Urine and Fecal Microbiota in a Canine Model of Bladder Cancer

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Abstract

Introduction: Urothelial carcinoma (UC) is the tenth most diagnosed cancer in humans worldwide. Dogs are a robust model for invasive UC as tumor development and progression is similar in humans and dogs. Recent studies on urine microbiota in humans revealed alterations in microbial diversity and composition in individuals with UC; however, the potential role of microbiota in UC has yet to be elucidated. Dogs could be valuable models for this research, but microbial alterations in dogs with UC have not been evaluated.

Objective: The objective of this this pilot study was to compare the urine and fecal microbiota of dogs with UC (n = 7) and age-, sex-, and breed-matched healthy controls (n = 7).

Methods: DNA was extracted from mid-stream free-catch urine and fecal samples using Qiagen Bacteremia and PowerFecal kits, respectively. 16S rRNA gene sequencing was performed followed by sequence processing and analyses (QIIME 2 and R).

Results: Canine urine and fecal samples were dominated by taxa similar to those found in humans. Significantly decreased microbial diversity (Kruskal-Wallis: Shannon, $p = 0.048$) and altered bacterial composition were observed in the urine but not feces of dogs with UC (PERMANOVA: Unweighted UniFrac, $p = 0.011$). The relative abundances of *Fusobacterium* was also increased, although not significantly, in the urine and feces of dogs with UC.

Conclusion: This study characterizes urine and fecal microbiota in dogs with UC, and it provides a foundation for future work exploring host-microbe dynamics in UC carcinogenesis, prognosis, and treatment.

Key words: Bladder Cancer, Urine, Feces, Dogs, Gastrointestinal Microbiome, Microbiota, Pilot Study

1. Introduction

Bladder cancer is the tenth most diagnosed cancer worldwide [1]. In 2020, the International Agency for Research on Cancer estimated over 573,000 new bladder cancer diagnoses would be confirmed worldwide [2]. Urothelial carcinoma (UC), also known as transitional cell carcinoma, is the most common type of bladder cancer. Age (being over age 55), race (white), sex (male), and some heritable mutations [3–10] are established risk factors for bladder cancer [11–13]. Bladder cancer is also strongly associated with environmental exposures such as smoking [14–17] or occupational exposure to chemicals like aromatic amines, pesticides, industrial dyes, or diesel fumes [18,19]. However, not all persons exposed to these chemicals develop urothelial carcinoma indicating that there are individualized host-environment interactions that mediate UC risk.

Clear host-environment (diet) interactions mediated through the gut microbiome have emerged in colorectal carcinogenesis [20,21] and environment-microbiome-carcinogenesis links have also begun emerging in lung cancer [22,23]. For example, diets high in animal fat can directly or indirectly impact microbial composition by increasing liver bile acid production and excretion into the intestines. Bile tolerant microbes or microbes that can metabolize primary bile acids expand in this bile-rich environment, and some of these microbes produce pro-inflammatory, cytotoxic, or genotoxic secondary metabolites that can contribute to colorectal carcinogenesis. Work on the gut microbiome has far outpaced and outnumbered studies on the urine / bladder microbiome; however, it has now become apparent that the urine microbiota play a key role in host health and may also be influencing bladder cancer development and

progression [24]. Alterations in urine microbiota have been reported in association with multiple genitourinary diseases including chronic kidney disease [25], chronic prostatitis, chronic pelvic pain syndrome [26], interstitial cystitis [27], sexually transmitted infections [28], urgency urinary incontinence [29], urinary tract infections [30], urinary stone disease [31], urogenital schistosomiasis [32], urogynecologic surgery [33], and vaginosis [34]. A few recent studies on the urine / bladder microbiome have also revealed subtle but intriguing differences in urine or bladder tissue microbial diversity and composition of individuals with and without UC (**Table 1**) [17,35–45], but approaches and results in these studies vary widely. Studies in relevant animal models could advance this research by offering a more controlled environment. Multiple animal models of UC have been described, with most being rodent models that have many limitations [46].

The focus of this study was on invasive UC utilizing a naturally-occurring canine model and comparing the urine and fecal microbiota of dogs with and without UC. While it can be difficult to produce the collective features of cancer heterogeneity, molecular features, aggressive cancer behavior, and host immunocompetence in experimental models, these features are present in the canine model [57-59]. In humans, approximately 25 % of all UC cases are muscle invasive [44] while in dogs with UC, over 90 % present with intermediate- to high-grade muscle invasive bladder cancer [47,48]. Moreover, humans and dogs share many of the same environmental exposures, and canine UC, like human UC, has been epidemiologically linked to chemical exposures including herbicides and pesticides [49,50]. Dogs also exhibit strong heritable (breed-specific) associations with UC offering unique opportunities for gene-environment studies [49–51]. Notably, the human microbiome is more similar to the dog microbiome compared to other

92 animal models, such as the rodent microbiome [52], making dogs a more suitable model for
93 studying microbiota in relation to UC.
94
95 2. Materials and Methods
96 **2.1 Sample Collection:** All dogs were recruited through Purdue University College of
97 Veterinary Medicine between September 2016 and October 2019 (Purdue IACUC: 1111000169;
98 Ohio State University IACUC: 2019A00000005). Urine and fecal samples were initially
99 collected from 57 dogs with biopsy-confirmed urothelial carcinoma (UC) and 56 age, sex, and
100 breed-matched healthy controls (**Figure 1**). Dogs with active urinary tract infections were
101 excluded. We additionally excluded any dog with a history of chemotherapy (vinblastine,
102 zebularine, vemurafenib, chlorambucil, mitoxantrone, and cyclophosphamide) or a history of
103 antibiotics within the previous 3 weeks due to the potential effects of these medications on the
104 microbiome [53–60]. We did not exclude dogs on non-steroidal anti-inflammatory drugs
105 (NSAIDs), including piroxicam and deracoxib, which are commonly used in dogs with UC.
106 Healthy dogs underwent physical exams and had no history of antibiotics (within the previous 3
107 weeks) or indications of gastrointestinal or urogenital disease.
108 In healthy dogs, urine was collected via mid-stream free catch. In dogs with UC, a variety
109 of urine collection methods were employed as deemed clinically appropriate including: mid-
110 stream free catch, catheter, or cystoscopy. Free catch urine can include bacteria from the bladder,
111 urethra, periurethral skin, prepuce, or vagina, while urine collected via catheterization or
112 cystoscopy primarily includes microbes from the bladder and limits the presence of genital and
113 skin microbes [41,61–63]. To determine if collection method could potentially influence our
114 results, we compared samples from dogs with UC collected via free catch (n = 8) to samples

115 collected via non-free catch methods (catheterization, cystoscopy) (n = 11) (**Supp. Table 1;**
116 **Supp. Figures 1,2,3**). We observed significant differences in microbial composition but not
117 diversity by collection method (Bray-Curtis PERMANOVA rarefied: $p = 0.008$; non-rarefied: p
118 = 0.005; **Supp. Figures, 1f,2f**). Moreover, *Staphylococcus* and *Streptococcus* – common skin
119 colonizers - were amongst the top genera in free catch urine but not amongst the top genera in
120 non-free catch urine (**Supp. Table 2**). Based on the compositional differences we observed by
121 collection method and on other studies that have reported differences in urine microbiota due to
122 collection method [41,61–65], we opted to limit the remainder of our analyses to samples
123 collected via free catch only. This allowed us to compare microbiota in urine from healthy dogs
124 and dogs with UC without introducing collection method as a potential confounder.
125 As such, after exclusions, urine samples from a total 7 dogs with UC and 7 age, sex, and
126 breed-matched healthy controls were compared in this study (**Table 2)**. Fecal microbiota from a
127 subset of these 14 dogs for which we had fecal samples (4 dogs with UC and 6 healthy controls)
128 were also compared [30,66,67]. All urine and stool samples were placed on ice immediately after
129 collection and then transferred into a -80°C freezer. Samples were transported on dry ice from
130 Purdue (West Lafayette, IN, USA) to the Ohio State University (Columbus, OH, USA), where
131 they were stored in at -80°C until extraction.
132
133 **2.2 DNA extraction and quantification:** Urine samples were extracted using QIAamp®
134 BiOstic® Bacteremia DNA Isolation Kit (Qiagen, Hilden, Germany) as described previously
135 [68]. Fecal samples were extracted using the QIAamp® PowerFecal® DNA Kit (Qiagen, Hilden,
136 Germany) following the manufacturer's instructions. Negative (no sample) controls were run
137 with each kit used for extraction. DNA concentrations were measured using a Qubit® 4.0

Fluorometer (Invitrogen, Thermo Fisher Scientific™, Carlsbad, CA, USA) and purity was assessed using Nanodrop One (Thermo Fisher Scientific™, Carlsbad, CA, USA).

2.3 16S rRNA sequencing and sequence processing: Library preparation, PCR amplification, and amplicon sequencing was performed at Argonne National Laboratory (DuPage County, Illinois). Likewise, negative controls underwent the full extraction, library preparation, and sequencing process. We amplified the V4 region of the 16S rRNA gene using primers 515F and 806R, and PCR and sequencing were performed as described previously (2 x 250bp paired-end reads, on an Illumina Miseq (Lemont, IL, USA)) [68–70]. Raw, paired-end sequence reads were processed using QIIME2 v. 2020.11 and DADA2 [71,72]. Taxonomy was assigned in QIIME2 using the Silva 132 99% database and the 515F / 806R classifier [73,74]. In the analysis comparing urine collection method in dogs with UC, we excluded samples with fewer than 1,000 reads and analyzed the data with rarefaction (at 1,000 reads) and without rarefaction. We included both analyses because rarefaction, especially at low read counts, can increase type 1 errors and mask potential differentially abundant taxa between samples [75]. In the analyses comparing urine and fecal microbiota from dogs with and without UC, samples with fewer than 7,000 reads were excluded; this cutoff allowed us to retain all but two urine samples while excluding all negative controls (**Figure 1**). Urine samples from dogs with and without UC were rarefied at 7,000 reads; fecal samples were rarefied at 9,233 reads, which included all fecal samples. Sequencing data for this project is available in SRA BioProject PRJNA76392.

2.4 Urine and fecal sequence data processing: Prior to analyses, we first removed singletons (Amplicon Sequence Variants (ASVs) with only one read in the dataset). ASVs are roughly

equivalent to a microbial species or strain. We then applied the R package decontam to identify and filter out putative contaminant ASVs based on their frequency and prevalence (0.5 threshold) as compared to negative controls (R package, v.1.10.0) [76]. In total, we identified and removed 13 putative contaminant ASVs from the urine samples and 8 from the fecal samples (**Supp. Table 3**). We also removed sequences aligned to chloroplasts, eukaryotes, mammalia, and mitochondria. In addition, in the urine samples, we removed taxa within the phylum Cyanobacteria and the class Chloroflexia. All six negative controls, which contained fewer than 7000 reads, were then removed from subsequent analyses.

2.5. Statistical analyses: Data were tested for normality using the Shapiro Wilk Normality Test in R version 3.5.2 [77]. We then compared DNA concentrations and read numbers between groups using Wilcoxon Rank Sum tests and two-sample t-tests, respectively. All alpha and beta diversity metrics were assessed using the R package phyloseq with a p-value cutoff of 0.05 adjusted using the Benjamini & Hochberg False Discovery Rates [78]. Alpha-diversity metrics included Shannon, Simpson, and Observed Features followed by Kruskal-Wallis Rank Sum Tests to compare metrics by group. Beta-diversity metrics included Bray-Curtis, Unweighted UniFrac, and Weighted UniFrac. Permutational Multivariate Analysis of Variance (PERMANOVA) were implemented in QIIME2 v. 2020.11 to compare bacterial community composition by group. An Analysis of Composition of Microbiome (ANCOM) was used to identify differentially abundant taxa by group.

3. Results

3.1 Urine microbiota in dogs with UC: We compared the urine microbiota of 7 dogs with UC to 7 age, sex, and breed-matched healthy controls. The total number of reads across all samples ranged from 7,232 – 36,692 with a mean of 20,010 ± 7,329 reads. Urine samples contained a total of 21 bacterial phyla, 308 genera, and 187 species. Urine DNA concentrations were significantly higher in dogs with UC as compared to healthy dogs (**Figure 2a**: Wilcoxon Rank Sum test, $p = 0.002$), but there was no significant difference in the number of 16S reads between dogs with and without UC (**Figure 2b**: two-sample t-test, $p = 0.99$).

Dogs with UC had significantly lower urine microbial diversity compared to healthy dogs as measured by the Shannon diversity index and Observed Features but not by the Simpson diversity index (Kruskal-Wallis: Shannon, $p = 0.048$; Observed Features, $p = 0.025$; Simpson, $p = 0.133$; **Figure 3a, Supp. Figure 4a,b**). Dogs with UC also had significantly different urine microbial composition than healthy dogs based on an Unweighted UniFrac distance matrix (**Figure 3b**; PERMANOVA, $p = 0.011$); although, no significant differences were observed by Bray Curtis ($p = 0.888$) or Weighted UniFrac ($p = 0.168$) distance matrices (**Supp. Figure 4c,d**). At the phylum level, Firmicutes (healthy: 61.1 %; UC: 79.5 %) Proteobacteria (healthy: 18.0 %; UC: 15.6 %), and Actinobacteria (healthy: 12.5 %; UC: 4.26 %) were the three most abundant phyla in the urine of healthy dogs and dogs with UC (**Figure 4a**). At the family level, Staphylococcaceae (healthy 42.6%; UC 48.6%) and Streptococcaceae (healthy 5.99 %; UC 14.8%) were amongst the most abundant taxa (**Figure 4b;** For genus and order level taxa see **Supp. Figure 5)**. Interestingly, *Fusobacterium* was present in the urine of dogs with UC but not in the urine of healthy dogs (relative abundance of *Fusobacterium* in healthy dogs: 0 %; in dogs with UC: 0.167 %). There were no differentially abundant taxa between healthy dogs and dogs with UC at the phylum, genus, or ASV levels.

3.2 Fecal microbiota in dogs with UC: We compared the fecal microbiota of a subset of dogs from the urine analyses for which we also had fecal samples: four dogs with and six dogs without UC. The total number of reads across all fecal samples ranged from 9,233 – 28,345 with a mean of 19,196 ± 6,100 reads. Fecal samples contained a total of 8 bacterial phyla, 92 genera, and 45 species. There was no significant difference in fecal DNA concentrations or number of 16S reads in dogs with UC as compared to healthy dogs; although, DNA concentrations were greater in dogs with UC (DNA concentration: Wilcoxon Rank Sum Test, $p = 0.136$; 16S reads: Two-sample t-test, $p = 0.322$; **Figure 5**).

Fecal microbial diversity and composition did not differ significantly in dogs with and without UC (Kruskal-Wallis: Shannon, $p = 0.67$; Unweighted UniFrac PERMANOVA, $p = 0.252$; **Figure 6**, **Supp. Figure 6**). The top three most abundant phyla across all fecal samples were Firmicutes (healthy: 72.6 %; UC: 32.9 %), Bacteroidetes (healthy: 10.6 %, UC 31.9 %) and Fusobacteria (healthy: 11.3 %, UC: 31.1 %) (**Figure 7; Supp. Figure 7**). At the family and genera levels, Fusobacterieacea (healthy: 11.4 %, UC: 31.7 %) and *Fusobacterium* (healthy: 12.0 %, UC: 33.1 %) were the most abundant taxa in UC but not healthy samples, respectively; although, these differences were not statistically significant. Only one *Bacteroides spp*. was significantly increased in relative abundance in dogs with UC compared to healthy dogs (ANCOM, W = 25).

To determine how results from this subset of fecal samples compared to a larger sample set, we then analyzed the fecal microbiota of 30 dogs with UC and 30 sex, age, and breed-matched healthy controls (**Supp. Table 4**). Fecal DNA concentrations, 16S reads, and fecal microbial diversity and microbial composition again did not differ significantly between groups

(DNA concentration: Wilcoxon Rank Sum test, $p = 0.515$; 16S reads: two-sample t-test, $p = 0.0697$; **Supp. Figure 8; Supp. Table 5**). Firmicutes, Bacteroidetes, and Fusobacteria also remained the most abundant phyla across both groups, and interestingly, Fusobacteriaceae (healthy: 17.4 %; UC: 28 %) and *Fusobacterium* (healthy: 18.5 %; UC: 29.2%) were still the most abundant family and genus in the fecal samples of dogs with UC (**Supp. Figure 9**); although, this difference was still not significant. In fact, no taxa were differentially abundant at the phylum, genus, or ASV levels between groups in the larger sample set (**Supp. Table 5**), suggesting that that *Bacteroides spp.* identified as differentially abundant in the subset was likely an artifact of small sample size.

3.3 Microbiota identified in both fecal and urine samples: As the gut can be a source for microbes in the urinary tract [30,67], we then combined urine and fecal data to determine what ASVs were present in both urine and fecal samples. There were a total of 1,204 ASVs across all urine and fecal samples combined. Sixty-six ASVs were identified in both urine and fecal samples from any dog (**Supp. Table 6**). The most common taxa found in both urine and fecal samples included taxa in the genera *Streptococcus* and *Blautia*. Notably, *Fusobacterium spp.*, *Porphyromonas spp.*, *Campylobacter spp.*, *Helicobacter spp.*, and *Clostridiodes difficile* were also found in both urine and fecal samples. Further, nine ASVs were identified in urine and fecal samples from the same dogs (**Supp. Table 7**). These ASVs included two *Escherichia* or *Shigella spp.*, two *Streptococcus spp.*, a *Clostridium sensu stricto 1 spp.*, *Actinomyces coleocanis*, *Streptococcus minor*, an *Enterococcus spp.*, and an uncultured *Peptoclostridium spp.*

4. Discussion

The purpose of our study was to characterize the urine and fecal microbiota in a naturally-occurring canine model of UC. We report a decreased urine microbial diversity and altered urine microbial composition in dogs with UC compared to healthy controls. We did not detect significant differences in fecal microbiota between dogs with and without UC; although, *Fusobacterium* was increased in dogs with UC. These results provide a foundation for further exploring the role of microbes in UC in a highly relevant animal model.

Urine and fecal microbiota associated with UC

The higher concentrations of DNA found in urine from dogs with UC is likely host DNA from epithelial or tumor cells being sloughed into the urine. Notably, urine microbial read numbers did not differ significantly between dogs with and without UC indicating similar amplicon sequencing depths despite differences in DNA concentrations. (Notably, efforts to remove host DNA from UC urine samples prior to sequencing may be beneficial in future microbiome studies employing shotgun metagenomics to ensure that the run is not overwhelmed with host sequences.)

Besides DNA concentrations, we also observed significant differences in urine microbial diversity (Shannon) and composition (Unweighted UniFrac) between dogs with and without UC. In this study, urine microbial diversity was greater in healthy dogs as compared to dogs with UC, a finding that aligns with several studies on urine microbiota in humans with UC [37,39]. However, there are also studies in humans that report no differences in microbial diversity or decreased diversity in urine from healthy individuals as compared to those with UC [17,35,36,38,42,44,79]. Differences in microbial composition (Unweighted UniFrac) have also been reported in previous human studies on UC [36,38,43,44]. In this study, the four most

abundant phyla in urine were Firmicutes, Actinobacteria, Bacteroides, and Proteobacteria. These phyla also dominate the urine microbiota in humans [17,36,38,40,44,45] and have been reported in previous studies on healthy dog urine [80,81]. In humans, taxa associated with UC vary widely across studies, but *Acinetobacter* and *Actinomyces* have been found at increased abundances in patients with UC across at least three studies [35,42,44]. In this study, we did not see *Acinetobacter* or *Actinomyces spp.* increased in relation to UC, which may be due to small sample sizes and reduced power to detect differentially abundant taxa, or differences between human and canine urine microbiota, or lack of a true link between these taxa and UC.

In relation to fecal microbiota, we did not observe any significant differences in dogs with and without UC. However, intriguingly, *Fusobacterium* was increased in relative abundance (although not significantly) in urine and fecal samples of dogs with UC. One previous study on bladder cancer also reported increased *Fusobacterium* in the urine of individuals (human) with UC [38]. Importantly, taxa in the phyla Fusobacteria are considered normal inhabitants of the canine gastrointestinal tract [82]; although, they are more typically associated with disease in humans. Studies in colorectal cancer have demonstrated direct links between Fusobacteria (*Fusobacterium nucleatum)* and carcinogenesis. Specifically, *Fusobacterium nucleatum* Fap2 protein can bind to host factor Gal-GalNAc which is overexpressed on tumor cells [83] - thereby localizing to tumors where Fap2 can impair host anti-tumor immunity [83]. *Fusobacterium nucleatum* can also induce the host Wnt / beta-catenin pathway resulting in upregulated host cellular proliferation [84]. Future studies are needed to elucidate the potential role of *Fusobacterium* in bladder cancer.

Microbiota present in both urine and fecal samples

298 Communication and migration of microbes between the gut and bladder can increase a host's
299 risk of UTIs and bacteriuria [30]. Microbes may migrate and ascend into the urogenital tract
300 externally from the rectum / anus, or internally via the blood stream [85,86]. In this study, 66
301 ASVs were shared between urine and fecal samples. Interestingly, ~ 59 % of those ASVs (39 /
302 66) are likely spore-formers (Bacilli, Clostridia, Negativicutes) suggesting that spore formation
303 may more readily enable exchange of microbes between body niches [87,88]. Among the
304 microbes (ASVs) found in both urine and fecal samples, there were multiple potentially
305 pathogenic taxa: *Campylobacter spp., Helicobacter canis, Clostridiodes difficile, Clostridium*
306 *baratii, Escherichia / Shigella spp., and Enterococcus spp*. There were also a few taxa that have
307 been associated with tumors or directly linked with tumor development or progression in
308 gastrointestinal, oral, and genital cancers: *Fusobacterium spp.* and *Porphyromonas spp.* [89–94].
309 The shared presence of two *Fusobacterium* ASVs between urine and fecal samples is particularly
310 of interest given the role of *Fusobacterium* in colorectal cancer.

311 This pilot study is a novel investigation of urine and fecal microbiota in a canine model
312 of UC. The dominant microbial taxa identified in canine urine and fecal samples were similar to
313 those reported in humans. Also, as in humans, altered microbial diversity and composition were
314 observed in dogs with UC as compared to healthy controls. This supports the idea that the
315 microbiota may play a role in UC development, progression, prognosis, or response to treatment,
316 as has been observed in other cancers. Moreover, *Fusobacterium* was increased – albeit not
317 significantly - in both urine and fecal samples of dogs with UC. *Fusobacterium* ASVs were also
318 shared between urine and fecal samples. Taken together, these results provide support for the use
319 of dogs as a model in UC microbiome studies. Additionally, these findings suggest that future

work evaluating the role of *Fusobacterium* in UC, and the gut as a potential source of this *Fusobacterium,* may be warranted.

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Author	Year	Sample Size	Collection Method	Microbial Diversity (α-diversity)	Microbial Composition (β-diversity)	Most Abundant Taxa
Xu et al.	2014	Healthy (n = 6) UC (n = 8)	not described	Increased number of genera in UC (statistical significance not indicated)	not described	*Acinetobacter* abundant in both healthy and UC groups **Increased in UC**: *Streptococcus, Pseudomonas,* and *Anaerococcus*
Bučević Popović et al.	2018	Healthy (n = 11 men), UC (n = 12 men)	mid-stream free catch	no differences detected	Bray Curtis: microbial composition differed by age but not between UC and healthy groups	**Increased in UC**: *Fusobacterium, Actinobaculum, Facklamia, Campylobacter, Subdoligranulum, Ruminococcaceae UCG-002, Campylobacter hominis , Actinobaculum massiliense* , and *Jonquetella anthropic* **Increased in Healthy**: *Veillonella , Streptococcus* , and *Corynebacterium*
Wu et al.	2018	Healthy (n = 18) UC (n = 31; MIBC = 5, NMIBC = 26)	mid-stream free catch	Observed Species, Chao1, and Ace indeces: cancer > healthy	Bray Curtis, Unweighted and Weighted UniFrac: microbial composition differed between UC and healthy groups	**Phyla dominant across all urine samples**: Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes **Genera increased in UC**: *Acinetobacter, Anaerococcus, Rubrobacter, Sphingobacterium, Atopostipes,* and *Geobacillus* **Genera increased in healthy**: *Serratia, Proteus, Roseomonas, Ruminiclostridium-6, Eubacterium-xylanophilum,* and *Laceyella* **Genera associated with UC recurrence**: *Herbaspirillum, Gemella, Bacteroides, Porphyrobacter, Faecalibacterium,* and *Aeromonas* **Genera associated with UC progression**: *Herbaspirillum, Porphyrobacter, Bacteroides,* and *Marmoricola*
Bi et al.	2019	Healthy (n = 26; men = 15, women = 11) UC (n = 29; men = 20, women = 9)	mid-stream free catch	UC > healthy (metric not specified)	Bray Curtis: microbial composition differed between UC and healthy groups	**Phyla increased in UC**: Tenericutes and Proteobacteria **Genera increased in healthy**: *Streptococcus , Bifidobacterium , Lactobacillus* , and *Veillonella* **Genera increased in UC:** *Actinomyces*
Liu et al.	2019	UC tissue (n = 22) adjacent normal tissue (n = 12)	intraoperative tissue collection	Shannon: normal > UC tissue, Evenness: normal > UC tissue	Weighted UniFrac: microbial composition differed between UC and normal tissue groups	**Phyla increased in UC tissue**: Proteobacteria and Actinobacteria **Phyla decreased in UC tissue**: Firmicutes and Bacteroidetes **Genera increased in UC tissue**: *Cupriavidus spp.* Unclassified *Brucellaceae, Acinetobacter, Escherichia-Shigella, Sphingomonas, Pelomonas, Ralstonia* , and *Anoxybacillus* **Genera increased in normal tissue**: *Lactobacillus, Prevotella 9,* and *Ruminococcaceae*
Mai et al.	2019	UC (n = 24; men = 18, women = 6)	mid-stream free catch	not described	not described	**Most abundant phyla**: Proteobacteria, Firmicutes, Actinobacteria, Tenericutes, and Bacteroidetes **Most abundant Classes**: Gammaproteobacteria, Bacilli, Actinobacteria, Mollicutes, Bacteroidia, Betaproteobacteria, and Clostridia **Most abundant Orders**: Enterobacteriales, Lactobacillales, Mycoplasmates, Actinomycetales, Xanthomonadales, Clostridiales, Bacillales, and Bacteroidales **Most abundant Families**: Enterobacteriaceae, Lactobacillaceae, Streptococcaceae, Mycoplasmataceae, Xanthomonadaceae, Corynebacteriaceae **Most abundant Genera**: unidentified Enterobacteriaceae genus, *Streptococcus, Lactobacillus, Ureaplasma, Corynebacterium, Stenotorphomonas, Enterococcus* , and *Staphylococcus* **Increased in UC (based on comparison to previously published healthy controls)**: *Acinetobacter, Rubrobacter, Geobacillus* , and *Rhizobiales*
Chipollini et al.	2020	Healthy (n = 10) UC (n = 27; MIBC, n = 15; NMIBC, n = 12)	mid-stream free catch	Evenness: Healthy > MIBC > NMIBC.	Weighted UniFrac: microbial composition did not differ between UC and healthy groups	**Increased in MIBC**: Bacteroides and Faecalibacterium **Increased in Healthy:** Bacteroides, Lacnoclostridium, and Burkholderiaceae
Mansour et al.	2020	UC urine (n = 10) UC tissue (n = 14)	urine = collected directly from bladder during surgery tissue = removed during transurethral resection	Shannon and Richness: male > female	No similarities in microbial composition between tissue and urine samples from same individual	**Phyla dominant across all urine and tissue samples**: Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, and Cyanobacteria **Most abundant genera in all urine**: *Lactobacillus, Corynebacterium, Streptococcus* , and *Staphylococcus* **Most abundant genera in tissue**: *Bacteroides, Akkermansia, Klebsiella* , and *Clostridium sensu stricto* **Genera increased in tissue compared to urine**: *Bacteroides, Akkermansia, Klebsiella, Clostridium Sensu Stricto,* and *Enterobacter*
Pederzoli et al.	2020	Healthy (n = 59; men = 24, women = 25) UC (n = 49; men = 36, women = 13)	mid-stream free catch. UC and healthy adjacent tissue collected at surgery	Richness: no difference between UC and healthy urine. UC urine > UC tissue and healthy tissue	Weighted UniFrac: microbial composition in urine samples differed by sex and UC vs. healthy groups. Tissue samples differed by sex but not UC	**Most abundant Phyla in urine samples**: Proteobacteria, Firmicutes, and Bacteroidetes **Taxa increased in UC urine (men)**: Acidobacteria-6, Opitutales, Opitutaceae **Taxa increased in UC urine (women)**: *Klebsiella* **Top 5 taxa increased in healthy urine (men)**: Tissierellaceae, Alphaproteobacteria, Rhizobiales, Sphingomonadales, Pasteurellales **Top 5 taxa increased in healthy urine (women)**: Betaproteobacteria, Burkholderiales, Pseudomonadales, Commomonadaceae, Moraxellaceae **Taxa increased in UC tissue**: *Burkholderia*
Zeng et al.	2020	Healthy (n = 19) UC: 62 + 40 NMIBC	mid-stream free catch	Observed Species, Chao1, and Ace indeces: cancer > healthy Shannon, Simpson: no difference	Bray-Curtis: microbial composition differed between UC and healthy groups	**Phyla dominant across all urine samples**: Firmicutes, Proteobacteria, Actinobacteria **Genera associated with UC recurrence**: *Anoxybacillus, Massilia, Thermomonas, Brachybacterium, Micrococcus, Nocardioides, Larkinella, Jeotgalibacillus, and Geomicrobium*
Chen et al.	2021	UC (n = 28; PD-L1 positive, n = 19; PD-L1 negative, n = 9)	mid-stream free catch	Ace index and Observed Species: PD-L1 positive > PD-L1 negative	Weighted and Unweighted UniFrac: microbial composition was distinct between PD-L1 positive and PD-L1 negative groups	**Increased in PD-L1 positive**: *Leptotrichia* **Increased in PD-L1 negative**: Bacteroidetes, Bacteroidia, Bacteroidales, Prevotellaceae, and *Prevotella*
Hussein et al.	2021	Healthy (n = 10) UC (n = 43)	healthy: mid stream free catch; UC: transurethral catheterization	Observed index, Chao1, Shannon, Simpson: no differences between UC and healthy or MNIBC and MIBC	Bray Curtis: microbial composition differed between UC and healthy groups	**Phyla most abundant in UC**: Actinobacteria and Proteobacteria **Phyla most abundant in Healthy**: Firmicutes and Deinococcus-Thermus **Genera most abundant in** UC: *Actinomyces, Achromobacter, Brevibacterium, Brucella* , and *Thermus* **Genera most abundant in Healthy**: *Salinococcus, Jeotgalicoccus, Escherichia-Shigella, Faecalibacterium* , and *Lactobacillus* **Taxa most abundant in MIBC**: Firmicutes, *Haemophilus* , and *Veillonella* **Taxa most abundant in NMIBC**: Proteobacteria and *Cupriavidus*
Oresta et al.	2021	Healthy (n = 10 men) UC (n = 51 men)	catheter, mid-stream free catch, bladder washout	Evenness: cancer > healthy; Richness, Chao1, Shannon, Simpson: no difference	Bray Curtis: microbial composition did not differ between UC and healthy groups. Mistream vs. catheter vs. bladder washout groups did not differ.	**Genera increased in UC**: *Veillonella* and *Corynebacterium* **Genera decreased in UC**: *Ruminococcus 1*

695 **Table 1**: **Key findings in 13 publications about the urine / tissue microbiota and urothelial**

696 **carcinoma**. MIBC = Muscle Invasive Bladder Cancer; NMIBC = Non-Muscle Invasive Bladder

697 Cancer; PD-L1 = Programmed Cell Death 1 Ligand 1; UC = Urothelial Carcinoma.

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Category	Healthy	UC
Sex, n (%)		
Females	5 (71.4 %)	5 (71.4 %)
spayed	4	4
non-spayed	1	1
Males	2 (28.6 %)	2 (28.6 %)
neutered	2	2
non-neutered	0	0
Age (mean ± SD)	10.1 ± 1	10.1 ± 0.7

Table 2: Demographics of dogs with and without urothelial carcinoma (UC). Urine samples were collected and analyzed from all dogs. Stool samples were collected and analyzed from a subset of these dogs including 6 healthy (4 females, 2 males), and 4 with UC (3 females, 1 male).

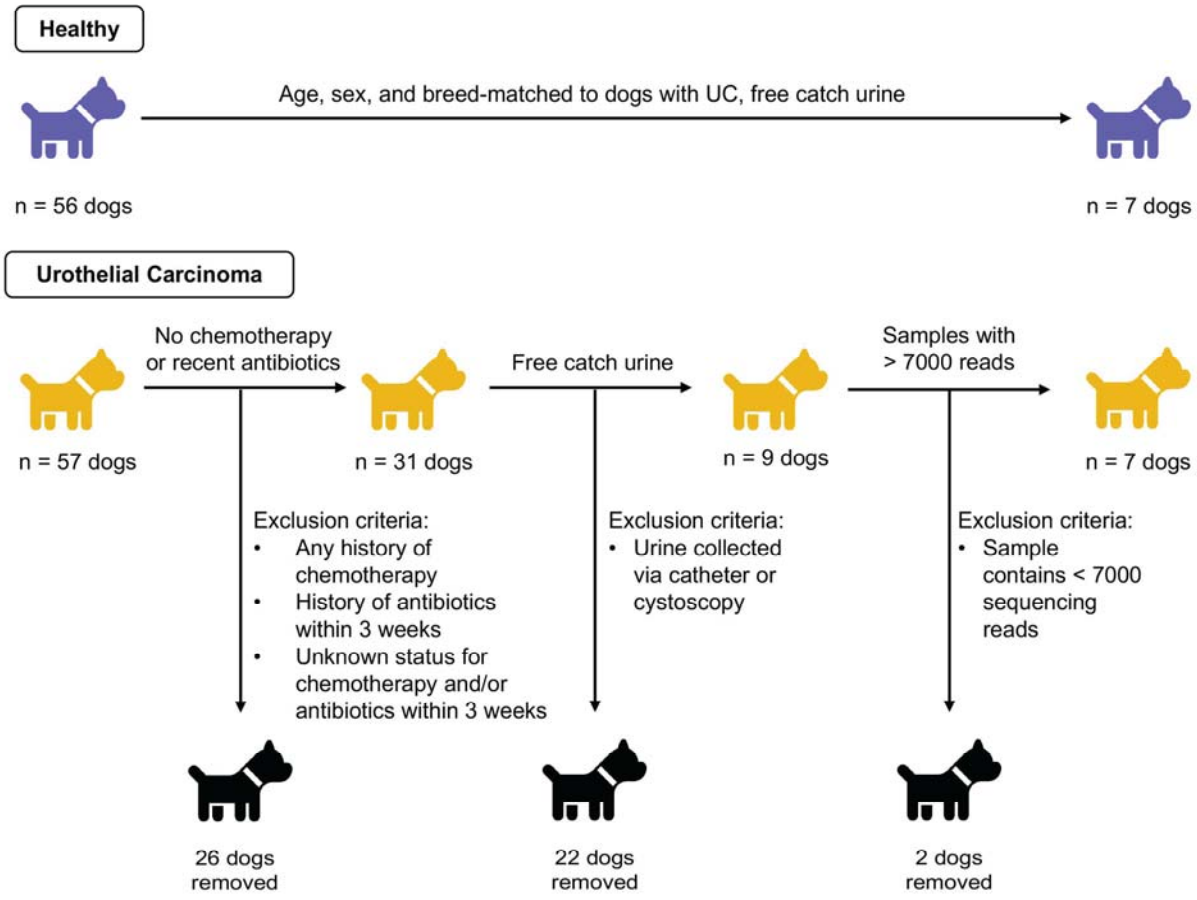
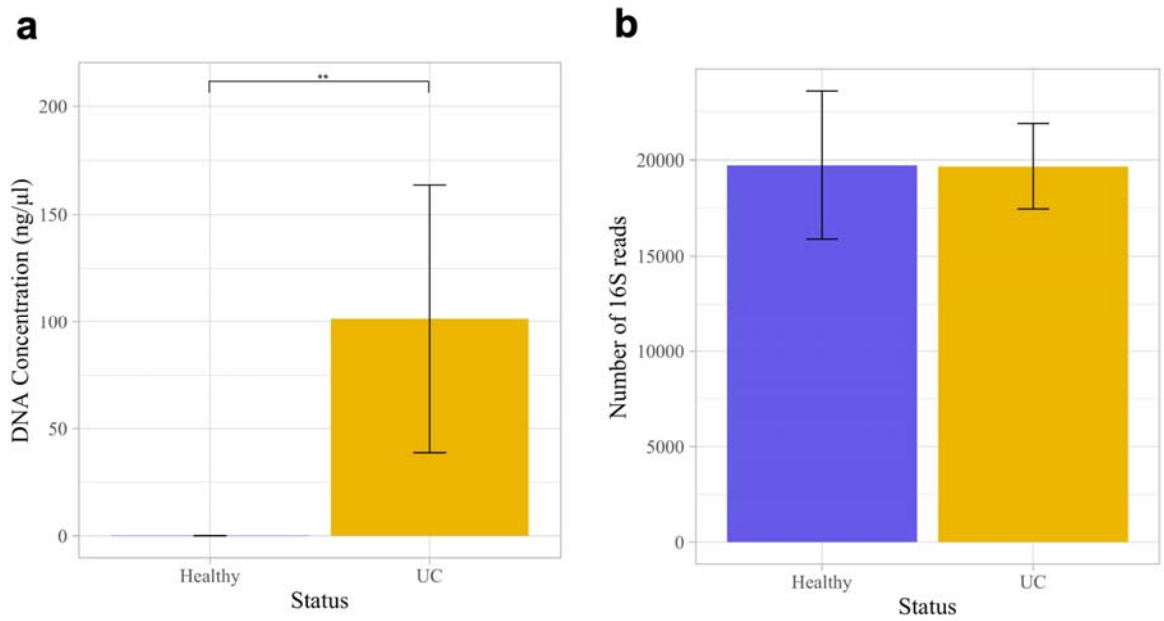


Figure 1: Experimental design



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740 **Figure 2: DNA concentrations and number of 16S reads in the urine samples of dogs with**
741 **and without urothelial carcinoma (UC).** (**a**) DNA concentrations were significantly greater in
742 dogs with UC than in healthy dogs (Wilcoxon Rank Sum test, $p = 0.002$). (**b**) The number of 16S
743 reads did not differ significantly between groups (two-sample t-test, $p = 0.99$). Error bars denote
744 standard error. Statistical significance is represented by stars: * < 0.05, ** < 0.001, *** < 0.0001

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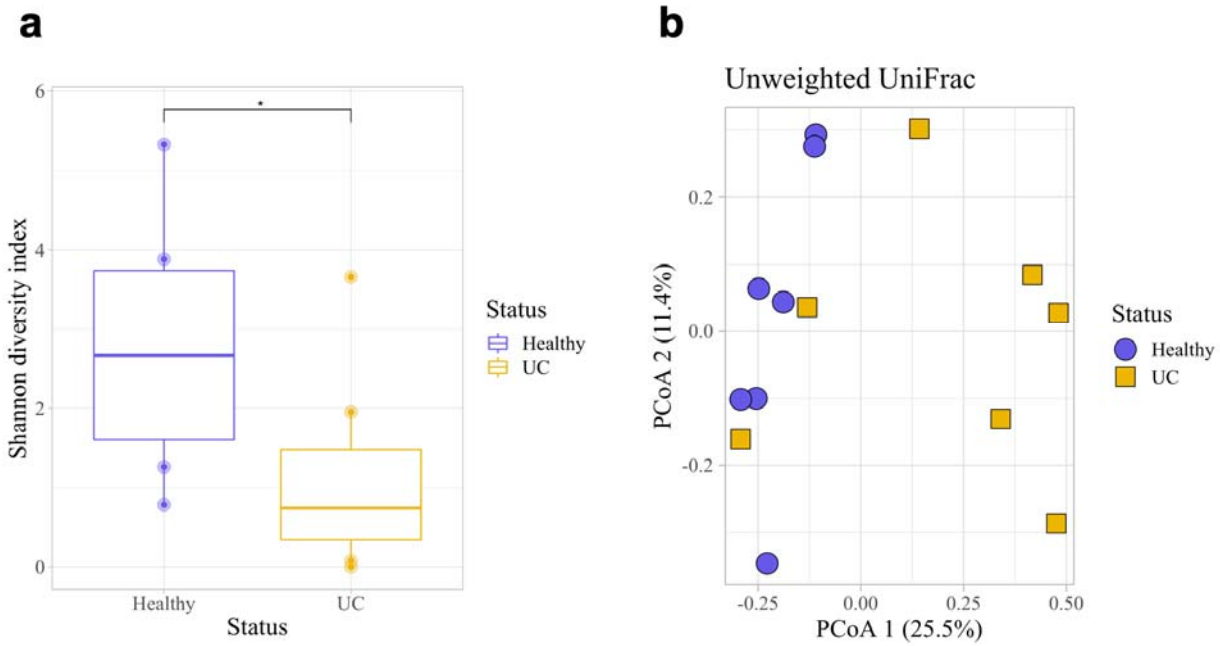

Figure 3: Microbial diversity and composition in the urine of dogs with and without UC. (**a**) Healthy dogs had a significantly higher microbial diversity compared to dogs with UC as measured by the Shannon diversity index (Kruskal-Wallis, $p = 0.048$). (**b**) Microbial composition between healthy dogs and dogs with UC also differed significantly (Unweighted UniFrac, PERMANOVA, $p = 0.011$). Error bars denote standard error. Statistical significance is represented by stars: * < 0.05, ** < 0.001, *** < 0.0001

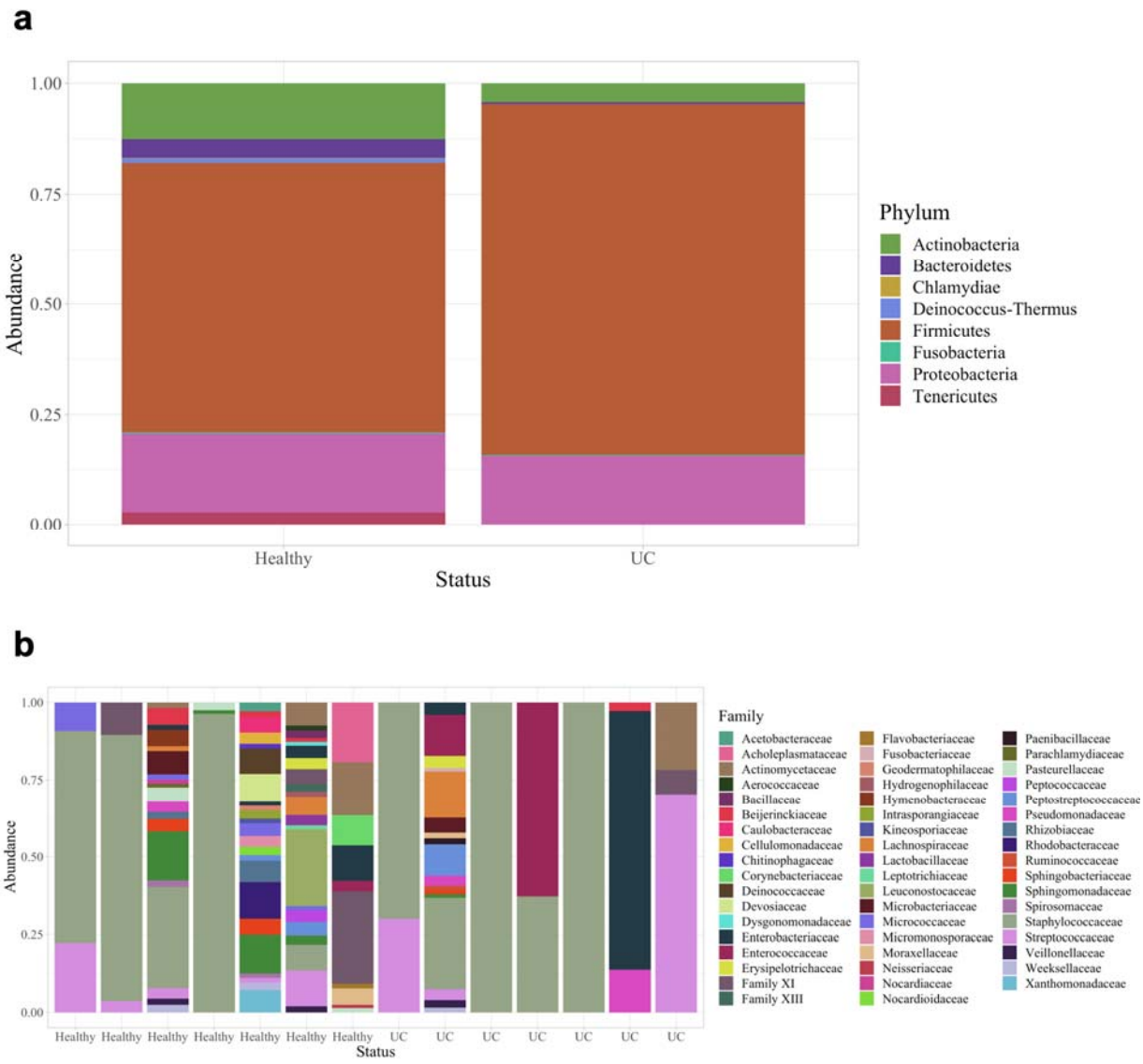

Figure 4: Phyla and family taxa bar plots of urine samples in dogs with and without UC. (**a**) Phyla and (**b**) family relative abundances. At the family level, the taxonomic composition of each sample is shown individually to demonstrate the variability across urine samples.

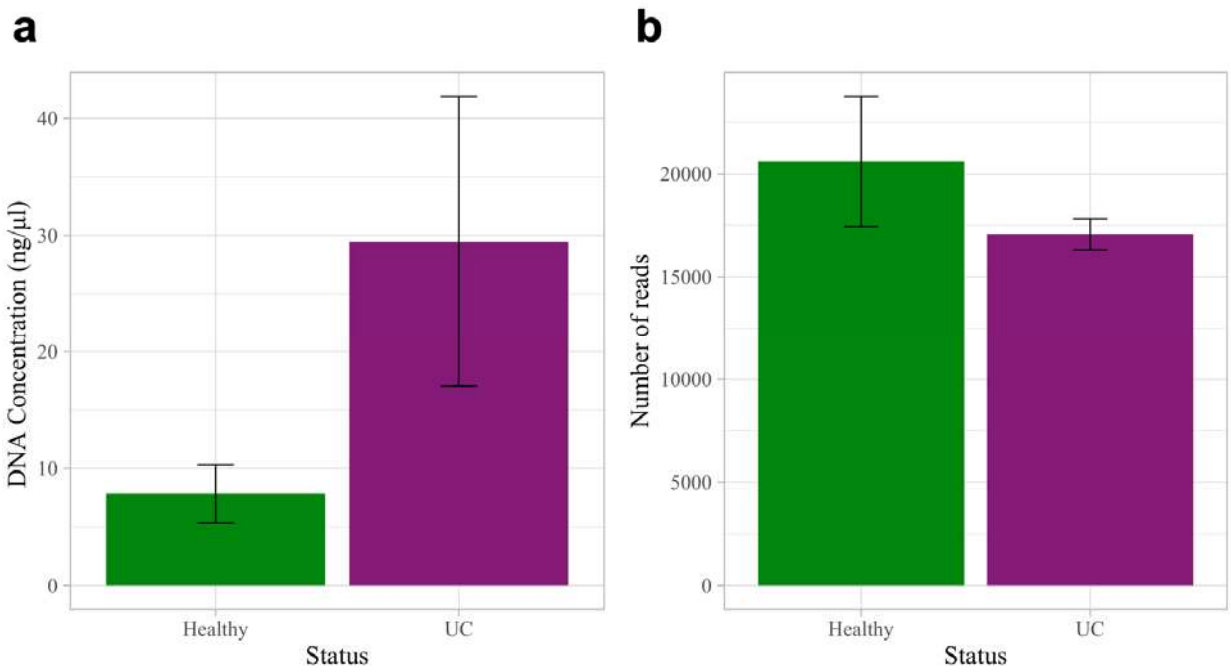

Figure 5: DNA concentrations and number of 16S reads in the fecal samples of dogs with and without UC. (**a**) DNA concentrations were greater (but not significantly) in dogs with UC as compared to healthy dogs (Wilcoxon Rank Sum Test, $p = 0.136$). (**b**) The number of 16S reads did not differ significantly between groups (two-sample t-test, $p = 0.322$). Error bars denote standard error.

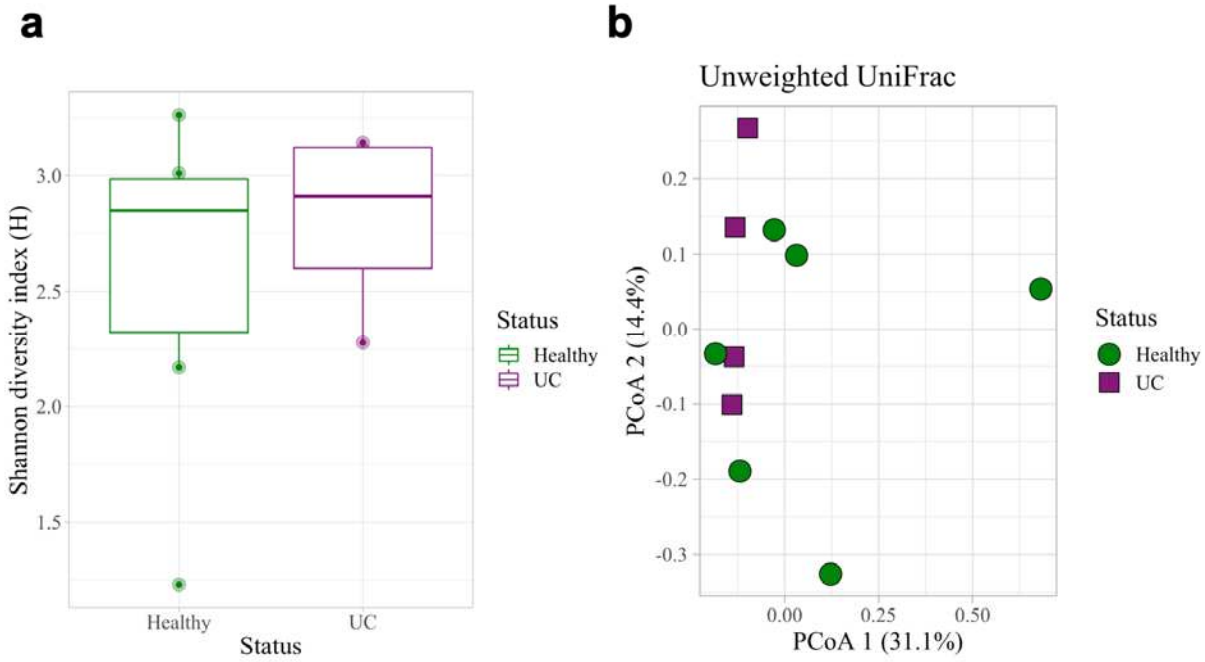

Figure 6: Microbial diversity and composition of fecal samples in dogs with and without UC. (**a**) Fecal microbial diversity did not differ significantly between dogs with and without UC (Kruskal-Wallis, $p = 0.67$). (**b**) Microbial composition also did not differ significantly between healthy dogs and dogs with UC (Unweighted UniFrac, PERMANOVA, $p = 0.252$). Error bars denote standard error.

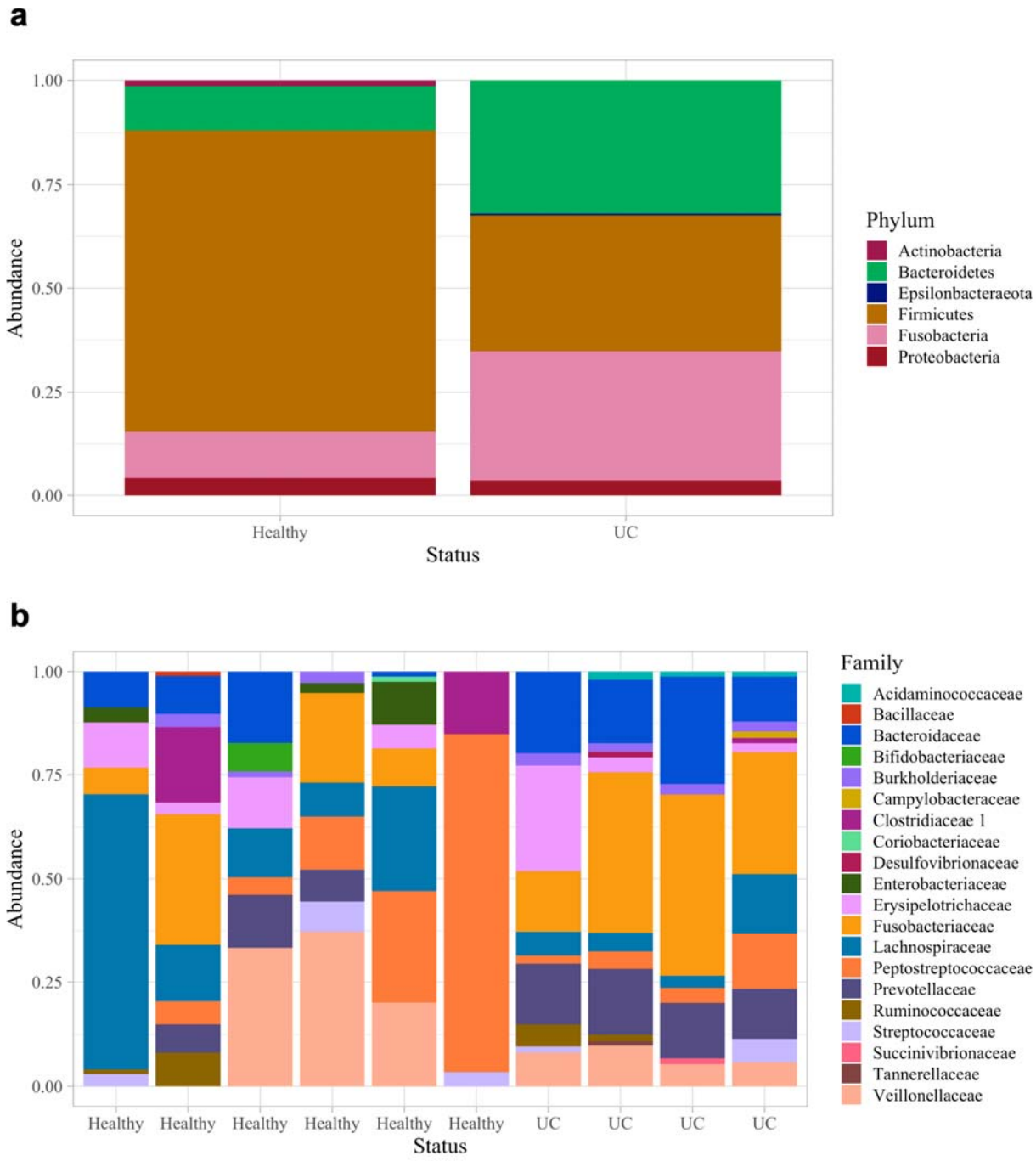

Figure 7: Taxa bar plots of fecal samples in dogs with and without UC. (**a**) Microbial phyla and (**b**) family relative abundances.

Supplemental Material:

Category	Free Catch	Non-Free Catch
Sex, n (%)		
Females	5 (62.5 %)	7 (62.6 %)
spayed	4	6
non-spayed	1	1
Males	3 (37.5 %)	4 (36.4 %)
neutered	3	4
non-neutered	0	0
Age (mean ± SD)	10.1 ± 2	9.6 ± 1.8

Supplemental Table 1: Demographics of dogs with urine samples collected via free catch and non-free catch methods. All dogs had urothelial carcinoma. Eight dogs had urine collected via mid-stream free catch while eleven dogs were sampled via non-free catch methods including cystoscopy or catheterization.

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Free Catch Urine		Non-free Catch Urine	
Phylum			
Firmicutes	70.3 %	Firmicutes	33 %
Proteobacteria	20.1 %	Tenericutes	26.7 %
Bacteroidetes	5.98 %	Proteobacteria	26.7 %
Genera			
Staphylococcus	43.2 %	*Mycoplasma*	18.3 %
Streptococcus	12.6 %	*Escherichia-Shigella*	18.1 %
Pantoea	11.4 %	*Enterococcus*	9.73 %

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832 **Supplemental Table 2: Dominant taxa in urine from dogs with UC by collection method.**

833 Relative abundance of the top three taxa in free catch and non-free catch urine at the phylum and

834 genera levels. All urine was collected from dogs with UC.

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Putative urine contaminants (ASVs)
D_1__Tenericutes;D_2__Mollicutes RF39;D_4__uncultured prokaryote;D_5__uncultured prokaryote;D_6__uncultured prokaryote
D_1__Deinococcus-Thermus;D_2__Deinococci;D_3__Thermales;D_4__Thermaceae;D_5__Thermus
D_1__Actinobacteria;D_2_Actinobacteria;D_3__Micrococcales;D_4__Micrococcaceae;D_5__Micrococcus
D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Burkholderiaceae;D_5__Cupriavidus
D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Betaproteobacteriales;D_4__Burkholderiaceae
D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella9;D_6__uncultured bacterium
D_1__Kiritimatiellaeota;D_2__Kiritimatiellae;D_3__WCHB1-41;D_4__uncultured rumen bacterium;D_5__uncultured rumen bacterium;D_6__uncultured rumen bacterium
D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae
D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Lactobacillus;D_6__Lactobacillus iners AB-1
D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Cytophaga
D_1__Verrucomicrobia;D_2__Verrucomicrobiae;D_3__Opitutaceae;D_4__Opitutaceae;D_5__Lacunisphaera;D_6__Opitutus sp. WS3(2011)
D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 9
D_1__Proteobacteria;D_2__Alphaproteobacteria;D_3__Rhizobiales;D_4__Xanthobacteraceae;D_5__Bradyrhizobium
Putative fecal contaminants (ASVs)
D_0__Bacteria
D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Veillonella
D_1__Firmicutes;D_2__Bacteoridia;D_3__Bacteroidales;D_4__Prevotellaceae;D_5__Prevotella 9
D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Staphylococcaceae;D_5__Staphylococcus
D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Atopobiaceae;D_5__Coriobacteriaceae UCG-002
D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae
D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Atopobiaceae;D_5__Coriobacteriaceae UCG-002

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838 **Supplemental Table 3: Contaminant ASVs.** Using the frequency and prevalence methods

839 (threshold value of 0.5) in the R package decontam v.1.10.0, putative contaminant ASVs were

840 identified and bioinformatically removed prior to further analyses.

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Category	Healthy	UC
Sex, n (%)		
Females	16 (53.3 %)	16 (53.3 %)
spayed	15	15
non-spayed	1	1
Males	14 (46.7 %)	14 (46.7 %)
neutered	11	11
non-neutered	3	3
Age (mean ± SD)	10 ± 1.76	10.4 ± 1.97

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844 **Supplemental Table 4: Demographics of larger canine cohort from which fecal samples**

845 **were collected.** Fecal samples were collected from dogs with UC (n = 30) and age-, sex-, breed-

846 matched healthy controls (n = 30).

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	Metric	Fecal samples from healthy dogs vs. dogs with UC
Alpha Diversity	Shannon Diversity Index Kruskal-Wallis	$p = 0.214$
	Simpson Diversity Index Kruskal-Wallis	$p = 0.506$
	Observed Features Kruskal-Wallis	$p = 0.336$
Beta Diversity	Bray Curtis PERMANOVA	$p = 0.468$
	UnWeighted UniFrac PERMANOVA	$p = 0.134$
	Weighted UniFrac PERMANOVA	$p = 0.0819$
Differentially Abundant Taxa	Phylum ANCOM	No differentially abundant taxa
	Genus ANCOM	No differentially abundant taxa
	ASV ANCOM	No differentially abundant taxa

Supplemental Table 5. Microbial diversity and composition of fecal samples from healthy dogs and dogs with UC. There were no significance differences in microbial diversity or composition between dogs with UC (n = 30) and sex-, age-, and breed-matched healthy controls (n = 30). ANCOM – Analysis of Composition of Microbiome.

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ASVs in both urine and fecal samples	Taxa
07124e5371867ec34213eb740707a0de	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnoclostridium
1345b73795b14ab0330b8ffb81b5b4aa	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia
181065d22563c4b1f591c6a5bbee7355	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales;D_4__Actinomycetaceae;D_5__Actinomyces;D_6__Actinomyces sp. canine oral taxon 374
1905e47315e57ce205d4505f1a5c5d67	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus;D_6__Streptococcus minor
1b3a2b9873a54f01302d629406b52aa9	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia
1cd1e7291e9803c9cdfe24a15309e043	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminiclostridium 5;D_6__uncultured organism
27046d59617e724675b68185aeb33d4a	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus
2a39faab1cf27e5068ef885794a3d1b1	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Microbacteriaceae
2cb64cfaa13ecebb8150698e244aa026	D_1__Epsilonbacteraeota;D_2__Campylobacteria;D_3__Camplybacterales;D_4__Helicobacteraceae;D_5__Helicobacter;D_6__Helicobacter canis
35815582b2cf31eb986673cddccb558c	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__peptoclostridium;D_6__uncultured bacterium
382cccf9f2613e42c602882e5efba519	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia
38ad78b86309fa98eaea53bac8579237	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Candidatus Arthromitus;D_6__uncultured bacterium
3acf68a82e28a71226cc15195277f39a	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured;D_6__uncultured organism
3c4c352e66306770ce10d3ac128d0ca8	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Lactococcus
42aa3a600f30a5267eea5a34d8655853	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured
4611ef696d9c9f16982f0886174522fe	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Epulopiscium
4952ad8a58b2e7d70d5315ce330442bb	D_1__Fusobacteria;D_2__Fusobacteriia;D_3__Fusobacteriales;D_4__Fusobacteriaceae;D_5__Fusobacterium
4a654a475be76c770508d1ea6a9771d9	D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichiaceae;D_5__Faecalitalea;D_6__Eubacterium sp. 1-5
4d74ef18790f690b2acf5fc60f89c222	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Ruminococcus] gauvreauii group
4f1d5517aa4ce179ae9241d5a5b3796d	D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Bacillaceae;D_5__Bacillus
52990f305d65b7df7dedd887cc08988f	D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Veillonellaceae;D_5__Megamonas
52ef51c7bec642ab72d7ce474821b108	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micrococcales;D_4__Micrococcaceae;D_5__Rothia
601426df62ac2005c0a78bbe617425a4	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales;D_4__Actinomycetaceae;D_5__Actinomyces;D_6__Actinomyces coleocanis
6019612a56660d54c57f12299224759d	D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Eryspielotrichales;D_4__Erysipelotrichaceae;D_5__Catenibacterium
61b2e2fc40303b1f0f19c1017f258bac	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__terrisporobacter;D_6__uncultured bacterium

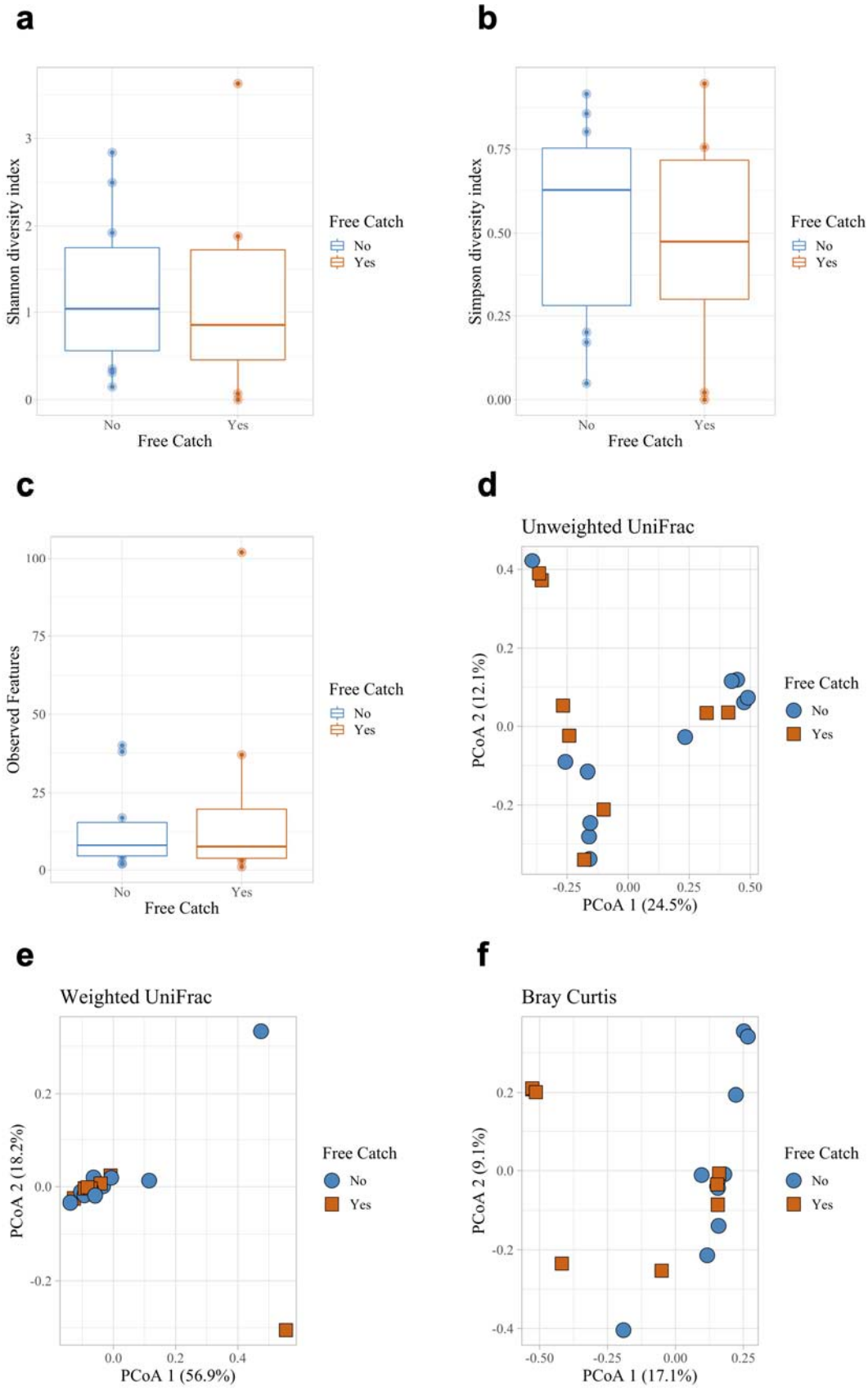
674e202dd30eab31fd826255caec43e1	D_1__Firmicutes;D_2__Negativicutes;D_3__Selenomonadales;D_4__Acidaminococcaceae;D_5__Phascolarctobacterium;D_6__uncultured Veillonellaceae bacterium
682c96e343759d3583a2a293fa4e0160	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Lachnoclostridium;D_6__Lachnospiraceae bacterium 2_1_46FAA
6a081f2b1b45ee5773bb947b977f5893	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__uncultured
6e441eb1e3bc74bb8a5ec4ff24b11147	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Lactobacillaceae;D_5__Lactobacillus
6fdb8a40fc3f65447a2ea0b3c21bbd68	D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Bacteroidaceae;D_5__Bacteroides;D_6__Bacteroides stercoris ATCC 43183
730125adfc6eae51053161e4a29f2bc9	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Enterococcaceae;D_5__Enterococcus
7439a1dc0a2e589a4605cefd7fcc6cb4	D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Collinsella
7510965009242aaa1cde47a1a2c1b998	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia;D_6__uncultured Blautia sp.
75300d9701d85567f711799e6dc01dce	D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichiaceae;D_5__Faecalitalea;D_6__Erysipelatoclostridum
76815f71f41950d2e2d481b6b730f3d8	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Faecalibacterium
777de77e069f708364a08b2b03f8eae9	D_1__Firmicutes;D_2__Bacilli;D_3__Bacillales;D_4__Bacillaceae;D_5__Bacillus
7cd06cbcae217263f67621482303de07	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus
84e088771adb5cfc2e134c9bad18c76a	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Peptostreptococcaceae;D_5__Clostridioides;D_6__Clostridioides difficile
877d42a21d6e5694161ea485ce3dacf8	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Flavonifractor
87a5ae82db511f591c640d9ad67321fc	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Micromonosporales;D_4__Micromonosporaceae;D_5__Actinoplanes
91beca23d467a7cb152b78f9505e650e	D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichiaceae;D_5__Allobaculum;D_6__Allobaculum stercoricanis DSM 13633
9d135cd7fd9b670ce5fdccfce8851183	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia
a3000823e9ab005bb353ff4e1e20eed8	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae 1;D_5__Clostridium sensu stricto 1
a3d3d817d8183e0d74175e4afbe65409	D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Pasteurellales;D_4__Pasteurellaceae;D_5__Pasteurella;D_6__Pasteurella multocida
a80abf00da9c833cb1faaa9707727dda	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus
ab9782e24971a281bf5c73c33d9ad73d	D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Faecalitalea;D_6__[Eubacterium] dolichum
b0d75fc101fefcde86c03b7cfdb39caf	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Corynebacteriales;D_4__Corynebacteriaceae;D_5__Corynebacterium 1
b7095a583ea62033ff918e2187652b27	D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Porphyromonadaceae;D_5__Porphyromonas;D_6__Porphyromonas sp. COT-052 OH4946
bd4017ad4efac59720e2d164da18ace4	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiaceae1;D_5__Clostridium sensu stricto 1;D_6__Clostridium baratii
c5073ccb362bfa533ad671fac3babb80	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__Blautia;D_6__Blautia sp. YHC-4
c6bedd5b82d0f92872c6e9d7435a172e	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Ruminococcaceae;D_5__Ruminococceae UCG-014;D_6__uncultured organism

c8f1df932d5f877f524cd2c16367e721	D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Coriobacteriaceae;D_5__Collinsella;D_6__Collinsella stercoris
cc8f83128875d60f9e1de433a207ce81	D_1__Epsilonbacteraeota;D_2__Campylobacteria;D_3__Camplybacterales;D_4__Campylobacteraceae;D_5__Campylobacter
d3d0bd88ddd06bf6e49cde1cdff07e9b	D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Erysipelatoclostridium
dae3d6aa2560755d958618047492c1f2	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus
e1002cca0084443ac173b037d6049d8b	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Ruminococcus] torques group;D_6__uncultured Clostridium sp.
e46e5d3e3462c7351e1dc52ec42e64cf	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Corynebacteriales;D_4__Corynebacteriaceae
e49f8561188c9050a9a3e3af2aa75c24	D_1__Bacteroidetes;D_2__Bacteroidia;D_3__Bacteroidales;D_4__Bacteroidaceae;D_5__Bacteroides;D_6__uncultured bacterium
ee10da4f77a1cf2cbf3146af2563a05c	D_1__Fusobacteria;D_2__Fusobacteriia;D_3__Fusobacteriales;D_4__Fusobacteriaceae;D_5__Fusobacterium;D_6__gut metagenome
f8b7aef6c94fcbe1b4793ffc3304bf0b	D_1__Firmicutes;D_2__Erysipelotrichia;D_3__Erysipelotrichales;D_4__Erysipelotrichaceae;D_5__Catenibacterium
f8cc743ae9448d9472ef8d3914262ccb	D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales;D_4__Enterobacteriaceae;D_5__Escherichia-Shigella
f957a7c9e0410797ffaa0be222cb0085	D_1__Actinobacteria;D_2__Coriobacteriia;D_3__Coriobacteriales;D_4__Eggerthellaceae;D_5__Slackia
fa0dcff3fde22b426ce94d8c91f56a17	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae;D_5__[Ruminococcus] gnavus group
fa4dd8c953b8a69498d1543bf15a4190	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Lachnospiraceae
fe9db134f6a44b3e5ac3ed1315920582	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae;D_5__Streptococcus
ffd03765b364ad4cdc17ebef2611ab72	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Bifidobacteriales;D_4__Bifidobacteriaceae;D_5__Bifidobacterium

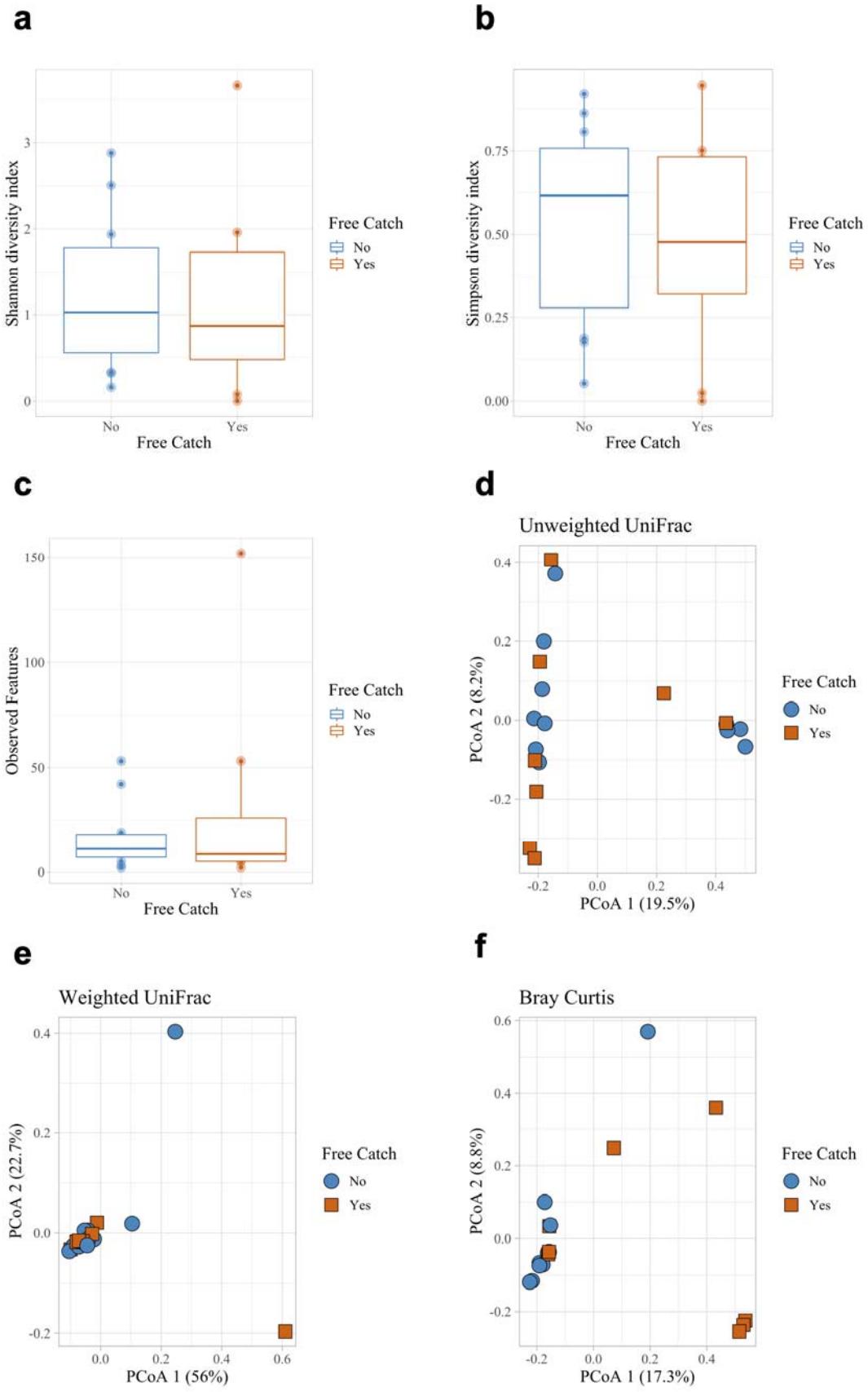
Supplemental Table 6: ASVs identified in both urine and fecal samples. There were 66 ASVs found in both urine and fecal samples of any dog.

ASVs in both urine and fecal samples by dog	Taxa
Dog 1 - UC	
f8cc743ae9448d9472ef8d3914262ccb	D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales; D_4__Enterobacteriaceae;D_5__Escherichia-Shigella
Dog 2 - UC	
27046d59617e724675b68185aeb33d4a	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae; D_5__Streptococcus
Dog 3 - Healthy	
f8cc743ae9448d9472ef8d3914262ccb	D_1__Proteobacteria;D_2__Gammaproteobacteria;D_3__Enterobacteriales; D_4__Enterobacteriaceae;D_5__Escherichia-Shigella
1878459013cf15f2993a81c14978c980	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae; D_5__Streptococcus
a3000823e9ab005bb353ff4e1e20eed8	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales;D_4__Clostridiales 1;D_5__Clostridium sensu stricto 1
601426df62ac2005c0a78bbe617425a4	D_1__Actinobacteria;D_2__Actinobacteria;D_3__Actinomycetales; D_4__Actinomyceteaceae;D_5__Actinomyces;D_6__Actinomyces coleocanis
1905e47315e57ce205d4505f1a5c5d67	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Streptococcaceae; D_5__Streptococcus;D_6__Streptococcus minor
Dog 4 - Healthy	
730125adfc6eae51053161e4a29f2bc9	D_1__Firmicutes;D_2__Bacilli;D_3__Lactobacillales;D_4__Enterococcaceae; D_5__Enterococcus
35815582b2cf31eb986673cddccb558c	D_1__Firmicutes;D_2__Clostridia;D_3__Clostridiales; D_4__Peptostreptococcaceae; D_5__Peptoclostridium;D_6__uncultured bacterium

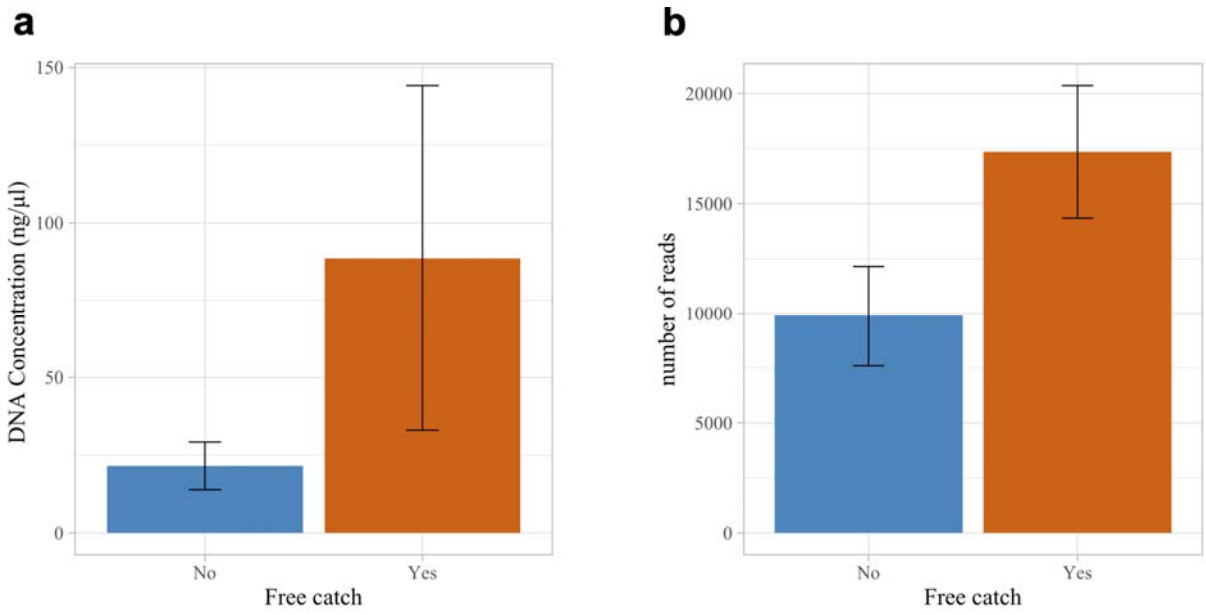
Supplemental Table 7: ASVs in urine and fecal samples from the same dog. Four dogs contained ASVs that were found in both their urine and fecal samples.



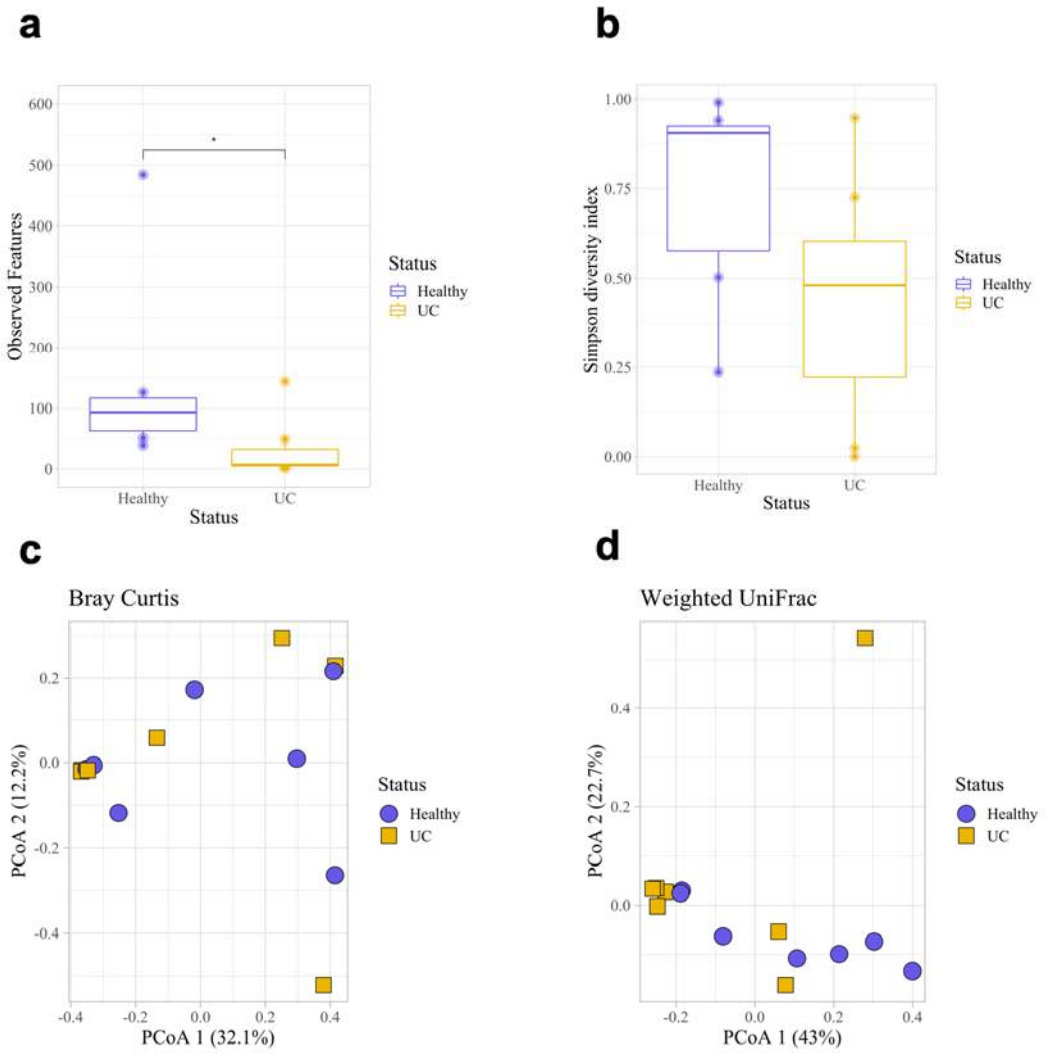
Supplemental Figure 1: Urine microbial community diversity and composition by collection method in dogs with UC (rarefied data). Dogs with UC were sampled via free catch (n = 8) and non-free catch (n = 11) methods. Samples were rarefied at 1000 reads. There were no significant differences in microbial diversity between collection methods as assessed via (**a**) Shannon (Kruskal-Wallis: $p = 0.62$) or **b**) Simpson diversity indices ($p = 0.68$) or (**c**) Observed Features (richness) ($p = 0.901$). The microbial composition of free-catch urine did not differ significantly from non-free catch urine based on (**d**) Unweighted (PERMANOVA, $p = 0.328$) or (**e**) Weighted UniFrac distance matrices ($p = 0.485$) but did differ significantly based on (**f**) Bray Curtis ($p = 0.008$). Error bars denote standard error.



Supplemental Figure 2: Urine microbial community diversity and composition by collection method in dogs with UC (unrarefied data). Dogs with UC were sampled via free catch (n = 8) and non-free catch (n = 11) methods. Data are non-rarefied. There were no significant differences in alpha diversity between collection methods as assessed using the (**a**) Shannon (Kruskal-Wallis: $p = 0.68$) or **b**) Simpson diversity indices ($p = 0.68$) or (**c**) Observed Features (richness) ($p = 0.901$). The microbial composition of free-catch urine did not differ significantly from non-free catch urine based on (**d**) Unweighted (PERMANOVA, $p = 0.342$) or (**e**) Weighted UniFrac distance matrices ($p = 0.54$) but did differ significantly based on (**f**) Bray Curtis ($p = 0.005$). Error bars denote standard error.



Supplemental Figure 3: DNA Concentrations and 16S reads by urine collection method. (**a**) Urine DNA concentrations and (**b**) 16S reads in dogs with UC sampled via free catch or non-free catch methods (cystoscopy, catheterization). DNA concentrations and 16S reads were greater, although not significantly, in mid-stream free catch urine samples (DNA concentration: Wilcoxon Test, $p = 0.778$; 16S reads: two-sample t-test, $p = 0.067$). Error bars denote standard error.



930

931 **Supplemental Figure 4: Urine microbial diversity and composition in dogs with and**

932 **without UC.** Dogs with UC had lower microbial diversity compared to healthy dogs based on

933 (**a**) Observed Features (richness) and the (**b**) Simpson diversity index; however, only Observed

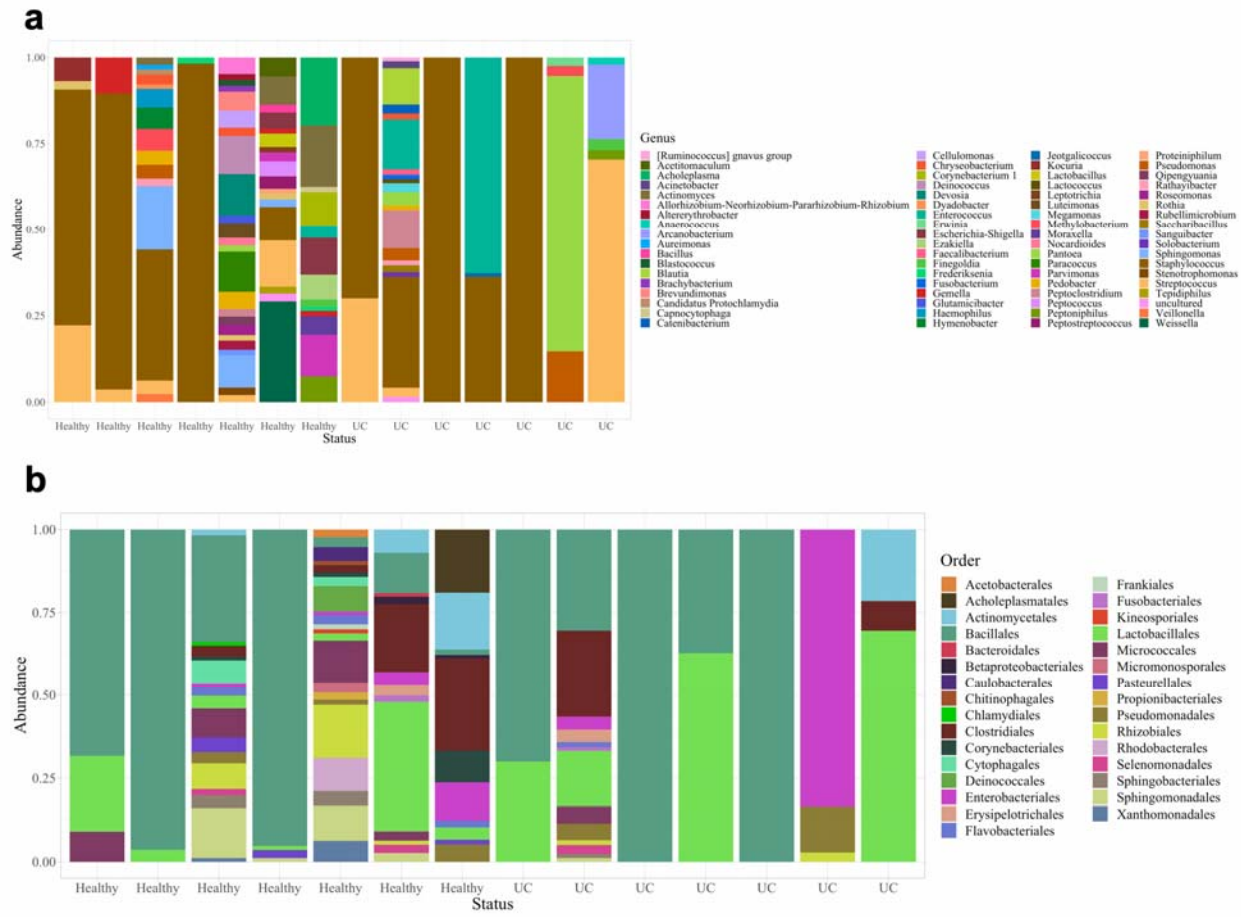
934 Features was statistically significant (Kruskal-Wallis: Observed Features, $p = 0.025$; Simpson, p

935 $= 0.133$). Microbial composition did not differ significantly based on (**c**) Bray Curtis or (**d**)

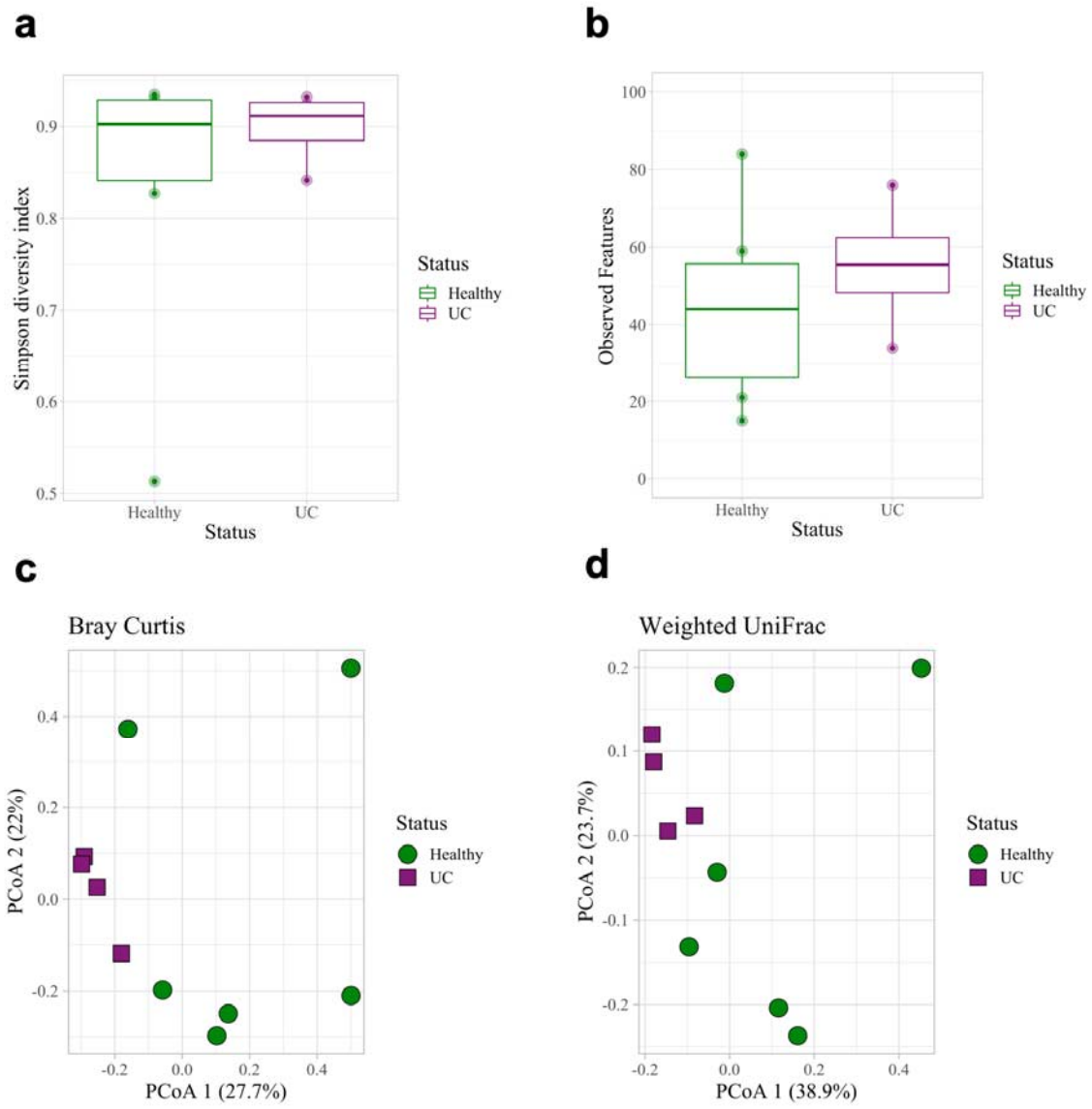
936 Weighted UniFrac distance matrices (PERMANOVA: Bray Curtis, $p = 0.888$; Weighted

937 UniFrac, $p = 0.168$). Error bars denote standard error. Statistical significance is represented by

938 stars: * < 0.05, ** < 0.001, *** < 0.0001



Supplemental Figure 5: Taxa bar plots of urine samples in dogs with and without UC. (**a**) Microbial genera and (**b**) order relative abundances.



948

949 **Supplemental Figure 6: Fecal microbial diversity and composition in dogs with and**

950 **without UC.** Fecal microbial diversity did not differ significantly in dogs with (n=4) or without

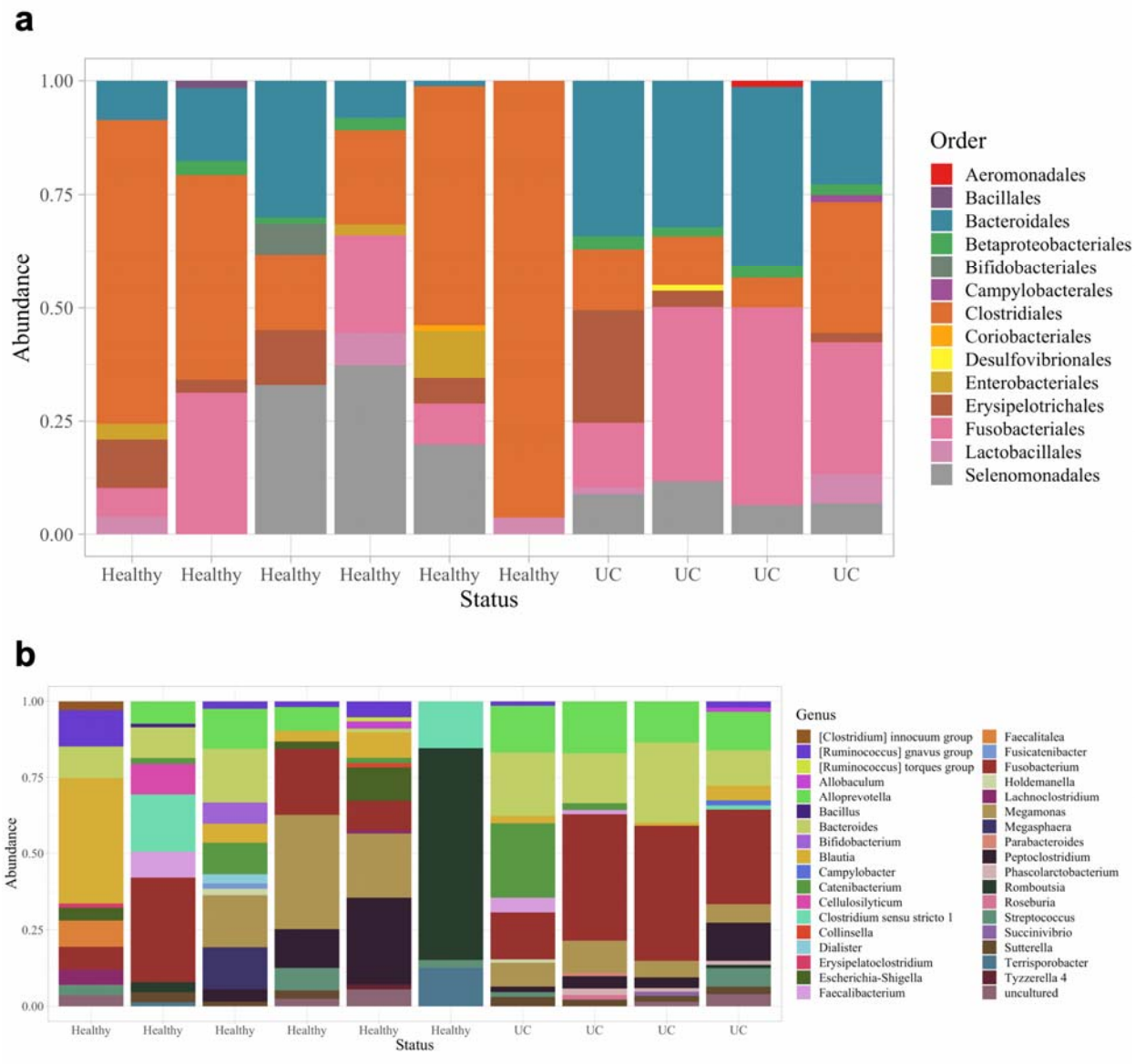
951 (n=6) UC based on (**a**) Observed Features (richness) and the (**b**) Simpson diversity index

952 (Kruskal-Wallis: Observed Features, $p = 0.67$; Simpson, $p = 0.522$). Microbial composition also

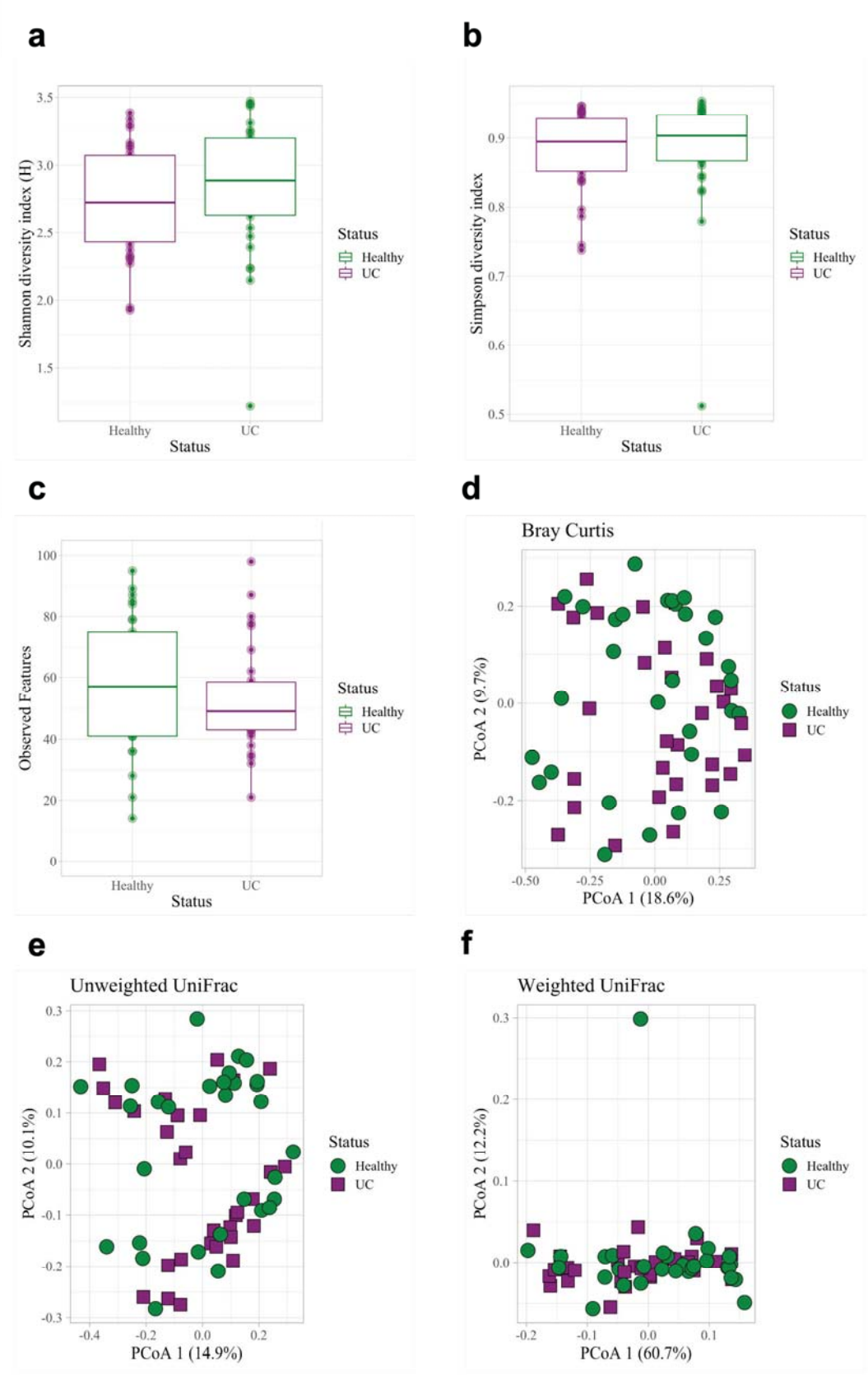
953 did not differ significantly based on (**c**) Bray Curtis or (**d**) Weighted UniFrac distance matrices

954 (PERMANOVA: Bray Curtis, $p = 0.06$; Weighted UniFrac, $p = 0.06$). Error bars denote standard

955 error.

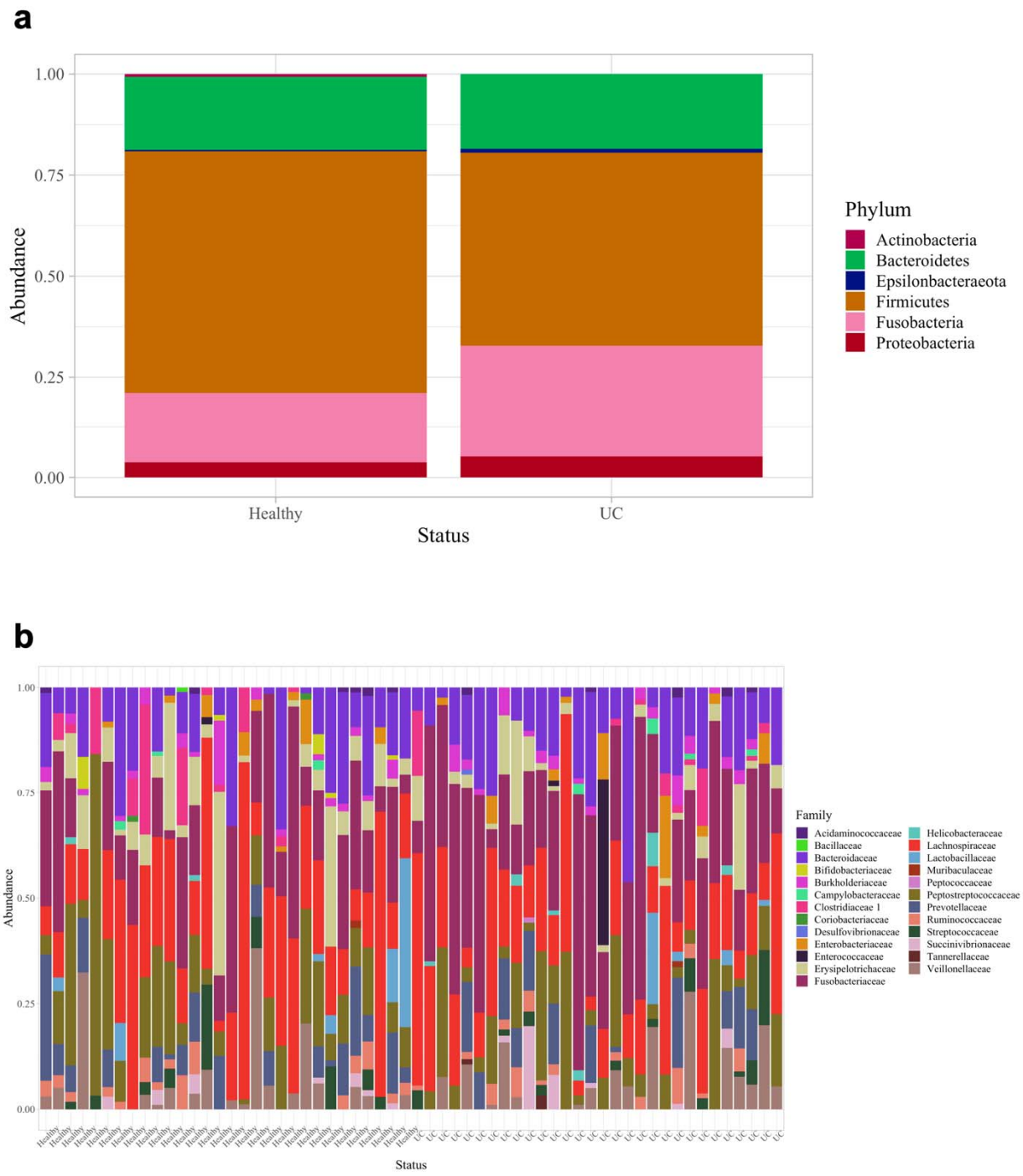


Supplemental Figure 7: **Taxa bar plots of fecal samples.** (**a**) Microbial order and (**b**) genera relative abundances in dogs with (n=4) and without UC (n=6).



964

965 **Supplemental Figure 8: Fecal microbial diversity and composition.** We compared fecal
966 microbiota in dogs with UC (n = 30) and sex-, age-, and breed-matched healthy controls (n =
967 30). There were no significant differences in microbial diversity by (**a**) Shannon (Kruskal-Wallis,
968 $p = 0.214$), (**b**) Simpson (Kruskal-Wallis, $p = 0.506$), or (**c**) Observed Features (Kruskal-Wallis,
969 $p = 0.336$). There were also no significant differences in microbial composition by (**d**) Bray
970 Curtis (PERMANOVA, $p = 0.468$), (**e**) Unweighted UniFrac (PERMANOVA, $p = 0.134$), or (**f**)
971 Weighted UniFrac distance matrices (PERMANOVA, $p = 0.0819$).
972



Supplemental Figure 9: Fecal microbial taxa bar plots. Relative abundances of fecal microbiota at the (**a**) phyla and (**b**) family levels from dogs with UC (n = 30) and age-, sex-, and breed-matched healthy controls (n = 30).