Evidence for an indigenous female mouse urobiome

Sidra Sohail [1], Daniel Bushnell [2], Mark Khemmani [1], Sridhar Narla [2], Olivia Lamana [3], Bhanu Sharma [2], Robert B. Moreland [1], Alan J. Wolfe [1*], Catherine S. Forster [2*]

[1] Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, IL, USA

[2] Department of Pediatrics, University of Pittsburgh School of Medicine and UPMC Children's Hospital of Pittsburgh, Pittsburgh, PA, USA

[3] Biomedical Research Institute, Rockville, MD, USA

***Co-Corresponding authors:**

Catherine Forster, MD, MS
4401 Penn Ave
FP 3133
Pittsburgh, PA 15224
Ph: 412-692-8337, Email: forstercs@upmc.edu

Alan J. Wolfe, PhD
2160 South First Ave
CTRE
Maywood, IL 60153
Email: awolfe@luc.edu

Keywords: 16S rRNA gene sequencing, Microbiome, Mouse, Urinary tract, Urobiome

Author contributions: AJW and CSF designed and implemented the original study protocol and obtained funding. DB, SN, OL, and BS did the mouse work. MK processed the samples for sequencing. SS analyzed the data. MK and SS constructed the figures. AJW, MK, and CSF interpreted data. AJW, CSF, MK, RBM, and SS wrote the manuscript. All authors reviewed and approved the manuscript.

Funding: CSF was supported by 1K23DK129783. AJW was supported by a contract with Pathnostics.

Disclosure statement from authors
AJW discloses membership on the Scientific Advisory Boards of Urobiome Therapeutics, Pathnostics, and Cerillo. He also discloses funding from the Craig H. Neilsen Foundation, Pathnostics, and an anonymous donor. The rest of the authors have no relevant disclosures.

Data availability: Raw sequencing reads are publicly available at the NCBI SRA database (BioProject ID: PRJNA1229522).

Abstract

Mice have been used as a valuable model of understanding pathophysiological mechanisms of urinary tract infection for almost six decades. Mice offer many advantages including genetic manipulation to test the role of genes and mechanisms, the availability of germ-free mice, and similarities to humans in innate immune defenses and the strain-dependent presence of vesicoureteral reflux. However, like with humans, the mouse bladder urine above the urinary sphincter has generally been assumed to be sterile. Yet, given the presence of urobiomes in other mammals and the emerging role of the human urobiome in the defense of the urinary bladder and upper urinary tract, the existence of a mouse urobiome should be critically examined as indigenous microbiota may influence experimental results. To determine if an indigenous murine urobiome exists, we obtained expressed urine from two sets of female C57BL/6J mice during three different intervals using two different extraction and sequencing methods and analyzed them simultaneously by a single method. For one set, we also obtained urine by suprapubic aspiration, which we compared to the paired expressed urine samples. We conclude that an indigenous murine urobiome exists and that expressed urine contains post-urethral microbes.

49 # Introduction

50 Urinary tract infections (UTI) are among the most common infections in clinical
51 practice (1). Commonly diagnosed with urine dipstick and in office urinalysis, UTIs
52 are responsible for the bulk of antibiotic prescriptions worldwide, leading to
53 antibiotic resistant microbes (2). UTIs, especially when they recur, are associated
54 with renal scarring and can lead to eventual renal failure (3, 4). In one autopsy study
55 of all-cause mortality, healed pyelonephritis was found in 18% of patients while a
56 total of 33% demonstrated evidence of active or healed pyelonephritis (3).
57 Consequently, understanding the pathophysiological mechanisms involved in UTIs
58 is of key importance (5).

59 Urine has long been believed to be sterile above the urethral sphincter (6, 7). This
60 belief is reinforced by false negative urine dipstick analyses, and the fact that most
61 standard urine cultures (SUC) exhibit no growth in the absence of symptoms.
62 However, much of the myth of urine sterility is due to SUC methods that favor
63 facultative anaerobes, such as members of the genera *Escherichia*, *Pseudomonas*,
64 *Klebsiella*, *Proteus*, *Staphylococcus*, and *Enterococcus*, with *Escherichia coli* by far
65 considered the predominant cause of UTI (5, 6, 8). We now know that human bladder
66 urine is not sterile and that appropriate culture conditions can culture 70% of the
67 genera detected by DNA sequencing methods (8, 9). Furthermore, negative SUC or

68 urine dipstick tests can miss many important emerging uropathogens, as well as lead
69 to unnecessary antibiotic use that destroys the indigenous healthy urinary
70 microbiome (also known as the urobiome) (1).

71 Research aimed at bettering our understanding of the virulence mechanisms and
72 host-pathogen interactions in UTI have relied on animal models (10). Key in
73 experiments with dogs, cats, rats and mice has been the assumption that urine above
74 the urethral sphincter is sterile and that introduction of test microbes reflects human
75 UTI. Recently, this notion has been disproved with low biomass, indigenous
76 microbiota in dogs (11-14), cats (15) and rats (16, 17). While defining low biomass
77 microbiota brings challenges associated with ruling out contamination (15, 18, 19),
78 the indigenous microbiota may play a role in the defense of the bladder and
79 understanding normal states and dysbiosis are key in any model of UTI, as well as
80 in human disease (20-23).

81 Mice have been used as a valuable model of understanding pathophysiological
82 mechanisms of UTI for almost six decades (10). Mice offer many advantages
83 including genetic manipulation to test the role of genes and mechanisms, the
84 availability of germ-free mice (which lack microbiota), and similarities to humans
85 in innate immune defenses and the strain-dependent presence of vesicoureteral
86 reflux (10, 24). However, like with humans, the mouse bladder urine above the
87 urinary sphincter has generally been assumed to be sterile. Yet, given the presence

88 of urobiomes in other mammals and the emerging role of the human urobiome in the
89 defense of the urinary bladder and upper urinary tract, the existence of a mouse
90 urobiome should be critically examined as indigenous microbiota may influence
91 experimental results. Indeed, two studies provide some evidence that a mouse
92 urobiome does exist.

93 One study examined microbes from mouse bladder urine sampled by suprapubic
94 aspirates using aerobic culture methods and viability assays (25). By aerobic culture,
95 they found 150 ± 220 CFU/ml (n=5); however, when cell viability was examined
96 using a BacLight LIVE/DEAD bacterial viability assay, they found $4.5 \pm 2.8 \times 10^6$
97 cells/mL. These were scored as "viable but not culturable." Another study examined
98 the effect of microbiota on the transcriptome and weight of the urinary bladder by
99 comparing germ-free (GF) and specific pathogen-free (SPF)-housed mice (26). The
100 GF urinary bladders had on average 25% lower weight than bladders from mice
101 housed under standard SPF conditions. These experiments were carried out in two
102 different facilities using two different C57BL/6 substrains. Histologically,
103 hematoxylin-eosin-stained sections of GF and SPF bladder tissue showed regular
104 bladder tissue composition. The difference in bladder weight was not correlated to
105 any tissue structural changes by histological assessment of stained sections. To
106 confirm that reduced bladder weight was not a microbiota-driven developmental
107 anomaly, the investigators depleted microbiota among SPF-housed mice using

108 broad-spectrum antibiotics. This continuous antibiotic treatment reproduced the
109 reduced bladder weight phenotype in SPF mice. Thus, reduction in bladder weight
110 was consistent with an adaptive bladder change in response to the absence of
111 microbiota or their products. This study suggests that microbiota can have a
112 profound effect on the host it inhabits. Since germ-free mice are devoid of gut and
113 bladder microbiota, it is impossible to distinguish the effect of urinary microbiota
114 from gut microbiota. However, removing all microbiota from mice affects urinary
115 bladder weight and size. Taken together, these two studies provide a compelling
116 reason to ask whether an indigenous murine urobiome exists.

117 To answer this question, we obtained expressed urine from two sets of female
118 C57BL/6J mice during three different intervals using two different extraction and
119 sequencing methods and analyzed simultaneously by a single method. For one set,
120 we also obtained urine by suprapubic aspiration, which we compared to the paired
121 expressed urine samples. We conclude that an indigenous murine urobiome exists
122 and that expressed urine contains post-urethral microbes.

123 Materials and methods

124 Ethics Statement

125 This study was carried out in strict accordance with the recommendations in the
126 Guide for the Care and Use of Laboratory Animals of the National Institutes of

127 Health. This study was approved by the institutional animal care and use committees
128 at the University of Pittsburgh (protocol 23124147) and the Biomedical Research
129 Institute (protocol 20-01) prior to initiation of the work. Suprapubic aspirations were
130 performed under inhaled isofluorane anesthesia, and all efforts were made to
131 minimize suffering.

132 **Mice.** Two sets of female C57BL/6J mice (Jackson Labs, Bar Harbor, ME; stock no.
133 000664) were analyzed. We allowed a three-week acclimation period prior to urine
134 collection for animals in set 1; the mice were 10 weeks at the time of urine collection.
135 For set 2, which were housed in a different institution, we allowed a one-week
136 acclimation period prior to urine collection; the mice were 10 weeks at the time of
137 urine collection. The mice in set 3 were some of the same mice in set 1, but they
138 were aged for an additional 4 months. There were no interventions or other
139 experimental procedures performed in the mice in set 3 while they aged. The mice
140 in set 3 were 27 weeks at the time of collection.

141 *Housing and husbandry:* All mice were socially housed with 5 mice per cage, with
142 standard enrichment in accordance with guidelines of the Association for
143 Assessment and Accreditation of Laboratory Animal Care. All animals within each
144 set were housed within the same room within the same vivarium and were given the
145 same chow and water from the same source. All mice for this work were
146 commercially-purchased: no mice were bred for this work.

147 *Animal Care and Monitoring:* Bladder expression for urine is not generally a painful
148 procedure and routine analgesia was not provided. Mice was anesthetized with
149 isoflurane prior to suprapubic aspiration. We had both ketoprofen and buprenorphine
150 available should mice display any signs of pain or distress, but no mice required
151 analgesia. There were no expected or unexpected adverse events. As this was an
152 observational study of the mouse urobiome, the endpoint was obtaining sufficient
153 urine for urobiome analysis. No mice were euthanized as part of this work.

154 **Urine Collection**

155 Expressed Urine. To collect expressed urine samples, we first scruffed each mouse
156 while holding the tail up in the palm of the collector's hand and used alcohol wipes
157 to clean the perineum. The mouse was then held over the open tube (sterile, DNase
158 and RNase-free microcentrifuge tubes), and suprapubic pressure applied to induce
159 voiding directly into the tube without any contact with the lid or rim. We had to
160 combine urine samples from multiple expressions to obtain sufficient urine volume
161 for sequencing, which we determined was a minimum of 0.5 mL. On average, each
162 voiding produced approximately 50 μL of urine. We added AssayAssure® (nucleic
163 acid preservative, Sierra Molecular Corp, Princeton, NJ, LOT G3030C) to the urine
164 in a 1:9 ratio and froze the sample at -80°C. For sets 1 and 2, only expressed urine
165 was obtained.

166 Suprapubic Aspiration. For set 3, we obtained paired urine samples collected by both
167 expression (as described above) and suprapubic aspiration. To minimize the time
168 between the expressed and aspirated samples for each mouse, we collected the
169 expressed samples immediately before collecting the aspirated samples. For
170 suprapubic aspiration, we anesthetized the mice with inhaled isoflurane prior to
171 aspiration. Once anesthesia was confirmed, we placed the mice on their backs and
172 used Nair™ (Church and Dwight Co, Ewing, NJ) to remove the fur on the suprapubic
173 region. We then thoroughly cleaned the area with alcohol swabs. We placed the
174 ultrasound probe (VisualSonics RMV 710B Ultrasound Imaging Scanhead 25 MHz
175 70 x 140 μm for Vevo 770, FUJIFILM VisualSonics Inc, Toronto, Canada) on the
176 abdomen with the ultrasound machine (Vevo 770 High-Resolution In Vivo Micro-
177 Imaging System, FUJIFILM VisualSonics Inc, Toronto, Canada) on the maximum
178 refresh rate. Once the bladder was in view, we inserted a 1mL syringe with a 30G
179 needle into the abdomen and into the bladder under ultrasound guidance. From the
180 bladder, we aspirated the urine and transferred it into a sterile Eppendorf tube (Fisher
181 brand Premium 1.5 mL MCT Graduated Natural Eppendorf Tubes, sterile,
182 RNase/DNase free, Cat No. 05-408-129). We added AssayAssure® (Sierra
183 Molecular Corp, Princeton, NJ, LOT G3030C) in a 1:9 ratio and stored the samples
184 at -80°C.

185 **Genome Extraction.** For sets 1 and 3, each mouse's multiple samples of urine were
186 thawed at 4°C and then pooled into a single microcentrifuge tube. Next, 0.5 mL of
187 urine was transferred to a deepwell plate, which was centrifuged for 10 mins at 1300
188 x g and all but 100 μL of supernatant was removed. Enzymatic treatment consisting
189 of 4 mg Lysozyme, 3 kU Mutanolysin, 4 μg Lysostaphin, and 200U
190 Achromopeptidase in buffer (20 mM Tris, 2 mM EDTA, 1.2% Triton, pH 8.0) were
191 added to each sample and incubated at 37°C, 5 hours, shaking intermittently at
192 1000rpm (Eppendorf Thermomixer with SmartBlock DWP1000). Samples were
193 then processed using the Dneasy 96 Blood & Tissue kit (Qiagen, Germantown, MD)
194 according to the manufacturer's instructions. To assess potential DNA
195 contamination, extraction negative controls (no urine) were processed with samples
196 and sequenced in every run. For set 2, genomic DNA was isolated using the DNeasy
197 PowerSoil Kit (Qiagen, Germantown, MD), according to kit instructions, with the
198 exception that DNA was eluted from the column with nuclease-free water (Sigma-
199 Aldrich, St. Louis, MO).

200 **Sequencing.** The urine samples from sets 1 and 3 were sequenced by SeqCenter
201 (Pittsburgh, PA), where they were prepared using Zymo Research's Quick-16S kit
202 with phased primers targeting the V4 regions of the 16S rRNA gene (5'-
203 GTGYCAGCMGCCGCGGTAA-3', 5'-GGACTACNVGGGTWTCTAAT-3') and
204 sequenced. Following clean-up and normalization, samples were sequenced on a P1-

205 600cyc NextSeq2000 Flowcell to generate 2 x 301 bp PE reads. Adapters were
206 trimmed.

207 The urine samples from set 2 were sequenced by the Genomics and Epigenomic
208 Shared Resource at the Lombardi Comprehensive Cancer Center at Georgetown
209 University. Library preparation and sequencing was performed according to
210 Illumina 16S Metagenomics Sequencing Library Sample Preparation Guide. In
211 brief, genomic DNA isolated from mouse urine samples was used to amplify the
212 16S V3-V4 region. The primers used for the amplification were 16S Amplicon
213 PCR Forward Primer (5'-
214 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGC
215 AG) and 16S Amplicon PCR Reverse Primer (5'-
216 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTAT
217 CTAATCC). The IDT-Illumina DNA-RNA UD Indices SetA Tagmentation index
218 kit was used for the index PCR. The amplicons were pooled and sequenced with
219 MiSeq v3 600 cycle reagent kit (Illumina) with 15% PhiX spike-in.

220 **Taxonomic Identification.** For sets 1, 2, and 3, high-quality reads were obtained by
221 trimming low-quality sequences using Cutadapt (27). The DADA2 software (28)
222 was then used to process the reads through filtering, dereplicating, removing
223 chimeras, and constructing an amplicon sequence variant (ASV) abundance table.
224 ASVs were annotated using the Bayesian LCA (BLCA) method (29) and the NCBI

225 database. ASVs with BLCA confidence scores below 70% were labeled "unknown."
226 For sets 1 and 3, ASVs linked to the genus *Lysobacter* were excluded due to its
227 recognized presence as a contaminant in the achromopeptidase enzyme used during
228 the DNA extraction process; for set 2, ASVs linked to *Lysobacter* were excluded for
229 comparability to sets 1 and 3. Additionally, ASVs with total counts below the sample
230 number threshold were excluded (n=48/52/54 for sets 1, 3, and 2, respectively).
231 Contaminant ASVs were identified using control samples and were determined via
232 a scoring method that encompasses results from Decontam (30), nonparametric
233 statistical testing (Kruskal-Wallis $p<0.5$), mean comparison (i.e., mean counts of
234 samples were less than mean counts of extraction controls), and whether sample read
235 counts were less than the 5x greater than extraction controls counts. Decontaminated
236 ASV counts were used for downstream analysis.

237 **Analysis.** For each set, the downstream analyses included beta diversity as measured
238 by Principal Coordinate Analysis (PCoA) of decontaminated ASV counts.
239 Following taxonomic identification, histograms and heatmaps were constructed
240 showing the 49 most relatively abundant genera plus the categories “other” and
241 “unknown.” With the histograms, we included total read count for each genus. These
242 analyses were performed in R (R 4.3.1). For the PCoA, the Bray-Curtis distance was
243 calculated using the vegdist function in the vegan library version 2.6.6.1 (31) and
244 the resulting Bray-Curtis distance matrix was input to the pcoa function in the ape

245 library version 5.8 to perform PCoA. The first two axes of the PCoA output were
246 graphed using the plotly library version 4.10.4. The Bray-Curtis distance matrix was
247 used as input to the PERMANOVA function in the PERMANOVA library version
248 0.2.0 to calculate the *p*-value. The relative abundance graphs were created by
249 aggregating ASVs at the genus level and applying the transform function from the
250 microbiome library (version 1.26.0) to transform the sample counts and visualizing
251 the relative abundance using the plotly library (version 4.10.4). The heatmap
252 analysis was graphed using the Heatmap function in the ComplexHeatmap library
253 version 2.20.0 (32). To directly compare expressed urine samples from all three sets,
254 the decontaminated ASV count tables from each set were combined into one
255 database. Suprapubic aspirate samples were excluded. To directly compare paired
256 suprapubic aspirates and expressed urine from the mice of set 3, the decontaminated
257 ASV count tables were combined into one database. For both analyses, beta
258 diversity, heatmap, and taxa histograms were performed as described above. For
259 biplot analysis, genus level PCA was performed with the prcomp function
260 (stats library v. 4.4.1) and visualized as a 3D biplot using factoextra (v.1.0.7).

261 # Results

262 We first determined a minimal urine volume for mouse urobiome studies, comparing
263 16S rRNA gene sequencing results across a range of urine volumes aliquoted from
264 the same pooled urine from 4 different female mice. We determined that 500 μL was
265 the smallest volume sufficient to extract and sequence enough DNA to adequately
266 assess the urobiome while accounting for urine collection feasibility **(Supporting**
267 **Fig. 1).**

268 **Supporting Figure 1. Determination of the smallest volume sufficient to extract**
269 **and sequence enough DNA to adequately assess the urobiome while accounting**
270 **for urine collection feasibility.** Histogram showing the 49 most relatively abundant
271 genera detected in the extraction controls and expressed urine from 5 groups of mice.
272 The category "Other" is an amalgamation of all other genera; the category
273 "Unknown" represent all sequences that could not be identified. For each group of
274 mice, different urine volumes were tested (125, 250, 500, 1000 μL). Note that the
275 yellow line, which depicts total normalized arbitrary units (i.e., total reads per
276 sample), correlates with sample volume, with 125μL producing numbers of reads
277 apporximately the same as the extraction controls, while 1000 μL produced the
278 largest number of reads. 500 μL were chosen for subsequent study as producing
279 sufficiently high read numbers while balancing the feasiblity of obtaining urine from
280 mouse bladders.

281 To determine whether the bioinformatically decontaminated data from each set of
282 mice differed from their respective extraction controls, we performed PCoA using
283 Bray-Curtis distances. For example, see **Supporting Fig. 2** for the analysis of the
284 expressed urine samples of the set 1 mice. Although the samples differed
285 significantly from the extraction controls (PERMANOVA, $p = 0.003$), some samples

286 appeared similar to the controls. However, a histogram of aggregated ASVs at the
287 genus level and total read counts revealed substantial differences in terms of
288 composition with total read counts of samples 10- to 100-fold greater than those of
289 the extraction controls (**Supporting Fig. 3**). Based on these results, we decided to
290 include all samples in the downstream analyses.

291 **Supporting Figure 2. Example beta (between sample) diversity analysis used to**
292 **determine whether expressed urine samples from set 1 (blue) differ from**
293 **extraction controls (red).** Several samples appear to be quite similar to the
294 extraction controls.
295
296 **Supporting Figure 3. Example histogram comparing the compositions of**
297 **samples and extraction controls from set 1.** The histogram shows the 49 most
298 relatively abundant genera plus plus the categories "other" and "unknown" detected
299 in the samples and extraction controls from Supplemental Figure 2. Left Y-axis,
300 relative abundance; right Y-axis, $\log_{10}$ of total units (i.e., reads). Note that the
301 compositions of the samples differ from those of the extraction controls and that the
302 samples contain at least 10-fold more reads than the controls. On the basis of this
303 analysis, we decided to keep all samples for downstream analyses.

304 As a first step towards determining if an indigenous murine urobiome exists, we first
305 determined whether the expressed samples from the three sets of mice contained
306 bacteria (**Fig. 1, Fig. 2**). **Figure 1** is a set of three histograms (one for each set of
307 mice), showing the 49 most relatively abundant genera across all three sets of mice,
308 plus the categories "other" and "unknown." **Figure 2** is a heatmap that presents the
309 expressed urine samples from all three sets of mice. Taken together, these two
310 figures show that the expressed urines contained DNA evidence of bacteria. The
311 most prevalent and predominant genus in all three sets was *Staphylococcus.* Other

312 less prevalent but predominant genera included *Enterococcus* (all sets) and
313 *Mammaliicoccus* (sets 1 and 3).

314 **Figure 1. Three histograms showing the 49 most relatively abundant genera**
315 **detected in each of the 3 sets of mice.** The category "Other" is an amalgamation of
316 all other genera; the category "Unknown" represent all sequences that could not be
317 identified.
318
319 **Figure 2. Heatmap showing the 49 most relatively abundant genera detected in**
320 **the 3 sets of mice.** The category "Other" is an amalgamation of all other genera; the
321 category "Unknown" represent all sequences that could not be identified. Set 1, light
322 blue; set 2, dark blue; set 3, green.

323 To compare the compositions of the expressed samples, we performed a PCoA. The
324 three sets differed significantly whether tested together (**Fig. 3,** PERMANOVA, p =
325 0.001) or pairwise (**Supporting Fig. 4A-C**, PERMANOVA, p = 0.001 for all
326 comparisons). Thus, the young mice housed in 2 different facilities (10 weeks of
327 age; sets 1 and 2) differed significantly as did the young mice (set 1) and aged mice
328 (6 ½ months of age; set 3). However, there appeared to be substantial overlap
329 between the three sets. Sets 1 and 2 (both which included samples from young mice)
330 had considerable overlap (**Supporting Fig. 4A**), as did sets 1 and 3 (young and aged
331 mice) (**Supporting Fig. 4C**). Even sets 2 and 3 had some overlap (**Supporting Fig.**
332 **4C)**.

333 To identify the genera most responsible for distinguishing each set from the others,
334 we performed a biplot analysis, which identified them as *Staphylococcus,*
335 *Mammaliicoccus, Ligilactobacillus, Enterococcus, Ralstonia, Burkholderia,*

336 *Phenylobacterium, Escherichia,* and *Bradyrhizobium* plus the categories "other" and
337 "unknown" (**Fig. 4**).

338 **Figure 3. Beta (between participant) diversity analysis comparing all 3 sets of**
339 **mice.** Principal coordinate analysis of Bray-Curtis distances with P-value calculated
340 using PERMANOVA.
341
342 **Supporting Figure 4. Beta (between participant) diversity analysis doing a**
343 **pairwise comparison of all 3 sets of mice.** Principal coordinate analysis of Bray-
344 Curtis distances with P-value calculated using PERMANOVA. (A) Set 1 versus set
345 2, (B) set 1 versus set 3, and (C) set 2 versus set 3.
346
347 **Figure 4. Biplot analysis identifying the taxa that differentiate the 3 sets of mice**
348 **from each other.**

349 Because some of these genera are considered to be environmental species, we
350 hypothesized that expressed urine contained post-urethral microbes and thus
351 compared the expressed urine samples of set 3 with their paired aspirated samples.
352 First, we determined that the decontaminated aspirated samples differed
353 significantly from the six extraction controls (**Supporting Fig. 5A**, PERMANOVA,
354 p=0.001). One sample (483) seemed to resemble some of the controls, but when a
355 genus level histogram was constructed, it differed sufficiently to be included
356 downstream analyses (**Supporting Fig. 5B**). Next, we compared the two sample
357 types, which differed significantly as determined by PCoA (**Fig. 5A,**
358 PERMANOVA, p=0.001). This also was true when comparing the relative
359 abundance of taxa between each pair of samples (**Fig. 5B**). The differences in
360 bacterial taxa between aspirated and expressed samples are also illustrated in the

361 heatmap (**Fig. 5C**). While the samples with the highest relative abundance of
362 potential post-urethral microbes (e.g., *Staphylococcus, Ralstonia, Burkholderia)* are
363 from expressed samples, some aspirated samples also contain these microbes,
364 although at lower relative abundance compared to expressed samples.

365 **Supporting Figure 5. Comparison of suprapubic aspirate samples to extraction**
366 **controls for set 3.** (A) Principal coordinate analysis of Bray-Curtis distances with
367 P-value calculated using PERMANOVA. Note that one sample (483) appears to
368 resemble the extraction controls. (B) Histogram showing the 49 most relatively
369 abundant genera plus the categories "other" and "unknown" detected in the samples
370 and extraction controls from panel A. Left Y-axis, relative abundance; right Y-axis,
371 $\log_{10}$ of total units (i.e., reads). Note that the composition of sample 483 differed
372 from those of the extraction controls. On the basis of this analysis, we decided to
373 keep all samples for downstream analyses.
374
375 **Figure 5. Comparison of urobiome composition in expressed urine versus**
376 **paired suprapubic aspirate from the mice in set 3.** (A) Principal coordinate
377 analysis of Bray-Curtis distances with P-value calculated using PERMANOVA. (B)
378 Histograms showing the 49 most relatively abundant genera detected in the paired
379 expressed urine (top) and suprapubic aspirate (bottom) for each mouse in set 3. The
380 category "Other" is an amalgamation of all other genera; the category "Unknown"
381 represent all sequences that could not be identified. (C) Heatmap showing the 49
382 most relatively abundant genera detected in the paired expressed urine and
383 suprapubic aspirate for each mouse in set 3. The category "Other" is an
384 amalgamation of all other genera; the category "Unknown" represent all sequences
385 that could not be identified. Light blue, expressed urine; dark blue, suprapubic
386 aspirate.

387 # Discussion

388 Here, we provide the first comprehensive report of the female mouse urobiome. We
389 document similarity between the urobiome from the same strain of mice housed at
390 different institutions, and an effect of age on the urobiome. Finally, we used

391 suprapubic aspiration to demonstrate that there is a mouse urobiome that persists
392 after removal of suspected contaminants, and that samples collected via aspiration
393 significantly differ from those collected by manual expression.

394 An important aspect of this work is the data necessary to support the design of future
395 research involving the mouse urobiome. First, we demonstrated that the urobiome is
396 relatively consistent between mice housed in different sites. The mice in sets 1 and
397 2 were located in different institutions and had different facilities perform both the
398 DNA extraction and sequencing. Despite these differences and significant
399 differences based on PCoA analysis, there was substantial overlap between these 2
400 groups, suggesting some similarity between the sets. Second, we determined that 0.5
401 mL is an ideal urine volume for mouse urobiome studies. While this may not always
402 be feasible and requires pooling of urine collected at different time points, this
403 volume yielded sufficient sequencing depth. Third, we showed that aged mice before
404 the onset of reproductive transition (~6 ½ months) have urobiomes that differ
405 significantly from their younger adult selves (10 weeks) but with substantial
406 similarities. Finally, we showed that urine collected by manual expression has a high
407 proportion of microbes from post-urethral sources and that collection of urine by a
408 method that minimizes the risk of contamination, including aspiration, will reduce
409 the inclusion of these microbes in the urine sample. Thus, when feasible, urine for

410 urobiome studies from mice should be collected using a method that minimizes the
411 potential for contamination, such as suprapubic aspiration.

412 Our work shows a small effect of age on the mouse urobiome. This is consistent with
413 results from adult women, where the urobiomes of younger continent adult females
414 were more likely to be predominated by members of the genus *Gardnerella*, whereas
415 older continent adult females were more likely to be predominated by *Escherichia*
416 (33). The authors hypothesized that the differences in the urobiome that
417 corresponded to age were attributable to hormonal changes associated with aging.
418 The mice in our work, while older, had not yet reached the age of reproductive
419 transition, which correlates to menopause in women. This may partially explain the
420 observed differences, but still there is a substantial overlap between our aged and
421 non-aged mice. Further work that involves mice past the age of reproduction will
422 provide further insights on the effect of age and hormonal status on the mouse
423 urobiome.

424 There is limited literature with which to compare our results. However, there are a
425 few works that document the rat urobiome. For example, Liu et al. (16) examined
426 the effects of antibiotics on the urobiome of female rats. They collected urine by
427 urethral catheterization and used 16S rRNA gene sequencing to describe the
428 urobiome. Their control animals, which did not have any interventions, had a
429 urobiome. While there are several genera in common between the urobiome of their

430 control animals and our mice, it is notable that their most abundant genus, *Bacillus*,
431 was not within the 51 most abundant genera that we identified in mice. Chen et al.
432 also examined the rat urobiome but used autoclaved metabolic cages for urine
433 collection (17). Their genus level microbiome results from their female control rats
434 have several genera in common with what we report in mice, including *Escherichia,*
435 *Streptococcus,* and *Acinetobacter.* Thus, while not directly comparable given the
436 differences in species, these reports that document the urobiome in rats lend
437 credence to our data documenting the urobiome in mice.

438 One of the most relevant implications of our work, as well as our documentation of
439 the presence of the host urobiome, is related to preclinical *in vivo* UTI work. There
440 is a vast body of preclinical studies using the mouse model of UTI that have yielded
441 critical information about the pathobiology of uropathogenic *Escherichia coli* and
442 host immune responses related to UTI (10, 24, 34). However, a notable aspect of this
443 model is that mice require a large number of bacteria, usually 10^7 colony forming
444 units of *E. coli,* to induce more than transient bacteriuria. Furthermore, not all mice
445 that have that large number of bacteria instilled into their bladders develop
446 bacteriuria. One previously overlooked factor into the heterogeneity of UTIs in mice
447 is the native urobiome and its role in colonization resistance. The concept of
448 colonization resistance has been described in the context of probiotics in the gut
449 microbiome in which features of the gut microbiome can predict subsequent

450 colonization by exogenous bacteria. Colonization resistance is built on the concept
451 that a microbiome is an ecologically stable community of bacteria (35). Disrupting
452 an ecologically stable community requires alteration of the host microbiome such as
453 through exposure to antimicrobials, alteration of the host to affect availability of
454 resources, or provision of a large influx of exogenous microbes that subsequently
455 outcompetes the native community, such as occurs in the UTI model. Considering
456 the role of the native urobiome in UTI susceptibility is critical to development of
457 more translatable models (22, 23).

458 Limitations of this work include a small sample size of mice in each group.
459 Furthermore, we only included female C57BL/6 mice, and our results cannot be
460 generalized to either male mice or female mice of other strains. It is likely that both
461 sex- and strain-based differences exist within the urobiome that should be described
462 in future work. Also, we used short-read (V4) 16S rRNA gene sequencing, which
463 typically yields only confidently identifies taxonomy at the family or genus levels.
464 Future work should include long read 16S rRNA gene sequencing for confident
465 species level identification. Despite these limitations, our work does have several
466 strengths. We obtained data from two different sets of mice in two different facilities,
467 used two different sequencing methods, but analyzed all the sequence data
468 simultaneously with a single analytic approach. We compared expressed versus
469 aspirated urine to describe the effects of post-urethral microbes on expressed

470 urobiome samples. We used an improved DNA isolation method that uses an extra
471 enzyme (lysostaphin) with longer incubation times to increase cell wall breakage.
472 Finally, in the analytic step, we used a multi-step decontamination procedure.

473 Conclusions

474 There is a native mouse urobiome that is largely stable in the same strain of mice.
475 There is a small effect of age in female mice who are capable of reproduction.
476 Finally, use of suprapubic aspiration, or a similar technique, to bypass post-urethral
477 microbes, is critical for accurately assessing the mouse urobiome.

478

479 Acknowledgements

480 We gratefully acknowledge Melline Fontes Noronha for help with bioinformatics.

481 Author ORCIDs

482 Sridhar Narla http://orcid.org/0000-0001-9066-2072
483 Olivia Lamanna http://orcid.org/0000-0003-4479-6752
484 Robert B. Moreland http://orcid.org/0000-0001-8175-9219
485 Catherine Foster http://orcid.org/0000-0002-6233-1070
486 Alan J. Wolfe http://orcid.org/0000-0003-4532-0545

References

1. Moreland RB, Brubaker L, Tinawi L, Wolfe AJ. Rapid and accurate testing for urinary tract infection: new clothes for the emperor. Clin Microbiol Rev. 2025;38(1):e0012924.
2. Simoni A, Schwartz L, Junquera GY, Ching CB, Spencer JD. Current and emerging strategies to curb antibiotic-resistant urinary tract infections. Nat Rev Urol. 2024;21:707–22.
3. MacDonald RA, Levitin H, Mallory GK, Kass EH. Relation between pyelonephritis and bacterial counts in the urine. N Engl J Med. 1957;256(20):915–22.
4. JawetzE. Urinary tract infections; problems in medical management. California Med. 1953;79(2):99–102.
5. Timm MR Russell SK, Hultgren SJ. Urinary tract infections: pathogenesis, host susceptibility and emerging therapeutics. Nat Rev Microbiol. 2025;23(2):72–86.
6. Brubaker L Chai T, Horsley H, Khasriya R, Moreland RB and Wolfe AJ. Tarnished gold—the “standard” urine culture: reassessing the characteristics of a criterion standard for detecting urinary microbes. Front Urol. 2023;3:1206046.
7. Wolfe AJ, Brubaker L. Discovering the Urinary Microbiome. American Scientist. 2024;112(3):168–75.
8. Price TK, Dune T, Hilt EE, Thomas-White KJ, Kliethermes S, Brincat C, Brubaker L, Wolfe AJ, Mueller ER, Schreckenberger PC. The Clinical Urine Culture: Enhanced Techniques Improve Detection of Clinically Relevant Microorganisms. J Clin Microbiol. 2016;54(5):1216–22.
9. Du J, Khemmani M, Halverson T, Ene A, Limeira R, Tinawi L, Hochstedler-Kramer BR, Noronha MF, Putonti C, Wolfe AJ. Cataloging the phylogenetic diversity of human bladder bacterial isolates. Genome Biol. 2024;25(1):75.
10. Murray BO, Flores C, Williams C, Flusberg DA, Marr EE, Kwiatkowska KM, Charest JL, Isenberg BC, Rohn JL. Recurrent Urinary Tract Infection: A Mystery in Search of Better Model Systems. Front Cell Infect Microbiol. 2021;11:691210.
11. Burton EN, Cohn LA, Reinero CN, Rindt H, Moore SG, Ericsson AC. Characterization of the urinary microbiome in healthy dogs. PLoS One. 2017;12(5):e0177783.
12. Coffey EL, Gomez AM, Burton EN, Granick JL, Lulich JP, Furrow E. Characterization of the urogenital microbiome in Miniature Schnauzers with

526 and without calcium oxalate urolithiasis. J Vet Intern Med.
527 2022;36(4):1341–52.
528 13. Coffey EL, Gomez AM, Ericsson AC, Burton EN, Granick JL, Lulich JP,
529 Furrow E. The impact of urine collection method on canine urinary
530 microbiota detection: a cross-sectional study. BMC Microbiol.
531 2023;23(1):101.
532 14. Coffey EL, Becker ZW, Gomez AM, Ericsson AC, Churchill JA, Burton
533 EN, Granick JL, Lulich JP, Furrow E. Dietary Features Are Associated with
534 Differences in the Urinary Microbiome in Clinically Healthy Adult Dogs.
535 Vet Sci. 2024;11(7):286.
536 15. Joubran P, Roux FA, Serino M, Deschamps JY. Gut and Urinary Microbiota
537 in Cats with Kidney Stones. Microorganisms. 2024;12(6):1098.
538 16. Liu F HL, Sheng J, Sun Y, Xia Q, Tang Y, Jiang P, Wei S, Hu J, Lin H, Xu
539 Z, Guo W, Gu Y, Feng N. Can antibiotics for enteritis or for urinary tract
540 infection disrupt the urinary microbiota in rats? Front Cell Infect Microbiol.
541 2023;28(13):1169909.
542 17. Chen X, Cheng Y, Tian X, Li J, Ying X, Zhao Q, Wang M, Liu Y, Qiu Y,
543 Yan X, Ren X. Urinary microbiota and metabolic signatures associated with
544 inorganic arsenic-induced early bladder lesions. Ecotoxicol Environ Saf.
545 2023;259:115010.
546 18. Karstens L Asquith M, Caruso V, Rosenbaum JT, Fair DA, Braun J,
547 Gregory WT, Nardos R, McWeeney SK. Community profiling of the urinary
548 microbiota: considerations for low-biomass samples. Nat Rev Urol.
549 2018;15(12):735–49.
550 19. Agudelo J, Miller AW. Impact of study design, contamination, and data
551 characteristics on results and interpretation of microbiome studies.
552 mSystems. 2025; Online ahead of print.
553 20. Bleich A FJ. The Mammalian Microbiome and Its Importance in Laboratory
554 Animal Research. ILAR J. 2015;56(2):153–8.
555 21. Jayalath S, Magana-Arachchi D. Dysbiosis of the Human Urinary
556 Microbiome and its Association to Diseases Affecting the Urinary System
557 Indian J Microbiol. 2022;62(2):133–66.
558 22. Pastuszka A, Tobor S, Łoniewski I, Wierzbicka-Woś A, Sielatycka K,
559 Styburski D, Cembrowska-Lech D, Koszutski T, Kurowicz M, Korlacka K,
560 Podkówka A, Lemiński A, Brodkiewicz A, Hyla-Klekot L, Skonieczna-
561 Żydecka K. Rewriting the urinary tract paradigm: the urobiome as a
562 gatekeeper of host defense. Mol Biol Rep. 2025;52(1):497.
563 23. Reasoner SA, Francis J, Hadjifrangiskou M. The urinary microbiome: the
564 next frontier of bacterial ecology. J Bacteriol. 2025;:e0010525(:e0010525):
565 Online ahead of print.

24. Carey AJ, Tan CK, Ipe DS, Sullivan MJ, Cripps AW, Schembri MA, Ulett GC. Urinary tract infection of mice to model human disease: Practicalities, implications and limitations. Crit Rev Microbiol. 2016;42(5):780–99.
25. Anderson M, Bollinger D, Hagler A, Hartwell H, Rivers B, Ward K, Steck TR. Viable but nonculturable bacteria are present in mouse and human urine specimens. J Clin Microbiol. 2004;42(2):753–8.
26. Roje B, Elek A, Palada V, Bom J, Iljazović A, Šimić A, Sušak L, Vilović K, Strowig T, Vlahoviček K, Terzić J. Microbiota Alters Urinary Bladder Weight and Gene Expression. Microorganisms. 2020;8(3):421.
27. Martin M. CUTADAPT removes adapter sequences from high-throughput sequencing reads. EMBNet J. 2011;17(1):10–2.
28. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13(7):581–3.
29. Gao X, Lin H, Revanna K, Dong Q. A Bayesian taxonomic classification method for 16S rRNA gene sequences with improved species-level accuracy. BMC Bioinformatics. 2017;18(1):247.
30. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ. Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome. 2018;6(1):226.
31. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Solymos P, Stevens MHM, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Borman T, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista HBA, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill MO, Lahti L, Martino C, McGlinn D, Ouellette MH, Cunha ER, Smith T, Stier A, Ter Braak JF, Weedon J. vegan: Community Ecology Package. R package version 2.2-1 2015 [Available from: http://CRAN.R-project.org/package=vegan.
32. Gu Z, Miller S, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics. 2016;32(18):2847–9.
33. Price TK, Hilt EE, Thomas-White K, Mueller ER, Wolfe AJ, Brubaker L. The urobiome of continent adult women: a cross-sectional study. BJOG. 2020;127(2):193–201.
34. Chieng CCY, Kong Q, Liou NSY, Khasriya R, Horsley H. . The clinical implications of bacterial pathogenesis and mucosal immunity in chronic urinary tract infection. Mucosal Immunol. 2023;16(1):61–71.
35. Wu G, Zhao N, Zhang C, Lam YY, Zhao L. Guild-based analysis for understanding gut microbiome in human health and diseases. Genome Med. 2021;13(1):22.

606 # Supporting Information

607 **Supporting Figure 1. Determination of the smallest volume sufficient to extract**
608 **and sequence enough DNA to adequately assess the urobiome while accounting**
609 **for urine collection feasibility.** Histogram showing the 49 most relatively abundant
610 genera detected in the extraction controls and expressed urine from 5 groups of mice.
611 The category "Other" is an amalgamation of all other genera; the category
612 "Unknown" represent all sequences that could not be identified. For each group of
613 mice, different urine volumes were tested (125, 250, 500, 1000 μL). Note that the
614 yellow line, which depicts total normalized arbitrary units (i.e., total reads per
615 sample), correlates with sample volume, with 125μL producing numbers of reads
616 apporximately the same as the extraction controls, while 1000 μL produced the
617 largest number of reads. 500 μL were chosen for subsequent study as producing
618 sufficiently high read numbers while balancing the feasiblity of obtaining urine from
619 mouse bladders.

620

621 **Supporting Figure 2. Example beta (between sample) diversity analysis used**
622 **to determine whether expressed urine samples from set 1 (blue) differ from**
623 **extraction controls (red).** Several samples appear to be quite similar to the
624 extraction controls.

625

626 **Supporting Figure 3. Example histogram comparing the compositions of**
627 **samples and extraction controls from set 1.** The histogram shows the 49 most
628 relatively abundant genera plus plus the categories "other" and "unknown" detected
629 in the samples and extraction controls from Supplemental Figure 2. Left Y-axis,
630 relative abundance; right Y-axis, $\log_{10}$ of total units (i.e., reads). Note that the
631 compositions of the samples differ from those of the extraction controls and that the
632 samples contain at least 10-fold more reads than the controls. On the basis of this
633 analysis, we decided to keep all samples for downstream analyses.

634

635 **Supporting Figure 4. Beta (between participant) diversity analysis doing a**
636 **pairwise comparison of all 3 sets of mice.** Principal coordinate analysis of Bray-
637 Curtis distances with P-value calculated using PERMANOVA. (A) Set 1 versus set
638 2, (B) set 1 versus set 3, and (C) set 2 versus set 3.

639

640 **Supporting Figure 5. Comparison of suprapubic aspirate samples to extraction**
641 **controls for set 3.** (A) Principal coordinate analysis of Bray-Curtis distances with
642 P-value calculated using PERMANOVA. Note that one sample (483) appears to
643 resemble the extraction controls. (B) Histogram showing the 49 most relatively

644 abundant genera plus the categories “other” and “unknown” detected in the samples
645 and extraction controls from panel A. Left Y-axis, relative abundance; right Y-axis,
646 $\log_{10}$ of total units (i.e., reads). Note that the composition of sample 483 differed
647 from those of the extraction controls. On the basis of this analysis, we decided to
648 keep all samples for downstream analyses.

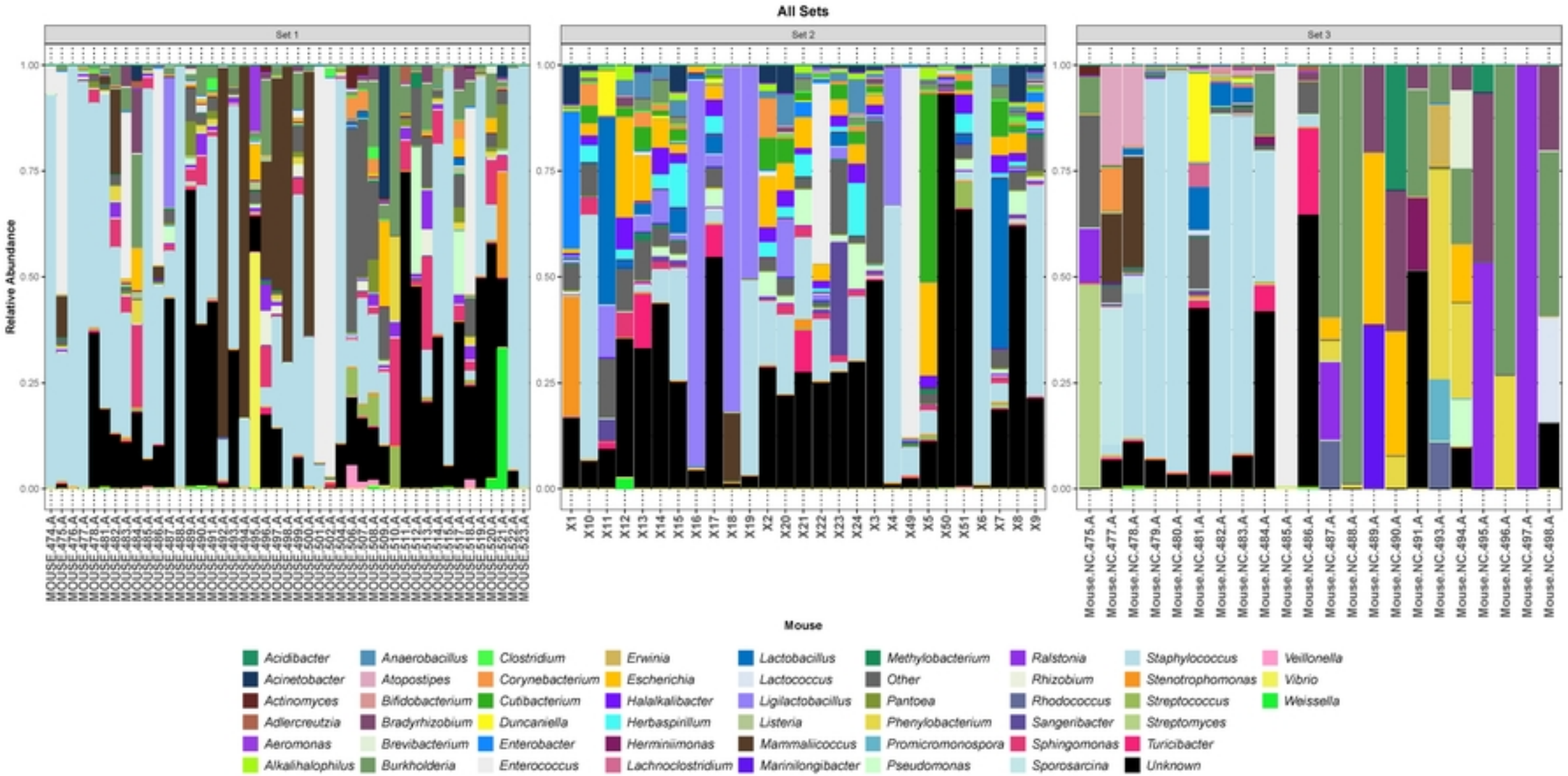
All Sets
Set 1
Set 2
Set 3
Relative Abundance
1.00
0.75
0.50
0.25
0.00
Mouse
Acidibacter
Acinetobacter
Actinomyces
Adlercreutzia
Aeromonas
Alkalihalophilus
Anaerobacillus
Atopostipes
Bifidobacterium
Bradyrhizobium
Brevibacterium
Burkholderia
Clostridium
Corynebacterium
Cutibacterium
Duncaniella
Enterobacter
Enterococcus
Erwinia
Escherichia
Halalkalibacter
Herbaspirillum
Herminiimonas
Lachnoclostridium
Lactobacillus
Lactococcus
Ligilactobacillus
Listeria
Mammaliicoccus
Marinilongibacter
Methylobacterium
Other
Pantoea
Phenylobacterium
Promicromonospora
Pseudomonas
Ralstonia
Rhizobium
Rhodococcus
Sangeribacter
Sphingomonas
Sporosarcina
Staphylococcus
Stenotrophomonas
Streptococcus
Streptomyces
Turicibacter
Unknown
Veillonella
Vibrio
Weissella

Figure 1

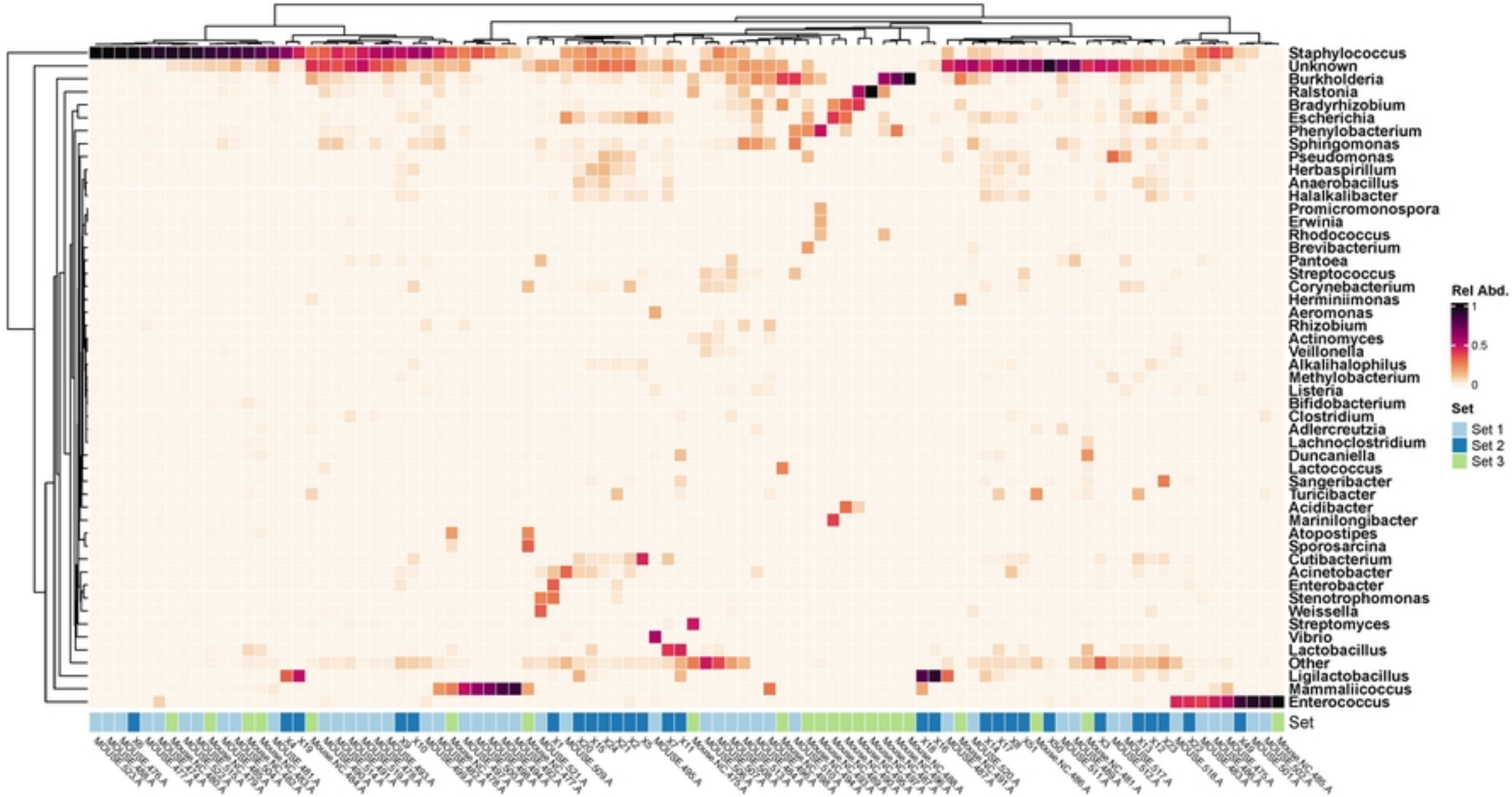
Staphylococcus
Unknown
Burkholderia
Ralstonia
Bradyrhizobium
Escherichia
Phenylobacterium
Sphingomonas
Pseudomonas
Herbaspirillum
Anaerobacillus
Halalkalibacter
Promicromonospora
Erwinia
Rhodococcus
Brevibacterium
Pantoea
Streptococcus
Corynebacterium
Herminiimonas
Aeromonas
Rhizobium
Actinomyces
Veillonella
Alkalihalophilus
Methylobacterium
Listeria
Bifidobacterium
Clostridium
Adlercreutzia
Lachnoclostridium
Duncaniella
Lactococcus
Sangeribacter
Turicibacter
Acidibacter
Marinilongibacter
Atopostipes
Sporosarcina
Cutibacterium
Acinetobacter
Enterobacter
Stenotrophomonas
Weissella
Streptomyces
Vibrio
Lactobacillus
Other
Ligilactobacillus
Mammaliicoccus
Enterococcus
Set
Rel Abd.
1
0.5
0
Set
Set 1
Set 2
Set 3

FIgure 2

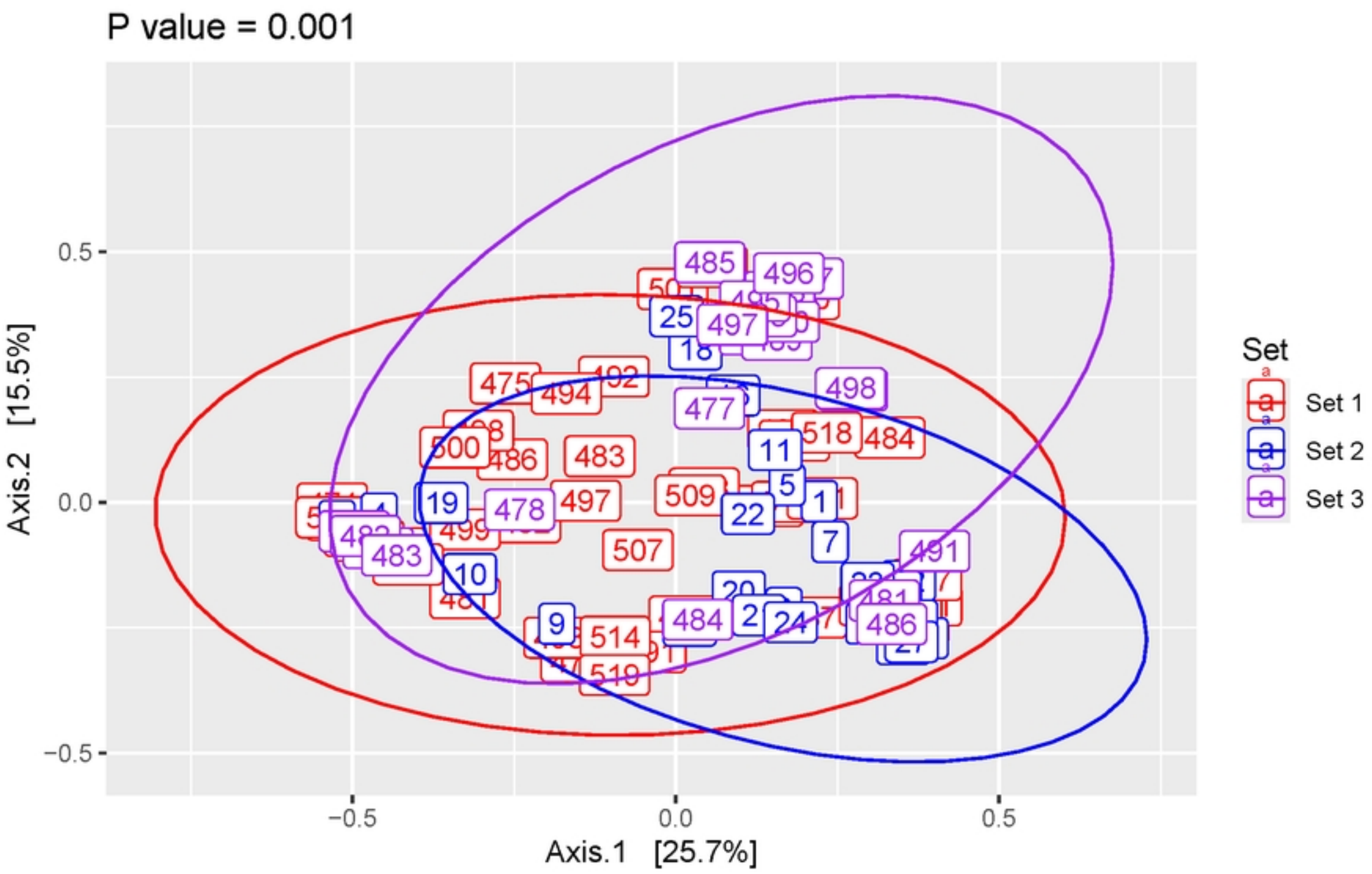
P value = 0.001
Axis.2 [15.5%]
Axis.1 [25.7%]
0.5
0.0
−0.5
−0.5
0.0
0.5
Set
Set 1
Set 2
Set 3

Figure 3

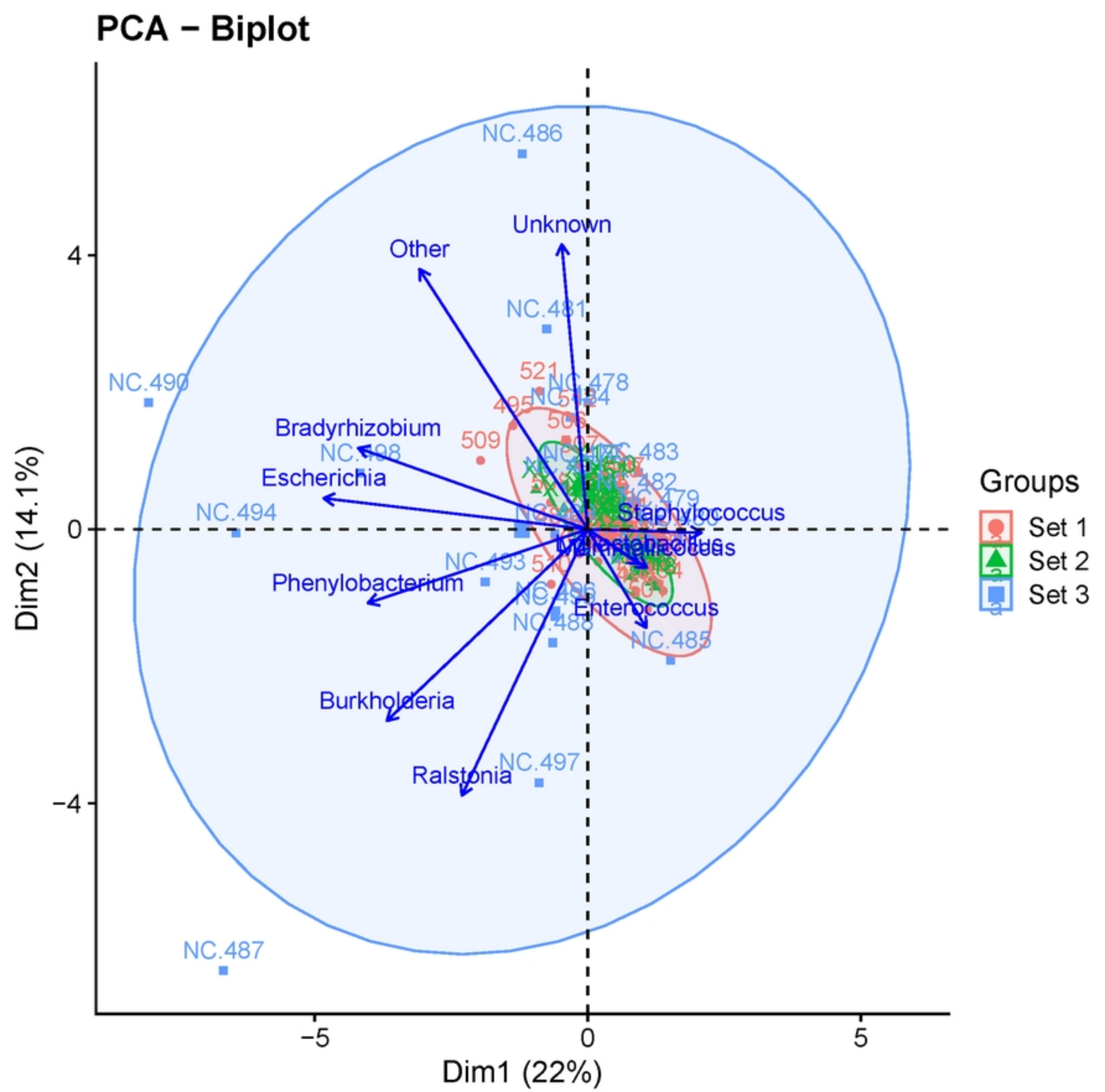
PCA – Biplot
Unknown
Other
Bradyrhizobium
Escherichia
Staphylococcus
Phenylobacterium
Enterococcus
Burkholderia
Ralstonia
NC.486
NC.481
NC.490
NC.478
NC.494
NC.483
NC.482
NC.479
NC.493
NC.488
NC.485
NC.497
NC.487
521
495
509
Groups
Set 1
Set 2
Set 3
Dim2 (14.1%)
Dim1 (22%)
4
0
−4
−5
0
5

Figure 4

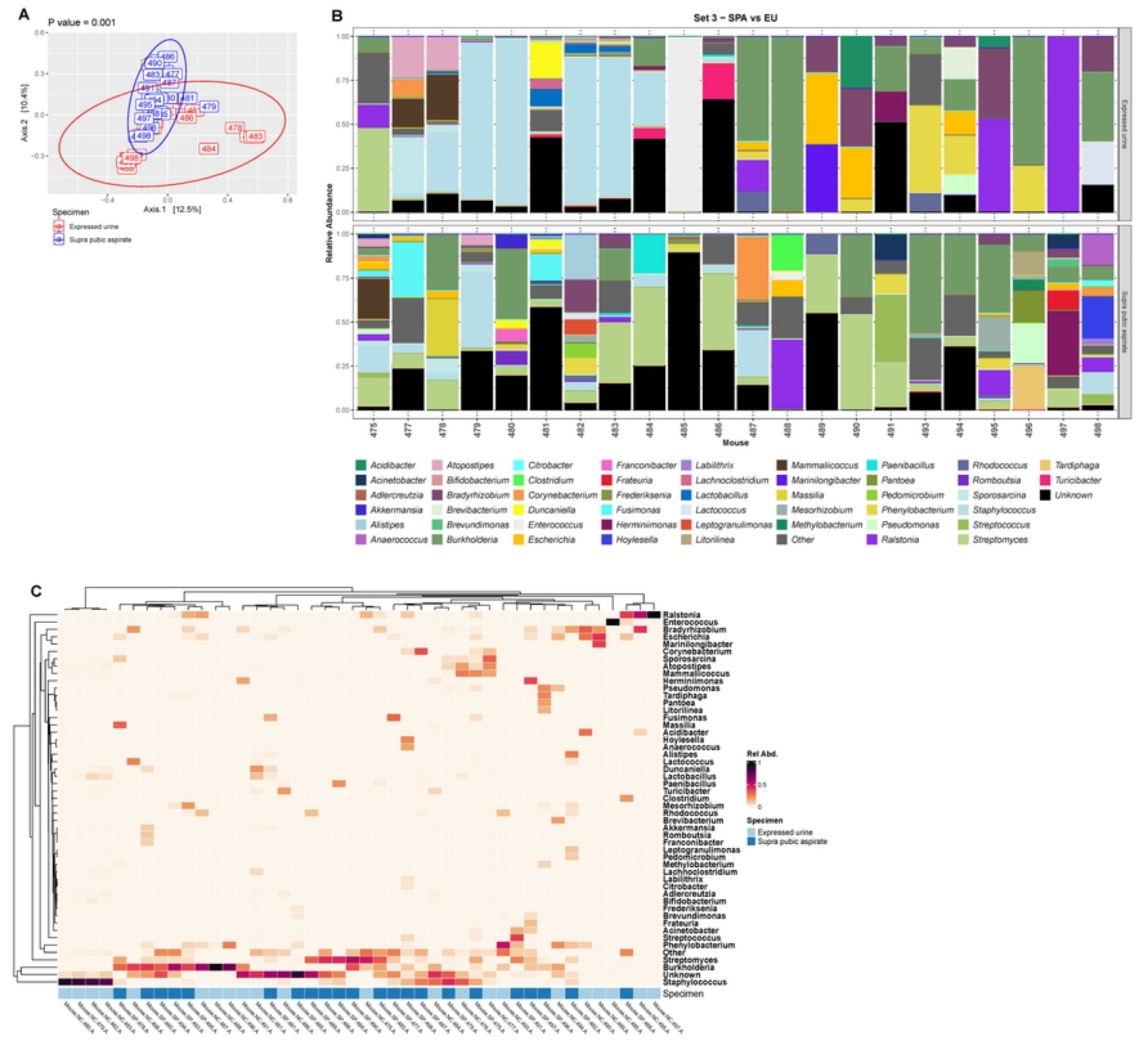
A
P value = 0.001
Axis.2 [10.4%]
Axis.1 [12.5%]
Specimen
Expressed urine
Supra pubic aspirate
B
Set 3 – SPA vs EU
Relative Abundance
Expressed urine
Supra pubic aspirate
Mouse
Acidibacter
Acinetobacter
Adlercreutzia
Akkermansia
Alistipes
Anaerococcus
Atopostipes
Bifidobacterium
Bradyrhizobium
Brevibacterium
Brevundimonas
Burkholderia
Citrobacter
Clostridium
Corynebacterium
Duncaniella
Enterococcus
Escherichia
Franconibacter
Frateuria
Frederiksenia
Fusimonas
Herminiimonas
Hoylesella
Labilithrix
Lachnoclostridium
Lactobacillus
Lactococcus
Leptogranulimonas
Litorilinea
Mammalicoccus
Marinilongibacter
Massilia
Mesorhizobium
Methylobacterium
Other
Paenibacillus
Pantoea
Pedomicrobium
Phenylobacterium
Pseudomonas
Ralstonia
Rhodococcus
Romboutsia
Sporosarcina
Staphylococcus
Streptococcus
Streptomyces
Tardiphaga
Turicibacter
Unknown
C
Rel Abd.
Specimen
Expressed urine
Supra pubic aspirate

Figure 5