# BMI and BPH correlate with urinary microbiome diversity and lower urinary tract symptoms in men

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40 **Abstract**

41 Several studies have identified bacteria and other microbes in the bladder and lower urinary

42 tract in the absence of infection. In women, the urinary microbiome has been associated with

43 lower urinary tract symptoms (LUTS), however, similar studies have not been undertaken in

44 large cohorts of men. Here we examine the urinary microbiome and its association with LUTS in

45 a subset of 500 men aged 65 to 90 years from the Osteoporotic Fractures in Men (MrOS) study.

46 We identified significant associations between benign prostatic hyperplasia (BPH), age, and

47 body mass index (BMI) with several diversity metrics. Our analysis revealed complex

48 relationships between BMI, BPH, LUTS, and alpha diversity which give insight into the intricate

49 dynamics of the urinary microbiome. By beginning to uncover the interrelationships of BPH,

50 BMI, LUTS, and the urinary microbiome, these results can inform future study design to better

51 understand the heterogeneity of the male urinary microbiome.

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## Introduction

The bladder and urinary tract have long been considered sterile in the absence of infection; however, several studies have found evidence of a resident microbiome in both males and females.[1–3] The urinary microbiome encompasses the bacteria, archaea, fungi, and viruses that inhabit the bladder and urinary tract, and these microbes may play a role in diseases and disorders affecting the urogenital system. The majority of urinary microbiome studies have examined the female urinary microbiome and found there are various healthy microbiome compositions commonly dominated by *Lactobacillus, Gardnerella, Streptococcus, Staphylococcus, Corynebacterium,* and *Escherichia*.[4,5] Urinary microbiome studies in men have revealed several of the same bacteria found in women, such as *Escherichia, Lactobacillus,* and *Streptococcus*, but the male urinary microbiome can also be dominated by genera such as *Prevotella* and *Enterococcus*.[6–8]

Importantly, differences in the types of bacteria found in the bladders of women have been associated with recurrent urinary tract infections,[9] as well as lower urinary tract symptom (LUTS) such as urge incontinence (the sudden urge to urinate followed by leakage of urine).[10,11] The composition of the female urinary microbiome has also been found to be predictive of response to urgency urinary incontinence treatment, with a positive treatment response observed in patients with a less diverse microbiome.[9] These studies have identified specific associations between the urinary microbiome and urological diseases, and indicate more broadly that the urinary microbiome may play a role in human health and disease.

Despite the numerous studies undertaken in women, far less is known about the urinary microbiome in men.[12] Similar to the female urinary microbiome, there are studies demonstrating that alterations in male urinary microbiome composition have been associated with diseases and disorders. A "male urinary microbiome" literature search in 2023 resulted in 22 primary research articles. Researchers have identified potential relationships between the male urinary microbiome and kidney stones,[12] benign prostatic hyperplasia (BPH),[13] as well as bladder and

92   prostate cancers,[14,15] among many other disorders. Nonetheless, the urinary microbiome

93   studies undertaken in men have had small cohorts (n < 100) and few have directly investigated

94   the potential link between body mass index (BMI), age, and the male urinary microbiome. These

95   associations could impact future study design and analysis. Larger studies are needed to both

96   study and evaluate the broad applicability of these findings in males.

97   Additionally, although clear associations have been found between the female urinary

98   microbiome and LUTS, the same associations have not been identified in a large cohort of men.

99   Approximately one-third of men over the age of 50 experience moderate to severe LUTS.[16] The

100   main cause of LUTS is thought to be BPH, which is hypothesized to press on the bladder and/or

101   urethra; however, this may not be the underlying etiology for all LUTS in all men. Here, we

102   evaluated the urinary microbiome from a large cohort of older community-dwelling men using

103   urine samples from the NIH-funded Osteoporotic Fractures in Men (MrOS) study [17].  Our goals

104   were to identify microbiome associations with clinical characteristics such as age, BMI, BPH,

105   and general medical history, as well as identify associations with LUTS, including symptoms

106   related to both urgency (irritation) and pushing or straining (obstruction).

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108   **Results**:

109   Clinical characteristics of study participants

110   We utilized urine samples and phenotypic data from 500 men at baseline as part of the MrOS

111   study.[17,18]  These 500 men were randomly selected from the overall cohort of 5994 MrOS

112   participants. The average age of the participants in the current analysis was $73.0 \pm 5.7$ years,

113   with an average BMI of $27.9 \pm 3.8$ kg/m$^2$. (**Table 1**). Participants completed a questionnaire

114   detailing medical history, current health status, and overall quality of life. All participants

115   provided a morning second-voided urine sample and completed a LUTS assessment using the

116   International Prostate Symptom Score (IPSS). Just over half of participants (55.8%) had IPSS

117     scores of 7 or lower and were designated as men with no/mild LUTS, while 44.2% of

118     participants had scores higher than 7 and were designated as men with moderate to severe

119     LUTS. All voided urine samples underwent 16S rRNA amplicon sequencing of the V4 region to

120     determine urinary microbiome composition (see Methods). None of the participants reported a

121     current urinary tract infection or recent use of antibiotics.

122             Grouped by LUTS status, the mean age in men with moderate to severe LUTS (73.7

123     years) was slightly but significantly higher (n=0.013) compared with those with mild to moderate

124     LUTS (72.4 years). There was a significant difference ($p < 0.001$) in the prevalence of reported

125     BPH between the two groups, with 60.5% of men with moderate to severe LUTS having a

126     history of BPH, compared with 38.1% in the no to mild LUTS group. Significantly more men with

127     moderate to severe LUTS also had a history of prostatitis (27.6%) compared with those with

128     no/mild LUTS (17.7%, $p = 0.014$).  The men with moderate to severe LUTS having both higher

129     rates of BPH and prostatitis is expected, as an enlarged prostate and inflammation are common

130     risk factors for more severe LUTS.[19] There was, however, no correlation between BMI and

131     LUTS severity, as both groups had a similar BMI (no to mild- 27.9 kg/m$^2$; moderate to severe-

132     28.0 kg/m$^2$) in contrast to one prior study.[16] There were also no significant differences in multiple

133     baseline clinical characteristics, such as prevalence of diabetes, which was approximately 14%

134     of the men in each group, or race (**Table 1**).

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136     <u>BPH, BMI, and age are drivers of male urinary microbiome diversity</u>

137     Of the total cohort, 25 samples were removed due to insufficient sequencing reads (<1000

138     reads), leaving a total of 475 samples for subsequent analysis. Like other body sites, the male

139     urinary microbiome composition varied between individuals and is highly heterogenous (**Figure**

140     **S1a, Figure S2**).  Twenty-one phyla and 571 genera were identified in the male urinary

141     microbiome, with 54 genera being core members (present in at least 10% of samples).

142     Firmicutes was the most abundant phyla with a mean of 40.2% ± 25.7%, followed by

143      Proteobacteria, Actinobacteria, and Bacteroidetes (27.4% $\pm$ 27.9%, 15.0% $\pm$ 15.4%, and 13.4%

144      $\pm$ 15.7%, respectively **Figure S1b**). In the majority of samples, either Firmicutes or

145      Proteobacteria was the dominant phyla, consistent with prior male urinary microbiome

146      studies.[1,20,21] The five most abundant genera identified were *Staphylococcus*, *Neisseria*,

147      *Corynebacterium*, *Prevotella*, and *Streptococcus* (**Figure S1c**).[1,21]

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149      **Table 1**. Clinical characteristics of the men from the MrOS study with urinary microbiome data.

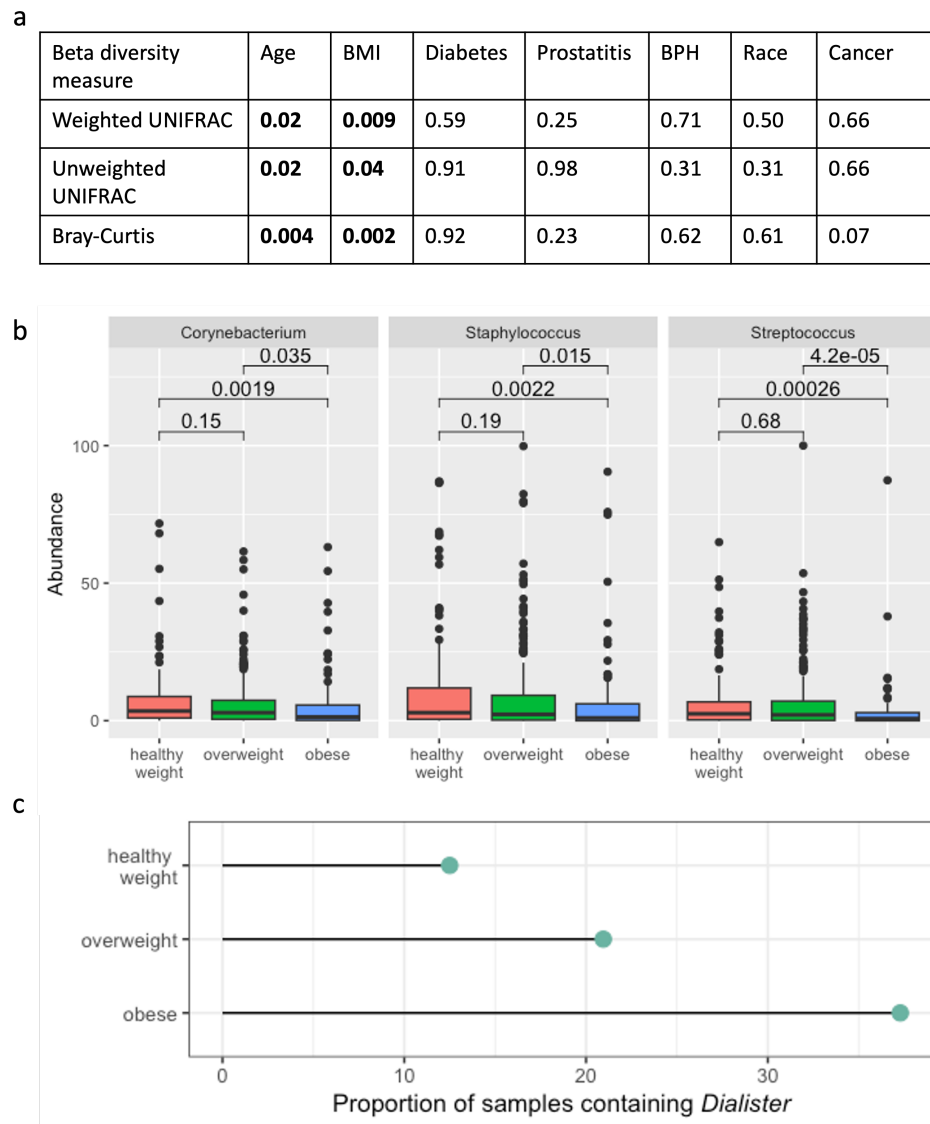| | **Participants (n=475)** | **No/mild LUTS (n=265)** | **Moderate/Severe LUTS (n=210)** | **p-value** |
|---|---|---|---|---|
| Age (y), mean (s.d.) | 73.0 $\pm$ 5.7 | 72.4 (5.2) | 73.7 (6.1) | **0.013** |
| BMI (kg/m$^2$), mean (s.d.) | 27.9 $\pm$ 3.8 | 27.9 (3.6) | 28.0 (4.1) | 0.693 |
| Race/ethnicity, white (%) | 420 (88.4%) | 231 (87.2%) | 189 (90.0%) | 0.416 |
| Diabetes (%) | 69 (14.5%) | 39 (14.7%) | 30 (14.3%) | 0.544 |
| No | 366 (77.1%) | 207 (78.1%) | 159 (75.7%) | |
| Missing | 40 (8.4%) | 19 (7.2%) | 21 (10.0%) | |
| Prostatitis history (%) | 105 (22.1%) | 47 (17.7%) | 58 (27.6%) | **0.014** |
| BPH history (%) | 228 (48.0%) | 101 (38.1%) | 127 (60.5) | **<0.001** |
| Cancer history (%) | | | | 0.736 |
| Other | 89 (18.7%) | 47 (17.7%) | 42 (20.0%) | |
| Prostate | 65 (13.7) | 35 (13.2) | 30 (14.3) | |

150

151        We next evaluated if the diversity of the male urinary microbiome was associated with

152    specific clinical characteristics (**Table Sup2, Figure 1a**). We identified significant associations

153    between BPH and several measures of alpha diversity of the urinary microbiome (Shannon,

154    Inverse Simpson, and Pielou indices, $p < 0.05$). We found that men with BPH tended to have

155    increased alpha diversity when compared with men without BPH. We did not identify any

156    significant associations between BPH and any beta diversity measures (weighted UNIFRAC,

157    Unweighted UNIFRAC, Bray-Curtis), but all three beta diversity measures were significantly

158    associated with both BMI and age (**Figure 1a**).  We also did not identify any significant

159    associations with urinary alpha diversity and age, BMI, diabetes, prostatitis, or race.

160        Based on the associations between BMI and beta diversity, we next compared the

161    relative abundances of the five most abundant phyla and 10 most abundant genera between

162    men with BMIs in the healthy, overweight, and obese ranges. We found no significant

163    differences in relative abundances at the phylum level. However, we identified differences at the

164    genus level for three genera (*Corynebacterium, Staphylococcus,* and *Streptococcus*, (**Figure**

165    **1b, Figure S3**). Overall, obese men had the lowest abundance of *Corynebacterium,*

166    *Staphylococcus,* and *Streptococcus*, whereas healthy men had the highest relative abundances.

167    All three genera are known members of a healthy male urinary microbiome,[1,8] and significant

168    differences in relative abundances of these taxa have been described in the gut microbiomes

169    between obese individuals and those with lower BMIs.[26] We also examined the 5 most

170    abundant phyla and 10 most abundant genera between individuals with and without BPH and of

171    different age groups (determined by tertile). We found no significant differences with BPH (data

172    not shown); however, the urinary microbiomes of men 76 to 90-years old had significantly higher

173    relative abundance of Actinobacteria compared to men 71 to 75-years-old ($p = 0.03$, **Figure**

174    **S4**).

175        We next determined how specific taxa in the male urinary microbiome were associated

176    with age, BMI, BPH, and the remaining clinical characteristics reported in **Table 1**. To

177    accomplish this, we employed an exploratory analysis using the machine-learning method,

178    Hierarchical All-against-All (HAllA) association testing, which is high-sensitivity pattern discovery

179    in large, paired multi-omic datasets.[27] HAllA detects possible associations with specific taxa and

180    accompanying metadata using a statistical method for discovery. After applying HAllA at the

181    phylum, family, and genus levels, we discovered a significant association between BMI and the

182    genus *Dialister* (p = 0.026). *Dialister* has previously been reported in both the female and male

183    urinary microbiome, as well as in the gut microbiome.[6,28] In the gut, higher abundances of

184    *Dialister* were found in people with a higher BMI and is associated with difficulty in losing

185    weight.[29–31] Similarly, in our data we found *Dialister* to be present in 23.2% of samples (**Figure**

186    **1c**), with the highest proportion in obese men, followed be overweight men, and lastly healthy

187    weight men (37.3%, 20.9%, and 12.5% respectively, p<0.0001, **Figure 1c**). We also identified

188    an association between the prevalence of diabetes and the phylum Bacteroidetes (p = 0.017),

189    which has been shown to have a similar association in the gut microbiome, but has not been

190    previously described in the male urinary microbiome.[32] We did not identify associations between

191    specific taxa and age or BPH using HAllA. Although age and BPH are significantly associated

192    with beta diversity and alpha diversity, respectively, these HAllA results do not point to any

193    specific bacteria. This suggests that multiple bacteria in the microbiome community likely

194    contribute to the differences seen above in diversity.

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a

| Beta diversity measure | Age | BMI | Diabetes | Prostatitis | BPH | Race | Cancer |
|---|---|---|---|---|---|---|---|
| Weighted UNIFRAC | **0.02** | **0.009** | 0.59 | 0.25 | 0.71 | 0.50 | 0.66 |
| Unweighted UNIFRAC | **0.02** | **0.04** | 0.91 | 0.98 | 0.31 | 0.31 | 0.66 |
| Bray-Curtis | **0.004** | **0.002** | 0.92 | 0.23 | 0.62 | 0.61 | 0.07 |



**Figure 1.** BMI is a dominant influence on beta diversity in this population. **a**) Table of p-values of PERMANOVA testing the relationship of the clinical characteristics with the beta diversity metrics of the urinary microbiome. BMI and age are both significantly associated with all three beta diversity metrics (bolded). **b**) Boxplots of relative abundance of *Corynebacterium, Staphylococcus,* and *Streptococcus* for men at healthy weight (red), overweight (green), and obese (blue) BMIs. **c**) Lollipop plot showing the percent of samples that contain *Dialister* by BMI grouping. A significantly higher proportion of obese individuals have *Dialister* in their urinary microbiomes as compared with both overweight and healthy weight individuals ($p < 0.00001$).

205    <u>Clustering reveals 8 "Urotypes"</u>

206    After examining specific taxa and their associations with clinical characteristics of this MrOS

207    cohort, we sought patterns in the overall urinary microbiome composition. In view of the

208    associations between the urinary microbiome and BPH, BMI, and age, we determined if the

209    associations between BMI, age, and BPH vary in the context of different microbiome

210    compositions. We used Dirichlet multinomial modeling (DMM) to cluster samples with similar

211    urinary microbiome compositions into "urotypes."[33] DMM clustering on members of the core

212    urobiome revealed eight urotypes each dominated by a specific bacterium (**Figure 2a**). Each

213    urotype contains between 19 and 89 samples. Urotypes 1, 2, and 7 were dominated by

214    *Staphyloccocus*, while the rest were dominated by *Corynebacterium*, *Neisseria*, *Prevotella*, and

215    *Anaerococcus*. Many clusters are dominated by a single bacterium while others are more

216    diverse – for example cluster 5 is predominantly *Neisseria*, while cluster 4 is more diverse with

217    high proportions of *Neisseria*, *Streptococcus*, and *Escherichia*.

218        Next, we investigated clinical characteristics within the urotypes, and observed no

219    significant differences in age, diabetes, prostatitis, BPH, or cancer. However, in line with our

220    previous findings which established BMI is correlated with the composition of the male urinary

221    microbiome, we determined the urotypes had significantly different proportions of samples

222    belonging to healthy weight, overweight, and obese individuals (p = 0.02, **Figure 2c**).  Earlier

223    we determined a relationship between *Dialister* and the urinary microbiomes of obese men;

224    thus, we wanted to explore that connection within these urotypes. We discovered that 88.1% of

225    the individuals of urotype 5, which has the highest percentage of obese men (42.4%) and lowest

226    percentage of healthy weight men (10.2%, **Figure 2c**), had *Dialister* in their urinary microbiomes

227    (**Figure 2d**). Interestingly, urotype 8 has the second most obese men (31.6%), yet none of

228    those men had *Dialister* in their urinary microbiomes.
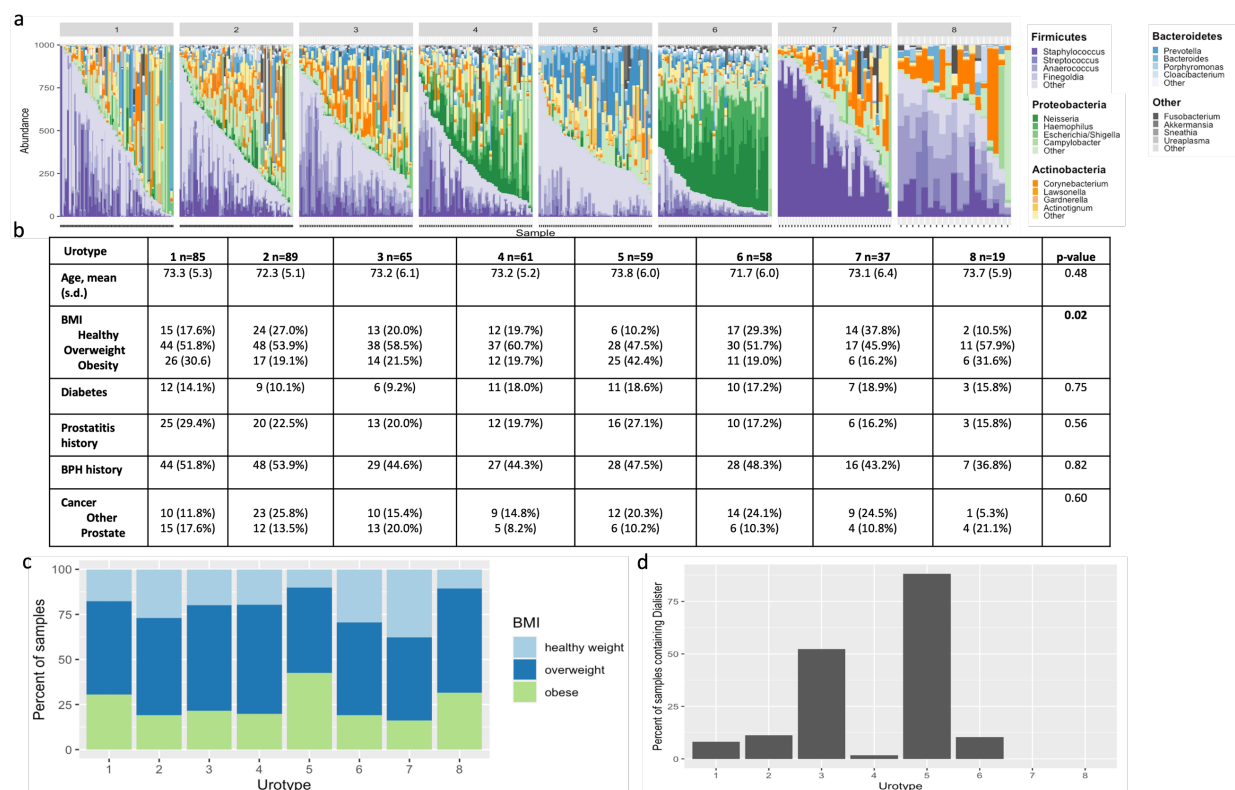
**Figure 2.** Distinct patterns of the male microbiome are present and are associated with BMI. **A)** Stacked bar plots of the eight urotypes created using Dirichlet Multinomial modeling. Each urotype is dominated by specific bacteria **b.)** Table summarizing the clinical characteristics for each urotype. BMI is significantly different between urotypes (p = 0.02, bolded) **c)** Traces of the percent of samples in each urotype colored by BMI group. **D.)** Bar graph of the percent of *Dialister* in each urotype. Urotype 5 has the highest percentage of *Dialister* and obese men.

## BMI, BPH and LUTS characteristics are associated with the diversity of the male urinary microbiome

Next, we wanted to determine if there was an association between LUTS and the male urinary microbiome. Since our results demonstrated that BPH, BMI, and age are associated with the overall male urinary microbiome composition, and previous studies have shown that BPH and BMI can also impact LUTS, we incorporated these variables into subsequent analysis.

243    To evaluate whether there are associations between the microbiome and LUTS, we first

244    looked at the complexity of the samples. Men with no to mild LUTS had an average of 16,369

245    reads per sample (minimum 1,018; maximum 101,017), while men with moderate to severe

246    LUTS averaged 19,367 reads per sample (minimum 1,184; maximum 81,167; p = 0.04, **Figure**

247    **S5**).

248    We then inspected the urinary microbiome compositions in men with no to mild LUTS

249    compared with those with moderate to severe LUTS and did not find significant differences

250    (**Figure 3a**). We also examined alpha diversity metrics (Kruskal-Wallis test) and beta diversity

251    metrics (PERMANOVA analysis) of the urinary microbiome composition in these two groups,

252    and found no significant differences were found even when adjusted for BPH, BMI, and age

253    (**Figure 3c and Figure 3d**).

254    LUTS is defined broadly, encompassing both irritative and obstructive symptoms, and

255    we hypothesized that there may be differences in the microbiome that were related to these

256    characteristics rather than overall severity. For example, studies on LUTS and the urinary

257    microbiome in women have found significant differences in women with stress incontinence

258    versus urgency incontinence;[25] however, differences are not typically found when examining

259    overall incontinence.[34]  After separating the IPSS questions into those addressing irritative

260    versus obstructive symptoms, we found the majority of men experienced some degree of both

261    symptoms. Fewer had irritative symptoms alone, while less than 1% of men experienced only

262    obstructive symptoms (**Figure 3b**). Irritative symptom scores ranged from 0 to 15, while

263    obstructive symptoms ranged from 0 to 18.

264    Taking a closer look at how obstructive and irritative symptoms are associated with

265    alpha and beta diversity, we used a Kruskal-Wallis Test and adjusted for BPH, BMI, and age.

266    We found the Inverse Simpson alpha diversity measures significantly associated with

267    obstructive symptoms and irritative symptoms independently (**Figure 3f**). Obstructive symptoms

268    were also associated with the Shannon index, while the association with irritative symptoms was
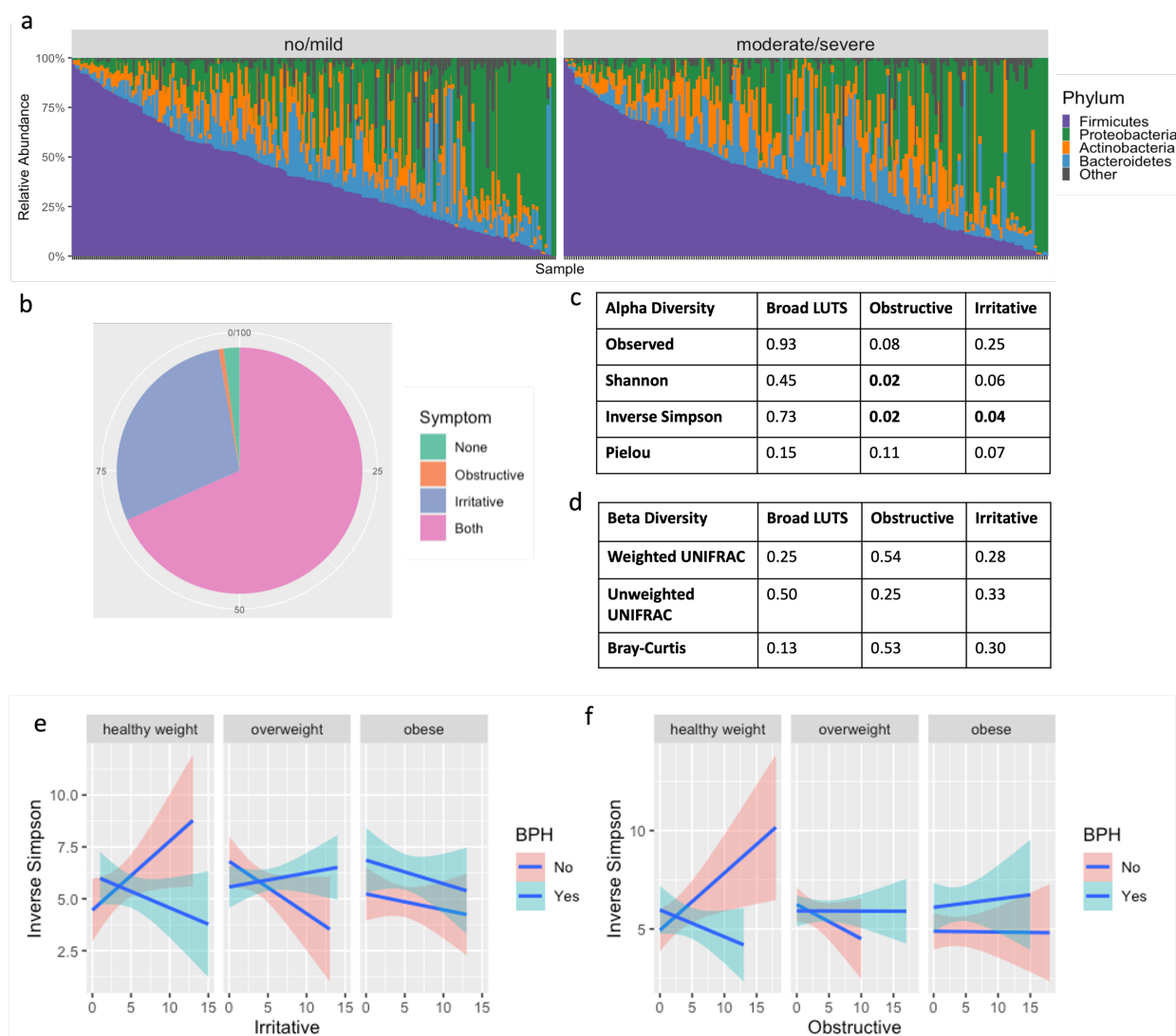
**Figure 3.** The alpha diversity of the male urinary microbiome is associated with irritative and obstructive LUTS when adjusted for BMI and BPH. **A)** Stacked bar plot of the male urinary microbiome subset by LUTS status. **B)** Pie chart showing percentage of men in the MrOS cohort who experience no symptoms, obstructive symptoms only, irritative symptoms only, and both obstructive and irritative symptoms. **C)** P-values for alpha diversity metrics and **d)** beta diversity metrics for broad LUTS (no to mild LUTS versus moderate to severe LUTS) and obstructive and irritative symptoms. Adjusted model includes interaction terms for BMI and BPH **e)** Significant interactions between BMI, BPH, and irritative or **f)** obstructive symptoms influence inverse Simpson index.

279    weaker, and not significant (Shannon, p = 0.06). In PERMANOVA comparisons, we found no

280    associations with beta diversity and obstructive nor irritative symptoms (**Figure 3d**).  In addition

281    to the alpha diversity, we also discovered significant interactions between irritative symptoms

282    and BPH and BMI (**Figure 3e**), as well as similar results with obstructive symptoms (**Figure 3f**).

283    In other words, men with BPH have varying associations between alpha diversity and symptom

284    severity depending on BMI, and there are different associations if the individual does not have

285    BPH. For example, in the healthy weight population, men with a higher Inverse Simpson index

286    tended to have increased irritative symptoms if they do not have BPH (**Figure 3e**). However, the

287    converse is true if they have BPH. We see the opposite trends for men in the overweight

288    category and found that BPH does not have the same impact in the obese group.

289

290    <u>Enterococcaceae and Caulobacteraceae are associated with irritative symptoms</u>

291    In light of irritative and obstructive symptoms and their potential role in the diversity of the male

292    urinary microbiome, we re-examined the eight urotypes and HAllA. There were no significant

293    differences in the obstructive and irritative symptom scores between urotypes (p>0.05), nor

294    when investigating broad LUTS severity.

295         To discover relationships with specific taxa and LUTS severity or irritative and

296    obstructive symptoms in the older male urinary microbiome, we again employed (HAllA)[27].

297    Supporting our previous results, no significant associations at any taxonomic resolution were

298    identified with overall LUTS score. We investigated irritative versus obstructive symptom scores

299    independently, which resulted in associations at the family level, between irritative symptoms

300    and the presence of Enterococcaceae (p = 0.002) and Caulobacteraceae (p < 0.001)

301    independently. Enterococcaceae has previously been described in the female urinary

302    microbiome of women with mild and moderate to severe LUTS.[35] Caulobacteraceae has been

303    found in the healthy canine urinary microbiome,[36] as well as the urinary microbiome of

304    spontaneously tolerant kidney-transplant recipients;[37] however, no research has shown any

305    relationship to urgency or LUTS.

306        We further assessed each of the questions from the American Urological Association

307    symptom index (AUA-SI) questionnaire for a more granular analysis and found an association at

308    the genus level between the urge to urinate frequently and *Mobiluncus* (p < 0.01). *Mobiluncus* is

309    primarily found in post-menopausal women and women with bacterial vaginosis and has not

310    previously been reported to persist in the male urinary microbiome.[38,39]

311

312    **Discussion**

313    Our analysis of a relatively large cohort of older community-dwelling men revealed that the male

314    urinary microbiome is heterogeneous and exhibits a great deal of inter-individuality in the

315    present microbes. We found that the male urinary microbiome is dominated by *Staphylococcus,*

316    *Corynebacterium, Prevotella,* and *Finegoldia*, which supports previous research (**Figure**

317    **S1c**).[1,40]  We also identified complex relationships between the male urinary microbiome and

318    BMI, BPH, age, and LUTS. While previous studies have found age-specific compositional

319    differences in the urinary microbiome regardless of gender,[2] associations between BMI and the

320    urinary microbiome have been established only in women.[25]

321        A major finding of our study is the significant association between BMI and the male

322    urinary microbiome composition (**Figure 1a**). Similar associations have been identified in other

323    microbiome communities in the human body[41] and in the urinary microbiome in women, [25] but

324    have not yet been reported in the male urinary microbiome. This means that men with similar

325    BMIs have similar urinary microbiome compositions, compared to men with different BMIs

326    regardless of age, BPH, and medical history.

327        We found that BMI is linked to specific genera as well as to the overall microbiome

328    composition. Using a Kruskal Wallis rank sum test, the relative abundances of

329    *Corynebacterium*, *Staphylococcus*, and *Streptococcus* were significantly different in healthy

330    weight, overweight, and obese men; obese men had a decreased abundance of all three

331    genera, whereas healthy weight men had an increased abundance (**Figure 1b**). Since these

332    genera are typically found in a healthy urinary microbiome, and BMI is significantly associated

333    with beta diversity, we can hypothesize that the lower relative abundance of these three genera

334    allows other bacteria, like *Dialister*, to create a niche in those communities. A machine-learning

335    approach, HAllA, revealed a significant relationship between the abundance of *Dialister* and

336    higher BMI (**Figure 1c**), an association which has not been previously reported in the context of

337    the urinary microbiome.[29–31] In the gut, many studies have shown *Dialister* in individuals with

338    higher BMIs and who have difficulty losing weight.[26,30,41,42] One study surmised that *Dialister*

339    aggravates the host inflammatory response and insulin resistance by releasing more

340    lipopolysaccharides,[30] which are known to be an important feature in metabolic disease and

341    weight gain.[43] Interestingly, in the DMM clustering that revealed eight urotypes (**Figure 2**),

342    urotype 5 contained the highest percentage of obese men and the highest percentage of men

343    with *Dialister*. Although this mechanism has not been studied in the urinary microbiome, a

344    similar one could be at play. Interestingly, in the DMM clustering that revealed eight urotypes

345    (**Figure 2**), in the second highest cluster of obese men (urotype 7) *Dialister* was not detected.

346    We believe this is something to investigate further and examine if other bacterial communities in

347    the urinary microbiome could have an association with BMI.

348         We also observed that men with BPH tended to have a higher alpha diversity in their

349    urinary microbiome as compared to men without (**Table Sup2**) – a finding that had previously

350    not been reported in studies.[23] BPH has previously been associated with significant changes in

351    the urinary microbiota and in the microbes of the prostate tissue itself.[22,23] In rats, BPH has been

352    associated with the beta diversity of the gut microbiome; however, no such studies have been

353    undertaken in humans.[24]

354         We did not identify significant associations between overall LUTS score and the urinary

355    microbiome composition in men.  Previous work has shown an association between more

356    severe LUTS and the following genera: *Haemophilus, Staphylococcus, Dolosigranulum, Listeria,*

357    *Phascolarctobacterium, Enhydrobacter, Ruminococcus, Bacillus, Faecalibacterium, and*

358    *Finegoldia.*[23] We did not find *Listeria* nor *Ruminococcus* in our dataset, and there were no

359    associations between the other genera and LUTS severity. However, we believe the overall

360    IPSS severity score may not allow for the detection of associations with specific causes of

361    urinary symptoms (e.g., irritative and obstructive symptoms), which could have different

362    etiologies. We considered the hypothesis that individual elements of the LUTS symptoms score

363    could have different associations with the microbiome. For instance, irritative symptoms could

364    have more inflammatory bacteria, while obstructive symptoms could be the result of bacteria

365    more likely to create a biofilm which could create some blockage while voiding. In support of this

366    idea, we did find significant associations between alpha diversity metrics and specific

367    obstructive and irritative LUTS symptoms when the analyses were adjusted for BMI, BPH, and

368    age. The inverse Simpson index was significantly associated with both obstructive and irritative

369    symptoms; however, different trends were observed depending on BMI grouping and BPH

370    (**Figure 3e-f**). Similar to what was discussed earlier with *Dialister* and BMI, these results

371    suggest that there could be different mechanisms causing irritative or obstructive symptoms in

372    obese men as compared to overweight men with or without BPH.

373         HAllA unveiled associations at the family level between Enterococcaceae and

374    Caulobacteracaea with irritative symptoms; however, no associations were found at the phylum

375    or genus level, nor any associations with obstructive symptoms. The HAllA analysis was

376    restricted to 54 of the 571 genera, so this may have contributed to our limited findings. More

377    work is needed to determine which taxa are important for irritative and obstructive symptoms;

378    however, the heterogeneity of bacteria between individuals and other characteristics of the

379    microbiome may not yield consensus. In other words, biological functions may be more

380    homogenous and may be more indicative of phenotype or symptoms, despite being caused by a

381    number of different bacteria.

382     The large sample size of this study allowed us to investigate broad trends across the

383     urinary microbiome of older community-dwelling men. Although we had a rich dataset, there

384     were some limitations that may have affected our analysis. Elements of the phenotypic data

385     (e.g., history of diabetes and cancer) were self-reported and thus subject to errors. The samples

386     collected were voided urine, which have been reported to not represent only the male bladder

387     microbiome, but instead be a mixture of the bladder and urethral communities.[21] Also, as with

388     any 16S rRNA sequencing study, analysis was limited to the genus level, and resolution to the

389     species level may improve findings. For example, there are many different species of *Dialister*.

390     Finally, the LUTS score is based on a set of symptoms that may be the result of multiple

391     biological pathways, and thus difficult to disentangle.

392     In summary, the male urinary microbiome is complex and challenging to investigate.

393     There is considerable individual variation in the urinary microbiome in men, and that variation

394     may be related to the presence and character of LUTS. Our study indicates that considering the

395     specific type of LUTS is important in understanding the contribution of urinary microbiota to

396     urinary disorders in men. We also revealed there is an intricate connection between BMI, BPH,

397     urinary microbiome diversity, and severity of irritative and obstructive symptoms. More studies

398     are needed to further investigate these covariates, and we believe future urinary microbiome

399     studies in men should heavily consider BMI and BPH in study designs and analyses.

400

401     **Methods**

402     Study Population:

403     We acquired samples and data from 500 randomly selected participants from the prospective

404     NIH-funded MrOS study. The MrOS study recruited a total of 5,994 men between the ages of 65

405     and 100 years from 6 clinical sites in the United States to assess risk factors for fracture and

406     other conditions related to aging. The cohort and recruitment methods have been previously

407     described.[17] At baseline, participants were at least 65 years of age, able to consent, walked

408    without assistance of another person, and did not have bi-lateral hip replacement or any

409    condition that in the judgment of the site investigator would likely impair participation in the

410    study.[44] The Institutional Review Boards at all sites reviewed and approved the study, and all

411    participants provided written informed consent.  Enrolled participants completed a series of

412    medical questionnaires, including medical history of prostatitis and BPH and the AUA-SI

413    questionnaire, and provided specimens such as blood and urine which were banked for future

414    research. Participants were recruited between 2000 and 2002 and followed longitudinally.

415    Morning, second-voided urine specimens were collected in sterile containers and frozen at -80

416    °C for future analyses.

417

418    DNA isolation and Sequencing:

419    Bacterial DNA was isolated from 4-mL aliquots of urine specimens. The V4 region of the 16S

420    rRNA gene was selectively amplified to evaluate microbial composition of the urine. DNA from

421    the 500 urine samples was submitted to Baylor University for paired-end 250 base-pair

422    sequencing using Illumina MiSeq by Dr. Nadim Ajami.

423

424    Classification of LUTS:

425    LUTS was assessed using the IPSS. The IPSS is a seven-question examination that evaluates

426    both irritative and obstructive aspects of LUTS. Each question is given a score from zero to five

427    in terms of how severe that specific symptom is, and the scores for all questions are totaled.

428    No/mild LUTS were assigned to scores $0 \leq 7$, while moderate/severe LUTS corresponds to

429    scores greater than 7. LUTS was then divided into irritative versus obstructive symptoms for

430    sub-analyses based upon the IPSS questionnaire. Scores from questions 1, 3, 4, and 6 were

431    totaled for an overall obstructive symptom score, and the scores from the remaining questions

432    made up the irritative score.

433

434     <u>Bioinformatics and Statistical Analyses</u>:

435     Raw sequences were processed into amplicon sequence variants (ASVs) using DADA2. The

436     RDP Classifier was used to map the ASVs to the SILVA 128 16S rRNA reference set for

437     taxonomic identification. The 500 samples were rarefied to 1000 reads, which removed 25

438     samples and left 475 samples for downstream analyses. All subsequent analyses were done on

439     ASVs agglomerated at the genus level in R, except for application of HAllA which uses

440     Python.[27] Data were further processed using phyloseq (version 1.42.0) and visualized using

441     *microshades* (version 1.10).[45] The *Vegan* R package version 2.6.4 and *rstatix* version 0.7.2

442     were used for all statistical analyses.

443

444     <u>Testing for associations between taxa and clinical characteristics</u>

445     We implemented HAllA version 0.8.20 as an exploratory analysis to investigate associations

446     between individual taxa and the clinical characteristics. HAllA was carried out separately at the

447     genus, family, and phylum levels, and we exported both our metadata and ASV tables to text

448     files. The metadata included the clinical variables of Table 1, and separate ASV tables were

449     generated for each taxonomic level.

450

451     We also looked at relative abundance of the top 5 phyla and top 10 genera for BMI, BPH, and

452     age. A Kruskal-Wallis test was used to test for a significant association between relative

453     abundance and clinical characteristic of interest, and then a pairwise Wilcoxon Rank Sum test

454     with false discovery rate correction.

455

456     <u>Clustering of samples by microbiome composition</u>

457     Treating each urinary microbiome as a community, we decided to cluster based on these

458     communities and then look for associations with clinical characteristics and LUTS. We used the

459     *DirichletMultinomial* version 1.40.0 for DMM clustering on the "core" taxa, or the bacteria

460    present in at least 20% of samples, to improve processing speed. We evaluated the model fit for

461    two through 12 clusters using the Laplace approximation and chose eight clusters based on a

462    global minimum (**Figure S5**)[33] and found that qualitatively eight clusters exemplified eight

463    different community types dominated by different taxa (Figure 2a). The samples were separated

464    into eight clusters based on the core taxa, and the bacterial communities in each cluster were

465    tested for associations with clinical characteristics using the tableone R package (version

466    0.13.2).

467

468    **Author Contributions**

469    E.O. designed the study and assisted with data access and acquisition. M.G. guided data

470    analysis and interpretation with clinical input. L.K. designed and guided the data analysis, and

471    wrote the manuscript. K.B. analyzed the data and wrote the manuscript. All authors contributed

472    to interpretation of results and editing the manuscript.

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**References**

488

489  1.  Fouts, D. E. *et al.* Integrated next-generation sequencing of 16S rDNA and

490      metaproteomics differentiate the healthy urine microbiome from asymptomatic bacteriuria

491      in neuropathic bladder associated with spinal cord injury. *J Transl Med* **10**, 174 (2012).

492  2.  Lewis, D. A. *et al.* The human urinary microbiome; bacterial DNA in voided urine of

493      asymptomatic adults. *Front Cell Infect Microbiol* **3**, (2013).

494  3.  Khasriya, R. *et al.* Spectrum of Bacterial Colonization Associated with Urothelial Cells

495      from Patients with Chronic Lower Urinary Tract Symptoms. *J Clin Microbiol* **51**, 2054–

496      2062 (2013).

497  4.  Pearce, M. M. *et al.* The female urinary microbiome: A comparison of women with and

498      without urgency urinary incontinence. *mBio* **5**, (2014).

499  5.  Price, T. *et al.* The urobiome of continent adult women: a cross-sectional study. *BJOG*

500      **127**, 193–201 (2020).

501  6.  Perez-Carrasco, V., Soriano-Lerma, A., Soriano, M., Gutiérrez-Fernández, J. & Garcia-

502      Salcedo, J. A. Urinary Microbiome: Yin and Yang of the Urinary Tract. *Front Cell Infect*

503      *Microbiol* **11**, (2021).

504  7.  Modena, B. D. *et al.* Changes in Urinary Microbiome Populations Correlate in Kidney

505      Transplants With Interstitial Fibrosis and Tubular Atrophy Documented in Early

506      Surveillance Biopsies. *American Journal of Transplantation* **17**, 712–723 (2017).

507  8.  Gottschick, C. *et al.* The urinary microbiota of men and women and its changes in women

508      during bacterial vaginosis and antibiotic treatment. *Microbiome* **5**, 99 (2017).

509  9.  Vaughan, M. H. *et al.* The Urinary Microbiome in Postmenopausal Women with Recurrent

510      Urinary Tract Infections. *Journal of Urology* **206**, 1222–1231 (2021).

511    10.    Price, T. K. *et al.* Temporal Dynamics of the Adult Female Lower Urinary Tract

512           Microbiota. (2020) doi:10.1128/mBio.

513    11.    Karstens, L. *et al.* Does the Urinary Microbiome Play a Role in Urgency Urinary

514           Incontinence and Its Severity? *Front Cell Infect Microbiol* **6**, (2016).

515    12.    Xie, J. *et al.* Profiling the urinary microbiome in men with calcium-based kidney stones.

516           *BMC Microbiol* **20**, 41 (2020).

517    13.    Yu, S. H. & Jung, S. il. The Potential Role of Urinary Microbiome in Benign Prostate

518           Hyperplasia/Lower Urinary Tract Symptoms. *Diagnostics* **12**, 1862 (2022).

519    14.    Mansour, B. *et al.* Bladder cancer-related microbiota: examining differences in urine and

520           tissue samples. *Sci Rep* **10**, 11042 (2020).

521    15.    Ahn, H. K., Kim, K., Park, J. & Kim, K. H. Urinary microbiome profile in men with

522           genitourinary malignancies. *Investig Clin Urol* **63**, 569 (2022).

523    16.    Mondul, A. M., Giovannucci, E. & Platz, E. A. A prospective study of obesity, and the

524           incidence and progression of lower urinary tract symptoms. *Journal of Urology* **191**, 715–

525           721 (2014).

526    17.    Orwoll, E. *et al.* Design and baseline characteristics of the osteoporotic fractures in men

527           (MrOS) study — A large observational study of the determinants of fracture in older men.

528           *Contemp Clin Trials* **26**, 569–585 (2005).

529    18.    Taylor, B. C. *et al.* Prevalence, severity, and health correlates of lower urinary tract

530           symptoms among older men: The MrOS study. *Urology* **68**, 804–809 (2006).

531    19.    De Nunzio, C., Salonia, A., Gacci, M. & Ficarra, V. Inflammation is a target of medical

532           treatment for lower urinary tract symptoms associated with benign prostatic hyperplasia.

533           *World J Urol* **38**, 2771–2779 (2020).

534    20.    Nelson, D. E. *et al.* Characteristic Male Urine Microbiomes Associate with Asymptomatic

535           Sexually Transmitted Infection. *PLoS One* **5**, e14116 (2010).

536    21.    Bajic, P. *et al.* Male Bladder Microbiome Relates to Lower Urinary Tract Symptoms. *Eur*

537            *Urol Focus* **6**, 376–382 (2020).

538    22.    Yu, H. *et al.* Urinary microbiota in patients with prostate cancer and benign prostatic

539            hyperplasia. *Archives of Medical Science* **2**, 385–394 (2015).

540    23.    Lee, H. Y. *et al.* The impact of urine microbiota in patients with lower urinary tract

541            symptoms. *Ann Clin Microbiol Antimicrob* **20**, (2021).

542    24.    Li, L.-Y. *et al.* Alterations of gut microbiota diversity, composition and metabonomics in

543            testosterone-induced benign prostatic hyperplasia rats. *Mil Med Res* **9**, 12 (2022).

544    25.    Thomas-White, K. J. *et al.* Evaluation of the urinary microbiota of women with

545            uncomplicated stress urinary incontinence. *Am J Obstet Gynecol* **216**, 55.e1-55.e16

546            (2017).

547    26.    Pinart, M. *et al.* Gut Microbiome Composition in Obese and Non-Obese Persons: A

548            Systematic Review and Meta-Analysis. *Nutrients* **14**, 12 (2021).

549    27.    Ghazi, A. R. *et al.* High-sensitivity pattern discovery in large, paired multiomic datasets.

550            *Bioinformatics* **38**, i378–i385 (2022).

551    28.    Hrbacek, J., Morais, D., Cermak, P., Hanacek, V. & Zachoval, R. Alpha-diversity and

552            microbial community structure of the male urinary microbiota depend on urine sampling

553            method. *Sci Rep* **11**, 23758 (2021).

554    29.    Muñiz Pedrogo, D. A. *et al.* Gut Microbial Carbohydrate Metabolism Hinders Weight Loss

555            in Overweight Adults Undergoing Lifestyle Intervention With a Volumetric Diet. *Mayo Clin*

556            *Proc* **93**, 1104–1110 (2018).

557    30.    Yan, H. *et al.* Gut Microbiome Alterations in Patients With Visceral Obesity Based on

558            Quantitative Computed Tomography. *Front Cell Infect Microbiol* **11**, (2022).

559    31.    Gao, X. *et al.* Body Mass Index Differences in the Gut Microbiota Are Gender Specific.

560            *Front Microbiol* **9**, (2018).

561   32.   Wang, J. *et al.* Enterotype Bacteroides Is Associated with a High Risk in Patients with

562         Diabetes: A Pilot Study. *J Diabetes Res* **2020**, 1–11 (2020).

563   33.   Holmes, I., Harris, K. & Quince, C. Dirichlet Multinomial Mixtures: Generative Models for

564         Microbial Metagenomics. *PLoS One* **7**, e30126 (2012).

565   34.   Komesu, Y. M. *et al.* The urinary microbiome in women with mixed urinary incontinence

566         compared to similarly aged controls. *Int Urogynecol J* **29**, 1785–1795 (2018).

567   35.   Li, K. *et al.* Interplay between bladder microbiota and overactive bladder symptom

568         severity: a cross-sectional study. *BMC Urol* **22**, 39 (2022).

569   36.   Burton, E. N. *et al.* Characterization of the urinary microbiome in healthy dogs. *PLoS One*

570         **12**, e0177783 (2017).

571   37.   Colas, L. *et al.* Unique and specific Proteobacteria diversity in urinary microbiota of

572         tolerant kidney transplanted recipients. *American Journal of Transplantation* **20**, 145–158

573         (2020).

574   38.   Curtiss, N. *et al.* Age, menopausal status and the bladder microbiome. *European Journal*

575         *of Obstetrics & Gynecology and Reproductive Biology* **228**, 126–129 (2018).

576   39.   Chart, H. Vibrio, mobiluncus, gardnerella and spirillum. in *Medical Microbiology* 314–323

577         (Elsevier, 2012). doi:10.1016/B978-0-7020-4089-4.00045-7.

578   40.   Pohl, H. G. *et al.* The Urine Microbiome of Healthy Men and Women Differs by Urine

579         Collection Method. *Int Neurourol J* **24**, 41–51 (2020).

580   41.   Liu, R. *et al.* Gut microbiome and serum metabolome alterations in obesity and after

581         weight-loss intervention. *Nat Med* **23**, 859–868 (2017).

582   42.   Zhang, Q. *et al.* Comparison of gut microbiota between adults with autism spectrum

583         disorder and obese adults. *PeerJ* **9**, e10946 (2021).

584   43.   Cani, P. D. *et al.* Metabolic Endotoxemia Initiates Obesity and Insulin Resistance.

585         *Diabetes* **56**, 1761–1772 (2007).

586    44.    Blank, J. B. *et al.* Overview of recruitment for the osteoporotic fractures in men study

587            (MrOS). *Contemp Clin Trials* **26**, 557–568 (2005).

588    45.    Dahl, E. M., Neer, E., Bowie, K. R., Leung, E. T. & Karstens, L. microshades : An R

589            Package for Improving Color Accessibility and Organization of Microbiome Data.

590            *Microbiol Resour Announc* **11**, (2022).

591

592