2011). Metagenomic biomarker discovery and explanation. Genome Biol *12*, R60.

1350 Segata, N., Waldron, L., Ballarini, A., Narasimhan, V., Jousson, O., and Huttenhower, C. (2012).
1351 Metagenomic microbial community profiling using unique clade-specific marker genes. Nat
1352 Methods *9*, 811-814.

1353 Siddiqui, H., Nederbragt, A.J., Lagesen, K., Jeansson, S.L., and Jakobsen, K.S. (2011).
1354 Assessing diversity of the female urine microbiota by high throughput sequencing of 16S rDNA
1355 amplicons. BMC Microbiol *11*, 244.

1356 Stamm, W.E., and Norrby, S.R. (2001). Urinary tract infections: disease panorama and
1357 challenges. J Infect Dis *183 Suppl 1*, S1-4.

1358 Stapleton, A.E., Au-Yeung, M., Hooton, T.M., Fredricks, D.N., Roberts, P.L., Czaja, C.A., Yarova-
1359 Yarovaya, Y., Fiedler, T., Cox, M., and Stamm, W.E. (2011). Randomized, placebo-controlled
1360 phase 2 trial of a Lactobacillus crispatus probiotic given intravaginally for prevention of recurrent
1361 urinary tract infection. Clin Infect Dis *52*, 1212-1217.

1362 Suzek, B.E., Wang, Y., Huang, H., McGarvey, P.B., Wu, C.H., and UniProt, C. (2015). UniRef
1363 clusters: a comprehensive and scalable alternative for improving sequence similarity searches.
1364 Bioinformatics *31*, 926-932.

1365 Theriot, C.M., Koenigsknecht, M.J., Carlson, P.E., Jr., Hatton, G.E., Nelson, A.M., Li, B.,
1366 Huffnagle, G.B., J, Z.L., and Young, V.B. (2014). Antibiotic-induced shifts in the mouse gut
1367 microbiome and metabolome increase susceptibility to Clostridium difficile infection. Nat Commun
1368 *5*, 3114.

1369 Thomas-White, K., Forster, S.C., Kumar, N., Van Kuiken, M., Putonti, C., Stares, M.D., Hilt, E.E.,
1370 Price, T.K., Wolfe, A.J., and Lawley, T.D. (2018). Culturing of female bladder bacteria reveals an
1371 interconnected urogenital microbiota. Nat Commun *9*, 1557.

1372 Thomas-White, K., Taege, S., Limeira, R., Brincat, C., Joyce, C., Hilt, E.E., Mac-Daniel, L., Radek,
1373 K.A., Brubaker, L., Mueller, E.R., *et al.* (2020). Vaginal estrogen therapy is associated with

1374 increased Lactobacillus in the urine of postmenopausal women with overactive bladder
1375 symptoms. Am J Obstet Gynecol *223*, 727 e721-727 e711.

1376 Thomas-White, K.J., Kliethermes, S., Rickey, L., Lukacz, E.S., Richter, H.E., Moalli, P., Zimmern,
1377 P., Norton, P., Kusek, J.W., Wolfe, A.J.*, et al.* (2017). Evaluation of the urinary microbiota of
1378 women with uncomplicated stress urinary incontinence. Am J Obstet Gynecol *216*, 55 e51-55
1379 e16.

1380 Vaishnava, S., Yamamoto, M., Severson, K.M., Ruhn, K.A., Yu, X., Koren, O., Ley, R., Wakeland,
1381 E.K., and Hooper, L.V. (2011). The antibacterial lectin RegIIIgamma promotes the spatial
1382 segregation of microbiota and host in the intestine. Science *334*, 255-258.

1383 van der Berg, C.L., Venter, G., van der Westhuizen, F.H., and Erasmus, E. (2020). Data on the
1384 optimisation of a solid phase extraction method for fractionating estrogen metabolites from small
1385 urine volumes. Data Brief *29*, 105222.

1386 Vaughan, M.H., Mao, J., Karstens, L.A., Ma, L., Amundsen, C.L., Schmader, K.E., and Siddiqui,
1387 N.Y. (2021). The Urinary Microbiome in Postmenopausal Women with Recurrent Urinary Tract
1388 Infections. J Urol *206*, 1222-1231.

1389 Waller, T.A., Pantin, S.A.L., Yenior, A.L., and Pujalte, G.G.A. (2018). Urinary Tract Infection
1390 Antibiotic Resistance in the United States. Prim Care *45*, 455-466.

1391 Whittaker, P.G., Morgan, M.R.A., Dean, P.D.G., Cameron, E.H.D., and Lind, T. (1980). SERUM
1392 EQUILIN, ŒSTRONE, AND ŒSTRADIOL LEVELS IN POSTMENOPAUSAL WOMEN
1393 RECEIVING CONJUGATED EQUINE ŒSTROGENS ('PREMARIN'). The Lancet *315*, 14-16.

1394 Wingett, S.W., and Andrews, S. (2018). FastQ Screen: A tool for multi-genome mapping and
1395 quality control. F1000Res *7*, 1338.

1396 Wolfe, A.J., Toh, E., Shibata, N., Rong, R., Kenton, K., Fitzgerald, M., Mueller, E.R.,
1397 Schreckenberger, P., Dong, Q., Nelson, D.E.*, et al.* (2012). Evidence of uncultivated bacteria in
1398 the adult female bladder. J Clin Microbiol *50*, 1376-1383.
1399

Table 1

Cohort	N-Value	Age $\pm$ SD	BMI $\pm$ SD	Urine pH $\pm$ SD
No UTI History	25	71.3 ± 9.41	25.4 ± 4.37	6.4 ± 1.06
rUTI Remission	25	70.4 ± 8.25	24.6 ± 4.18	6.0 ± 1.08
rUTI Relapse	25	75.7 ± 7.62	26.6 ± 4.30	5.64 ± 0.88

Figure 1

A rUTI Cycle

B COHORT STRUCTURE

No UTI History (n=25)	✗ History of rUTI	✗ Active UTI
rUTI Remission (n=25)	✓ History of rUTI	✗ Active UTI
rUTI Relapse (n=25)	✓ History of rUTI	✓ Active UTI

Whole Genome Metagenomic Sequencing

Clinical Metadata

Curated Bacterial and Fungal Isolate Biobank

C

Prevotella
Spike-in
Escherichia
Klebsiella
Gardnerella
Bifidobacterium
Polyomavirus
Proprionimicrobium
Varibaculum
Actinobaculum
Lactobacillus
Streptococcus
Corynebacterium
Aerococcus
Facklamia
Enterococcus
Staphylococcus
Finegoldia
Anaerococcus
Peptoniphilus

D

WGMS
7

Bacteria
22 Genera
51 Species

Eukaryota
1 Genus
2 Species

93.9% Culturing Coverage

2

Urine Culture

Figure 2

A No UTI History | rUTI Remission | rUTI Relapse | Bacterial Genera

Lactobacillus, *Aerococcus*, *Klebsiella*, *Escherichia*, *Enterococcus*, *Bifidobacterium*, *Streptococcus*, *Gardnerella*, *Staphylococcus*, *Actinobaculum*, *Finegoldia*, *Corynebacterium*, *Propionimicrobium*, *Facklamia*, *Anaerococcus*, Other

B No UTI History | rUTI Remission | rUTI Relapse | Bacterial Species

L. crispatus, *L. delbrueckii*, *L. gasseri*, *L. iners*, *A. urinae*, *K. oxytoca*, *K. pneumoniae*, *E. coli*, *Escherichia spp...*, *E. faecalis*, *B. breve*, *B. dentium*, *B. longum*, *S. agalactiae*, *S. anginosus*, *S. parasanguinis*, *G. vaginalis*, *S. epidermidis*, *S. haemolyticus*, *A. schaalii*, *F. magna*, *C. aurimucosum*, *C. pyruviciproducens*, *C. striatum*, *P. lymphophilum*, *F. hominis*, *F. ignava*, *A. hydrogenalis*, *A. lactolyticus*, *A. prevotii*, Other

Figure 3

A

	PPI
Aerococcus	*0.97*
Lactobacillus	*0.96*
Peptoniphilus	*1.00*
Prevotella	*1.00*
Actinomyces	*0.98*
Finegoldia	*1.00*
Oscillibacter	*0.99*
Enterococcus	*1.00*
Fusobacterium	*1.00*
Brevibacterium	*1.00*
Escherichia	*1.00*
Eggerthella	*0.97*
Clost. Fam. XIII Inc. Sedis	*1.00*
Bacteroides	*1.00*
Gemella	*1.00*
Klebsiella	*0.99*

○ No UTI History
● rUTI Remission

log$_{10}$(Posterior Effect Size)

B

	PPI
Lactobacillus vaginalis	*1.00*
Lactobacillus crispatus	*1.00*
Anaerococcus lactolyticus	*1.00*
Aerococcus urinae	*0.98*
Anaerococcus vaginalis	*0.99*
Staphylococcus epidermidis	*0.99*
Anaerococcus prevotii	*0.97*
Finegoldia magna	*1.00*
Facklamia hominis	*0.98*
Actinomyces europaeus	*1.00*
Corynebacterium pseudogenitalium	*0.99*
Peptoniphilus lacrimalis	*0.99*
Staphylococcus hominis	*1.00*
Enterococcus faecalis	*1.00*
Prevotella timonensis	*1.00*
Actinomyces turicensis	*1.00*
Anaerococcus hydrogenalis	*1.00*
Brevibacterium massiliense	*1.00*
Bacteroides uniformis	*1.00*
Ureaplasma parvum	*0.97*

○ No UTI History
● rUTI Remission

log$_{10}$(Posterior Effect Size)

Figure 4

A EHT(-) EHT(+) Genera

Lactobacillus, Gardnerella, Anaerococcus, Ruminococcus, Aerococcus, Staphylococcus, Prevotella, Other, Klebsiella, Actinobaculum, Peptoniphilus, Escherichia, Finegoldia, Alloscardovia, Enterococcus, Corynebacterium, Varibaculum, Bifidobacterium, Propionimicrobium, Actinomyces, Streptococcus, Facklamia, Atopobium

B EHT(-) EHT(+) Species

L. crispatus, S. anginosus, A. schaalii, A. prevotii, Other, L. gasseri, S. parasanguinis, E. coli, F. hominis, L. iners, G. vaginalis, K. pneumoniae, F. ignava, L. delbrueckii, S. haemolyticus, P. lymphophilum, A. omnicolens, B. longum, F. magna, E. faecalis, V. cambriense, B. breve, C. striatum, P. timonensis, A. vaginae, B. dentium, C. pyruviciproducens, P. harei, P. bivia, S. agalactiae, C. aurimucosum, A. lactolyticus, A. neuii

C P=0.0012

D P=0.0032

E P=0.0011

F P=0.0013

G EHT(-) EHT(+)

Streptococcus: P=0.0081, LDA=4.8

L. crispatus: P=0.0185, LDA=5.1

H PPI

A. vaginae 1.00
S. infantis 1.00
S. m/o/p 1.00
L. vaginalis 0.98
L. crispatus 1.00

EHT (-), EHT (+)

log₁₀(Posterior Effect Size)

Figure 5

A

Lactobacillus Relative Abundacne

P=0.0020
P=0.0055

EHT(-) | Oral | Patch | Vaginal
EHT(+)

B

E1 + E2 Conjugates

ng/µg Creatinine

P=0.0004

EHT(-) | Oral | Patch | Vaginal
EHT(+)

C

E1 Conjugates

ng/µg Creatinine

P=0.0003

EHT(-) | Oral | Patch | Vaginal
EHT(+)

D

E2 Conjugates

ng/µg Creatinine

P=0.0037

EHT(-) | Oral | Patch | Vaginal
EHT(+)

E

No UTI History

-Log(P-val)

A. prevotii
B. breve
L. iners
P. disiens
L. crispatus
F. nucleatum
Mobiluncus sp.
B. dentium
L. gasseri
P. anaerobius

Spearman R (Taxa vs Estrogen Conjugates)

F

rUTI Remission

-Log(P-val)

C. aurimucosum
D. microaerophilus
Dorea sp.
C. aerofaciens
R. obeum
F. prausnitzii
R. torques

Spearman R (Taxa vs Estrogen Conjugates)

G

No UTI History   rUTI Remission

E1+E2 Conjugates Log(ng/µg Creatinine)

B. breve | *L. iners* | *L. crispatus* | *A. prevotii*

Relative Abundance (CLR)

Figure 6

A

No UTI History
rUTI Remission
rUTI Relapse

PC2 (9.38%)

PC1 (26.01%)

B

No UTI History
rUTI Remission
rUTI Relapse

TCA Cycle

Demethylmenaquinol-8 biosynth.

Nucleotide biosynth. (*n*=8)

LPS biosynth. (*n*=4)

Fucose & Mannose deg.

Sucrose deg.

D-galacturonate deg.

L-lysine biosynth. II

SAM cycle

UDP-N-acetyl-D-glucosamine biosynth.

PC2 (9.38%)

PC1 (26.01%)

C

Pathway Enrichment

No UTI History | rUTI Remission

D-galactose deg. V
glucose & xylose deg.
L-rhamnose deg. I
heterolactic ferm.
L-1,2 propanediol deg.
glucose & glucose 1-P deg.
glycerol deg.
fucose deg.
β D-glucuronide & D-glucuronate deg.
2-methylcitrate cycle II
cinnamate & hydroxycinnamate deg.
2-methylcitrate cycle I
3-phenyl/hydroxyphenylpropeonate deg.
sulfoglycolysis
3-phenylpropanoate to 2-oxopent-4-enoate

Carbohydrate

ubiquinol 8 biosynth.
ubiquinol 7 biosynth.
ubiquinol 10 biosynth.
ubiquinol 9 biosynth.
menaquinol 6 biosynth. I
menaquinol 9 biosynth.
menaquinol 10 biosynth.
demethylmenaquinol 9 biosynth.

Electron Trans.

L-lysine biosynth. II
L-threonine biosynth.
L-ornithine biosynth.
L-arginine deg. II
L-tryptophan deg.

A.A.

LPS biosynth.
colanic acid biosynth.
ADP L-glycero β D-manno heptose biosynth.
GDP mannose derived O-antagen biosynth.

Cell Envelope

coenzyme A biosynth. I
panothenate & coenzyme A biosynth.
thiamine diphosphate biosynth. I
thiamine diphosphate biosynth.

Vitamin

4-deoxy L-threo hex-4-enopyranuronate deg.
hexuronide & hexuronate deg.
D-fructuronate deg.

P.S.

ppGpp biosynth.

Misc.

LDA Score

D

Pathway Enrichment

No UTI History | rUTI Relapse

UDP N-acetyl D-glucosamine biosynth. I
pyruvate ferm. to acetate & lactate II
L-rhamnose deg. I
glycolysis III from glucose
fucose deg.
glyoxylate cycle
hexitol ferm.
TCA cycle IV
TCA cycle V
glycolysis I from G6P
glucose & G1P deg.
fucose and rhamnose deg.

Carbohydrate

adenosine nucleotide biosynth. I
adenosine nucleotide biosynth. II
guanosine nucleotide biosynth. II
pyrimidine deoxyribonucleotide biosynth. III
pyrimidine deoxyribonucleotide biosynth. I
adenosine deoxyribonucleotide biosynth. II
guanosine deoxyribonucleotide biosynth. II
guanosine nucleotide deg. III
adenosine nucleotide deg. II

Nucleotide

L-lysine biosynth. II
threonine biosynth.
L-isoleucine biosynth. I
L-phenylalanine biosynth.
L-aspartate & L-asparagine biosynth.
L-tyrosine biosynth.

A.A.

peptidoglycan maturation
anhydromuropeptides recycling
dTDP-N-acetylthomosamine biosynth.
CMP-3-deoxy D-manno octulosonate biosynth.
phospholipid biosynth. I

Cell Envelope

D-fructuronate deg.
4-deoxy L-threo hex-4-enopyranuronate deg.

P.S.

S-adenosyl L-methionine cycle I
methylerythritol phosphate pathway I
NAD salvage pathway II
enterobactin biosynth.
sulfate reduction I assimilatory
cis vaccenate biosynth.

Misc.

LDA Score

Figure 7