Received: 24 January 2022 | Accepted: 15 June 2022

DOI: 10.1111/jvim.16482

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Characterization of the urogenital microbiome in Miniature Schnauzers with and without calcium oxalate urolithiasis

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Funding information
NIH ORIP K01 Mentored Research Scientist Development Award, Grant/Award Number: K01-OD019912; NIH T32 Comparative Medicine and Pathology Training Grant, Grant/Award Number: T32 OD010993-15; University of Minnesota's College of Veterinary Medicine Resident and Graduate Student Research Grant

Abstract

Background: Calcium oxalate (CaOx) uroliths are common in dogs. Humans with CaOx urolithiasis exhibit alterations of the urinary and urogenital microbiomes that might mediate urolith formation. Detection of urogenital microbes associated with CaOx in dogs could inform disease pathophysiology.

Objective: To identify compositional differences in the urogenital microbiome of Miniature Schnauzers with and without CaOx uroliths.

Animals: Nineteen midstream, voided urine samples from Miniature Schnauzers with (n = 9) and without (n = 10) a history of CaOx urolithiasis.

Methods: Analytical cross-sectional study. Microbial DNA was extracted from previously frozen urine samples and sequenced for the bacterial 16S rRNA V3-V4 hypervariable regions. Diversity and composition of microbial populations were compared between urolith formers and controls.

Results: Alpha and beta diversity measures were similar between groups. Five individual bacterial taxa differed in abundance (indicator values >0.5 and $P < .05$): *Acinetobacter*, 2 *Geobacillus* variants, and *Hydrogenophaga* were overrepresented in the urine of urolith formers, and *Sphingopyxis* was overrepresented in controls. Two distinct subtypes of urine microbial composition were observed based on beta diversity measures, independent of urolith status, and other clinical variables.

Conclusions and Clinical Importance: Although we did not detect a difference in the overall urogenital microbial composition between groups, observed differences in individual bacterial taxa might be clinically relevant. For example, *Acinetobacter* was overrepresented in urolith formers and is associated with CaOx urolithiasis in humans. Two unique clusters of the microbiome were identified, independent of urolith status, which may represent distinct urotypes present in Miniature Schnauzers.

KEYWORDS
canine, stones, urinary tract infection, urobiome

Abbreviations: ASV, amplicon sequence variant; BCS, body condition score; BG, blood glucose; CaOx, calcium oxalate; EQUC, expanded quantitative urine culture; iCa, ionized calcium; PCoA, principal coordinates analysis; UCa:Cr, urinary calcium-to-creatinine ratio; USG, urine-specific gravity.

1 | INTRODUCTION

Calcium oxalate (CaOx) urolithiasis is increasingly common and highly recurrent in dogs.[1,2] Various genetic, metabolic, and dietary variables contribute to urolith formation, but the etiology of CaOx urolithiasis remains incompletely understood.[3-8] Emerging interest in the role of the microbiome in CaOx urolith formation in humans has exposed new perspectives on disease pathogenesis. For instance, both microbiological culture and DNA sequencing methods confirm that CaOx uroliths themselves possess a unique microbiome.[9-11] Early investigations linking the microbiome and urolithiasis concentrated largely on the gut microbiome, with a particular focus on oxalate-degrading microbial populations.[12-16] Similar to humans, dogs with CaOx urolithiasis also experience shifts in the gut microbiome.[17]

More recently, distinct shifts in the diversity and composition of the urinary and urogenital microbiomes were observed in human urolith formers.[9,10,18] In fact, a comprehensive analysis of the gut and urogenital microbiomes determined that the urogenital microbiome exhibited more pronounced alterations by urolith status than did the gut microbiome.[9] Microbial communities also recently have been identified in the urine of healthy dogs,[19-21] but the role of these microbes in CaOx formation in dogs is unknown. Discovery of urogenital microbiome features that discriminate dogs with CaOx urolithiasis from healthy dogs could provide insight into disease pathogenesis. Microbial markers also could be applied in future research on urolith diagnosis, monitoring, or treatment.

Our primary objective was to identify differences in the urogenital microbiome of Miniature Schnauzers with and without CaOx uroliths using 16S rRNA amplicon sequencing. Miniature Schnauzers were selected because of the breed's high risk for CaOx urolithiasis.[3,4] We hypothesized that Miniature Schnauzer CaOx urolith formers would possess distinct microbial signatures as compared to urolith-free, breed-matched controls.

2 | METHODS

2.1 | Study population

Our study took place at the University of Minnesota College of Veterinary Medicine. Samples were selected from a biobank of frozen (−80°C) urine specimens collected from purebred Miniature Schnauzers for previous and ongoing studies on CaOx urolithiasis between September 2012 and July 2020.[5,22] Written, informed owner consent was obtained before sample collection with study protocols approved by the University of Minnesota's Institutional Animal Care and Use Committee (protocol #1207A17243, 1509-33019A, 1807-36213A). Medical records were reviewed, and urine specimens were included from dogs meeting the study criteria of either a CaOx urolith former or a urolith-free control. Urolith formers were defined as dogs with active or historical CaOx urolithiasis, defined as uroliths with a core consisting of 100% CaOx as determined by polarized light microscopy and infrared spectroscopy. Controls were defined as dogs that were at least 8 years of age (the average age of CaOx diagnosis) at the time of urine collection,[4] had no history of urolithiasis, and had no uroliths on screening abdominal radiographs. Control dogs with hypercalciuria, a known risk factor for CaOx urolith formation, were excluded.[5] Hypercalciuria was defined as a urinary calcium-to-creatinine (UCa:Cr) ratio of >0.05 mg/mg.[5]

In humans, urine collection method influences observed microbiota,[23-27] but the effect of collection method still requires investigation in dogs. Voided urine samples were used in the study that found differences in the microbiomes of humans with nephroliths versus healthy controls.[9] Therefore, only urine samples collected by midstream voiding were included, and results are classified as urogenital microbiome, as opposed to urinary microbiome.[28] Standard protocol was to freeze samples within 2 hours of urine collection, but the precise time intervals between collection and freezing were not routinely documented. Urinalyses, urine culture, and blood calcium concentration were not required for enrollment. However, in the dogs with these tests available for review, urine samples with pyuria (>5 WBC/hpf), cytologic bacteriuria, or a positive culture, and dogs with total or ionized hypercalcemia (>11.5 mg/dL or 5.9 mg/dL, respectively) were excluded. A medication history extending at least 1 month before sample collection was required in all dogs. Dogs that had received probiotics, immunosuppressive treatments, or antimicrobials within 1 month of urine collection were excluded. Dogs with diabetes mellitus, hyperadrenocorticism, lower urinary tract disorders other than CaOx urolithiasis, chronic gastrointestinal disease, or severe systemic comorbidities were excluded.

2.2 | DNA isolation and 16S rRNA amplicon sequencing

Frozen urine samples were shipped overnight on dry ice to Norgen Biotek Corporation (Thorold, Ontario) for molecular processing and sequencing. Microbial DNA isolation was performed using a commercial DNA extraction kit (Urine DNA Slurry Kit, Norgen Biotek Corp) in accordance with manufacturer instructions, followed by an additional DNA concentration step (DNA Clean-Up and Concentration Micro-Elute Kit, Norgen Biotek Corp). Total DNA concentrations were measured for all samples (Bioanalyzer DNA analysis solutions, Agilent, Santa Clara, California). Extraction of DNA and library preparation were performed in a single batch. Library preparation was performed (short amplicon Library Preparation Kit for Illumina, Norgen Biotek Corp), followed by amplification of the bacterial 16S rRNA V3-V4 region using the following amplicon primer sequences: forward primer 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG and reverse primer 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.[29]

Polymerase chain reaction was performed using a T100 thermal cycler (Bio-Rad Laboratories, Hercules, California) with the following conditions: 95°C for 3 minutes for denaturation, a total of 35 cycles with denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Amplicons were sequenced using the MiSeq

sequencing platform, 2 × 250 base pair paired-end reads, v2 chemistry (Illumina, San Diego, California),[30] followed by amplicon DNA quantification (Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen, Waltham, Massachusetts). Two negative controls were used for DNA isolation and library preparation, consisting of no template control nuclease-free water. Raw sequence data are available at the National Center for Biotechnology Information BioProject PRJNA798763.

2.3 | Processing of sequence reads

Primers were removed from raw, paired-end sequence reads using Cutadapt,[31] and sequences were further processed using QIIME2 (v. 2020.8)[32] for removal of low-quality sequence data, merging of forward and reverse reads, and taxonomic assignment. Sequences were truncated according to quality using the DADA2 plugin,[33] and taxonomy was assigned to high-resolution amplicon sequence variant (ASV) outputs using the Silva (v. 138) reference database.[34]

Sequences identified as chloroplasts, mitochondria, chimeras, or that were present in only a single sample were identified and removed from the dataset before downstream analysis. Additionally, sequences represented by <10 total reads across samples were filtered from the dataset, as previously described.[9,35] The R *decontam* package (v. 1.10.0)[36] was used to identify contaminant taxa using the following parameters: method = "combined," threshold = 0.5. The "combined" method employs a combination of 2 strategies for identification of putative contaminants. The first is based on the prevalence of microbes in negative controls as compared to true samples. The second method recognizes contaminant species by identifying inverse relationships between microbial sequence frequency and DNA concentrations.[36] All identified contaminants were removed before further analysis. Sequence data were transformed into relative abundance before downstream analysis.

2.4 | Statistical analysis

Statistical analyses were performed using various packages within R statistical software (v. 4.0.2). Normality of continuous variables was determined using the Shapiro-Wilk test. For normally distributed data, a Student's *t* test was used for comparison of data between experimental groups, and Wilcoxon rank sum tests were used for data lacking a normal distribution. Fisher's exact test was used for categorical data to compare proportions between groups. Variables related to the urine specimen or individual dog were tested to determine if they differed between 2 observed microbiome subtypes. These variables included urine volume, storage duration, total DNA concentrations, sequence reads, urine-specific gravity (USG), urine pH (classified as acidic if ≤7 versus alkaline if >7), UCa:Cr, age, sex, body weight, body condition score (BCS, 1-9 scale), serum biomarkers of renal function (BUN and serum creatinine concentrations), blood ionized calcium (iCa) concentration, blood glucose (BG) concentration, and serum triglyceride concentration. Urine specific gravity was measured using a digital veterinary refractometer (MISCO Palm Abbe, Solon, Ohio), and urine pH was determined using a urine dipstick chemistry test (Siemens MultiStix, Malvern, Pennsylvania). Urinalyses and blood testing must have been performed within 5 days of collecting the urine sample for microbiome analysis. Given incomplete dietary information for many dogs, incorporation of nutrient analysis was not possible. The Benjamini-Hochberg procedure was used to determine statistical significance, defined as a false discovery rate <.05.

Alpha diversity analyses were performed using the R *vegan*[37] and *phyloseq*[38] packages and included the Shannon diversity index, inverse Simpson diversity index, and observed richness. Wilcoxon rank sum tests were performed to compare alpha diversity between experimental groups. Specific alpha diversity metrics assess microbial communities with respect to richness (the number of unique ASVs), evenness (the distribution of ASV abundance), or both. For instance, observed richness compares the number of organisms present within different groups (regardless of abundance), and higher observed richness scores indicate that higher numbers of distinct organisms are present.[39,40] Both Shannon diversity and inverse Simpson diversity index evaluate a combination of richness and evenness using different statistical methods, but Shannon index offers more sensitivity to rare or singleton organisms.[39] Bray Curtis, Weighted UniFrac, and Unweighted UniFrac distance matrices were used to assess beta diversity. Similarly, different strategies for measuring beta diversity assess specific components of the data. UniFrac is a method that accounts for phylogenetic relatedness of the microbes present in a population, whereas genetic relatedness is not considered in Bray Curtis dissimilarity.[41,42] Furthermore, Unweighted UniFrac only considers the simple presence or absence of organisms, whereas both Weighted UniFrac and Bray Curtis also account for evenness, or relative abundance distribution of organisms.[41,42] Permutational multivariate analysis of variance using 1000 permutations was performed to assess differences in microbial composition between experimental groups.

Discriminant taxa were identified between groups using indicator species analysis[43] in the R *labdsv* package.[44] Indicator species analysis assigns an indicator value to individual taxa, which represents the product of the relative frequency and the relative abundance of that taxon in an experimental group. Values range from 0 to 1, and higher indicator values specify that a taxon has both high abundance within samples of a group and is frequent across samples of that group, as compared to an alternative group of interest.[43] Taxa with an indicator value >.5 and a *P* value for the specified indicator value <.05 were reported, as previously described.[45] Wilcoxon rank sum tests were performed on taxa meeting these criteria.

3 | RESULTS

3.1 | Study population

A total of 78 urine samples collected from 71 Miniature Schnauzers were screened for study eligibility. The reasons for excluding samples are summarized in Figure 1. Nineteen urine samples (9 cases and 10 controls) from 19 dogs met the criteria for enrollment and were

included. Metadata for the case and control groups are summarized in Table 1. No statistically significant differences in age, sex, BCS, volume of urine, specimen storage duration, USG, urine pH, or iCa were detected between groups. Seventeen dogs had concurrent urinalyses performed. Of these, no dogs had glucosuria, bacteriuria, or pyuria. Three dogs had hematuria (>5 RBC/hpf), and all were in the case group. Only 3 dogs had a urine bacterial culture and sensitivity performed, which were all negative for growth. All dogs had UCa:Cr results available. Seven of 9 samples (78%) from case dogs were classified as hypercalciuric (median, 0.06 mg/mg; range, 0.01-0.18 mg/mg). A blood iCa concentration determined at the time of urine sample collection was available for 15/19 dogs, and the remaining 4 samples had a total serum calcium concentration available.

In the case group, most (6/9, 67%) samples were obtained from dogs with active urolithiasis, and the remaining samples (3/9, 33%) were from dogs with historical CaOx urolithiasis. All 9 case dogs had cystoliths, 2 with concurrent nephroliths. Five of the 9 urine samples from urolith formers were from dogs with recurrent CaOx urolithiasis.

Dogs consumed variable commercial diets. Description of the primary diet fed was available for 18 of 19 dogs, and the diet was different for each dog. Twelve dogs were fed an over-the-counter commercial diet, and 5 dogs were fed prescription veterinary diets, including diets formulated for urinary health, gastrointestinal health, weight loss, a hydrolyzed protein diet, and 1 dog consumed a mixture of a renal health and weight loss diet. The remaining dog was reported to normally eat a commercial diet, but in the days leading up to the visit was eating a combination of home-cooked chicken and rice and a prescription diet for gastrointestinal health. Fifteen samples were obtained from dogs not receiving any medications or supplements at the time of urine collection. Two control dogs were receiving vitamins or nutraceutical supplements, 1 dog was receiving gabapentin and was reported to occasionally receive carprofen (Rimadyl, Zoetis, Parsippany, New Jersey), and 1 dog was receiving tramadol.

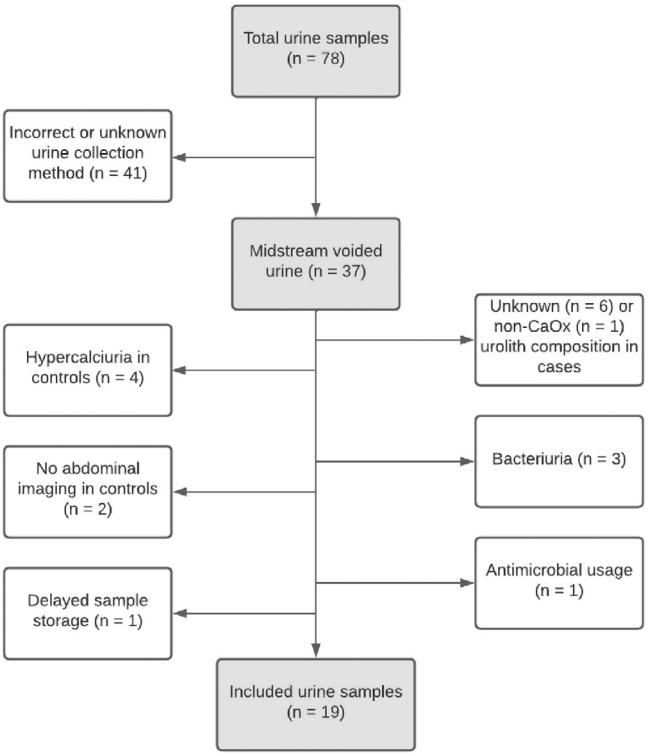

FIGURE 1 Flow diagram showing the indications for excluding urine samples. A total of 78 urine samples were evaluated for inclusion. Nineteen urine samples from 19 dogs met the inclusion and exclusion criteria and were included in this study.

3.2 | Bacterial composition of urine samples

Total DNA concentrations ranged from 0.1 to 8.7 ng/μL (median, 0.4 ng/μL). Sequence reads ranged from 65 055 to 221 943 total reads in samples. The 2 negative controls contained 2664 and 4085 sequence reads, respectively, comprised of 7 unique ASVs. No correlation between total DNA concentration and sequence reads was identified (Spearman's correlation; $\rho = .02$, $P = .95$). Twenty-eight taxa were identified as contaminants using the R package *decontam*,[36]

TABLE 1 Summary of metadata for 19 urine samples from Miniature Schnauzers with and without CaOx urolithiasis

Variable	Cases (n = 9)	Controls (n = 10)	*P* value	FDR-adjusted *P* values (q-values)
Age (years)	10.2 ± 1.5	10.4 ± 1.3	.72	.96
Sex	8M, 1F	8M, 2F	1.0	1.0
BCS (scale 1-9)	6.1 ± 1.17	5.7 ± 1.64	.53	.96
Sample volume (mL)	2.0 (1-9)	3.75 (1.5-10.5)	.28	.75
Storage duration (years)	6.2 (0.73-8.0)	5.8 (1.5-6.9)	.68	.96
USG	1.017 ± 0.010 [7]	1.032 ± 0.010	.01	.08
Urine pH	6.7 ± 1.0 [7]	6.8 ± 0.82	.86	.98
iCa (mg/dL)	5.7 ± 0.24 [8]	5.5 ± 0.21 [7]	.12	.48

Notes: *P* values for Student's *t* tests, Wilcoxon rank-sum tests, or Fisher's exact test are reported. Normally distributed data is reported as the mean ±SD. Data that did not follow a normal distribution are reported as median (range). The number of samples available for measurement is listed in brackets if not performed in all dogs. The Benjamini-Hochberg procedure was used to determine statistical significance, defined as a false discovery rate <.05.
Abbreviations: BCS, body condition score; USG, urine-specific gravity; iCa, ionized calcium.

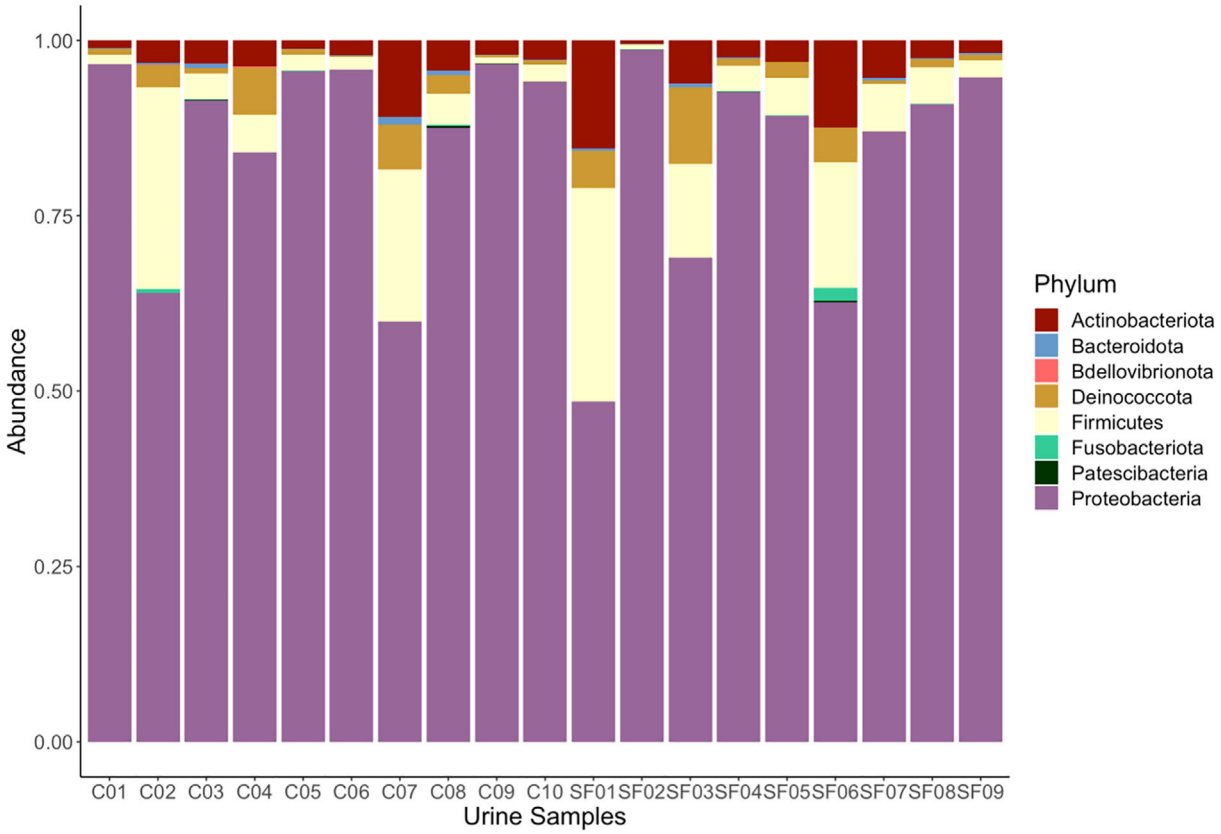

FIGURE 2 Barplots of bacterial phyla present in the urogenital microbiome of urolith-free control Miniature Schnauzers (n = 10, C01-C10) and Miniature Schnauzers with a history of CaOx urolithiasis (n = 9, SF01-SF09). Phylum-level bacterial relative abundance of individual urine samples. Samples C01 through C10 represent urine samples from control dogs. Samples SF01 through SF09 represent urine samples in the case group. Eight bacterial phyla are represented across samples.

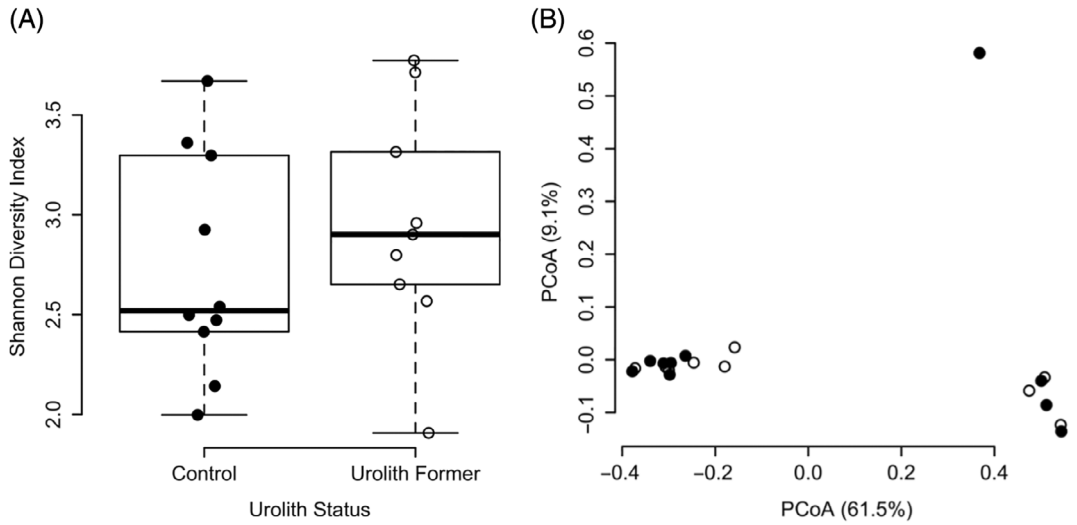

FIGURE 3 Alpha and beta diversity of the urogenital microbiome in urolith-free control Miniature Schnauzers (n = 10) and Miniature Schnauzers with a history of CaOx urolithiasis (n = 9). Urolith-free controls are represented by closed circles, and urolith formers are represented by open circles. A, Boxplot of alpha diversity as measured by the Shannon diversity index is reported for control and urolith former groups ($P = .28$). B, Principal coordinates analysis (PCoA) plot of beta diversity as measured by Bray Curtis dissimilarity matrix is reported for control and urolith former groups ($P = .85$). In (A), the boxes represent the 25th and 75th percentiles and whiskers represent 1.5 times the interquartile range. In (B), the proportion of variation explained by each axis is listed in parentheses.

and these sequences were removed from the dataset before downstream analysis (Table S1). After bioinformatics processing and filtering, 266 unique ASVs were present among all samples (mean, 157 802 ± 43 666 reads/sample), composed of 8 phyla and 219 genera. The most common phylum was Proteobacteria, followed by Firmicutes and Actinobacteriota. This distribution was consistent in both case and control groups (Figure 2; Supplemental Figure 1). Consistent with other studies of dogs, urine from both groups contained several genera previously reported as potential uropathogens, including *Escherichia-Shigella*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Pseudomonas*, *Neisseria*, *Sphingomonas*, *Actinomyces*, *Anaerococcus*, *Acinetobacter*, *Stenotrophomonas*, *Moraxella*, and *Roseomonas*.[21,46-58]

Alpha diversity was determined using the Shannon diversity index (Figure 3A), inverse Simpson diversity index (Supplemental Figure 2A), and observed richness (Supplemental Figure 2B). No differences were detected between cases and controls with any alpha diversity metric (Shannon diversity index, $P = .28$; inverse Simpson diversity index, $P = .55$; observed richness, $P = .87$). Overall bacterial composition across groups was evaluated using Bray Curtis dissimilarity matrix (Figure 3B), Weighted UniFrac (Supplemental Figure 3A), and Unweighted UniFrac (Supplemental Figure 3B). No statistically significant differences were observed between cases and controls (Bray Curtis, $P = .85$, $R^2 = .02$; Weighted UniFrac, $P = .92$, $R^2 = .02$; Unweighted UniFrac, $P = .71$, $R^2 = .05$).

Two distinct clusters were observed on beta diversity plots: Bray Curtis (Figure 4A, $P = .001$, $R^2 = .66$), Weighted UniFrac (Supplemental Figure 4A, $P = .001$, $R^2 = .58$), and Unweighted UniFrac (Supplemental Figure 4B, $P = .001$, $R^2 = .14$). Additional analyses then were performed to investigate these clusters, referred to as subtypes 1 and 2. These groups contain both cases and controls within the clusters. The proportion of cases was 6/12 in subtype 1, compared to 3/7 in subtype 2 ($P = 1$). Subtype 2 demonstrated significantly higher alpha diversity than subtype 1 for both the Shannon diversity index (Figure 4B, $P = .007$) and inverse Simpson diversity index (Supplemental Figure 5A, $P = .009$), but not observed richness (Supplemental Figure 5B, $P = .42$). Subtype 1 was more heavily dominated by Proteobacteria, whereas subtype 2 demonstrated relatively lower proportions of Proteobacteria and relatively higher proportions of Firmicutes, Actinobacteriota, and

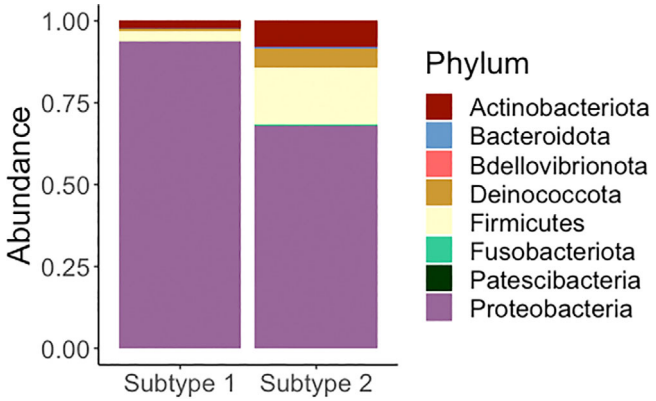

FIGURE 5 Barplots of bacterial phyla present in the urogenital microbiome of Miniature Schnauzers in subtypes 1 and 2. Phylum-level bacterial relative abundance grouped by subtypes 1 and 2. Eight bacterial phyla were represented in these samples. Subtype 2 demonstrated increased relative abundance of Firmicutes, Deinococcota, and Actinobacteriota compared to subtype 1.

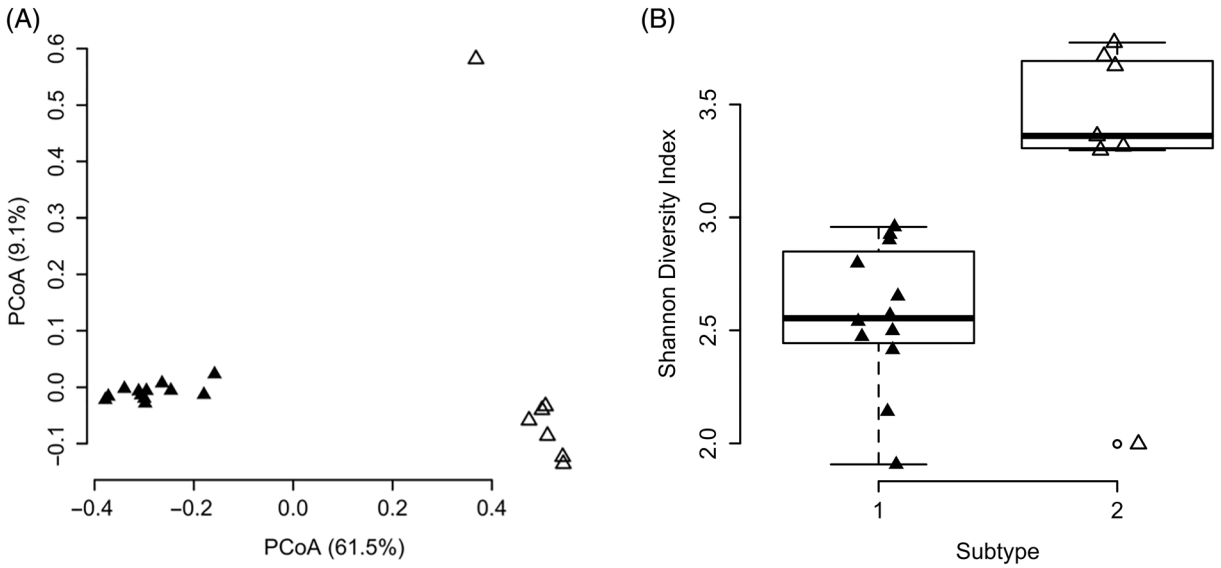

FIGURE 4 Alpha and beta diversity of the urogenital microbiome of Miniature Schnauzers with microbiome subtypes (subtypes 1 and 2). Subtype 1 is represented by closed triangles, and subtype 2 is represented by open triangles. A, Principal coordinates analysis (PCoA) plot of beta diversity as measured by Bray Curtis Dissimilarity Matrix is reported for subtypes 1 and 2 groups ($P = .001$). B, Boxplot of alpha diversity as measured by the Shannon Diversity Index is reported for microbiome subtype groups, subtypes 1 and 2 ($P = .007$). In (A), the proportion of variation explained by each axis is listed in parentheses. In (B), the boxes represent the 25th and 75th percentiles and whiskers represent 1.5 times the interquartile range.

TABLE 2 Results for univariate analysis of variables related to microbiome subtype (subtypes 1 vs 2)

Variable	Subtype 1 (n = 12)	Subtype 2 (n = 7)	*P* value (subtypes 1 vs 2)	FDR-adjusted *P* values (q-values)
Age (years)	10.4 ± 1.4	10.0 ± 1.4	.54	.83
Sex	1FS, 11MN	2FS, 5MN	.52	.83
Body weight (kg)	9.9 ± 3.4	9.3 ± 1.6	.63	.88
BCS (scale 1-9)	6.0 ± 1.5	5.7 ± 1.4	.68	.88
Sample volume (mL)	3.0 (1-9)	4.5 (1.5-10.5)	.28	.81
Storage duration (years)	5.3 (0.7-8)	6.2 (4.2-8)	.35	.81
DNA (ng/μL)	0.65 (0.3-8.7)	0.40 (0.1-2.6)	.10	.57
Sequence Reads	173 615 ± 33 258	130 696 ± 48 321	.07	.57
USG	1.025 ± 0.013 [10]	1.026 ± 0.013	.87	.92
Urine pH	6.9 ± 0.91 [10]	6.6 ± 0.89	.47	.83
Alkaline/acidic	5 acidic 5 alkaline [10]	6 acidic 1 alkaline	.30	.81
UCa:Cr (mg/mg)	0.037 (0.010-0.176)	0.043 (0.021-0.151)	.74	.88
iCa (mg/dL)	5.6 ± 0.27 [9]	5.6 ± 0.22 [6]	.93	.93
Creatinine (mg/dL)	0.80 (0.7-1.0)	0.90 (0.7-1.2)	.38	.81
BUN (mg/dL)	15.4 ± 4.2	10.9 ± 5.0	.07	.57
BG (mg/dL)	99.5 (97-115)	105 (100-124)	.15	.64
Triglycerides (mg/dL)	257 (52-973) [9]	292 (43-854) [6]	.78	.88

Notes: *P* values for Student's *t* tests, Wilcoxon rank-sum tests, or Fisher's exact test are reported. Normally distributed data is reported as the mean ±SD. Data that did not follow a normal distribution are reported as median (range). The number of samples available for measurement is listed in brackets if not performed in all dogs. The Benjamini-Hochberg procedure was used to determine statistical significance, defined as a false discovery rate <.05.
Abbreviations: BCS, body condition score; BG, blood glucose; iCa, ionized calcium; UCA: Cr, urine calcium-to-creatinine ratio; USG, urine-specific gravity.

TABLE 3 Differentially abundant bacterial taxa by CaOx stone status

Organism	Group overrepresented	Indicator value	*P* value (indicator value)	*P* value (Wilcoxon rank-sum tests)
Acinetobacter	Cases	0.69	.005	.007
Geobacillus[a]	Cases	0.63	.02	.03
Geobacillus[a]	Cases	0.56	.004	.009
Hydrogenophaga	Cases	0.53	.04	.04
Sphingopyxis	Controls	0.84	.04	.15

Notes: Indicator value represents the product of the mean relative abundance and frequency of a bacterial taxon by experimental group. Permutation tests are performed to calculate statistical significance to determine the *P* value for the indicator value. Five taxa were identified that had indicator values >.5 and *P* values <.05 when differentiating case and control groups. The *P* values for Wilcoxon rank-sum tests were then calculated between case and control groups for these taxa.
[a]Two unique ASVs of the *Geobacillus* genus were identified as differentially abundant.

Deinococcota than subtype 1 (Figure 5; Supplemental Figure 6). Sample and dog metadata were evaluated to test if known specimen and dog variables contributed to the differentiation between subtypes 1 and 2 (Table 2). None of the tested variables were found to differentiate between subtypes 1 and 2.

3.3 | Differential abundance of specific bacterial taxa

Determination of differentially abundant taxa between experimental groups (cases and controls) and between observed clusters (subtypes 1 and 2) was performed using indicator species analysis in the R package *labdsv*. Five ASVs were differentially abundant (indicator value >.5, *P* for indicator value <.05) between cases and controls (Table 3; Figure 6; Supplemental Figure 7). *Acinetobacter* was the taxon most strongly associated with the urolith former group (Figure 6), followed by *Geobacillus* (2 distinct ASVs) and *Hydrogenophaga* (Supplemental Figure 7).

A total of 54 differentially abundant ASVs were present between subtypes 1 and 2, with 15 overrepresented in subtype 1 and 39 overrepresented in subtype 2 (Table S2). Increased frequency and abundance of the genera *Delftia* and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was observed in subtype 1, whereas subtype

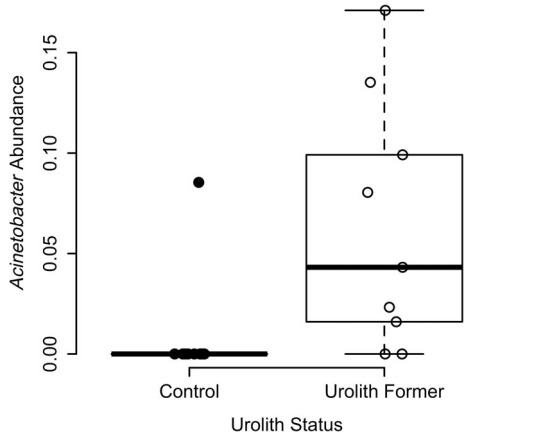

FIGURE 6 Relative abundance of *Acinetobacter* in urolith-free control Miniature Schnauzers (n = 10) and Miniature Schnauzers with a history of CaOx urolithiasis (n = 9). *Acinetobacter* was the most overrepresented taxon in the urine of cases as compared to urolith-free controls (indicator value = .69, *P* for indicator value = .005, *P* for Wilcoxon rank-sum test = .007). The boxes represent the 25th and 75th percentiles and whiskers represent 1.5 times the interquartile range.

2 demonstrated higher frequency and abundance of the genera *Diaphorobacter* and family *Comamonadaceae*. Exact indicator values and *P* values are presented in Table S2.

4 | DISCUSSION

Our results further confirm the presence of a urogenital microbiome in dogs. Although measures of alpha and beta diversity were similar between urine from CaOx urolith formers and urolith-free Miniature Schnauzers, several specific bacterial taxa differed in abundance between groups. Two of the discrepant taxa from our data also have been associated with urolith formation in studies of humans,[9,59] suggesting these individual taxa could be relevant to urolith formation, despite their low impact on overall microbiome composition. Whether these microbial alterations are a cause, consequence, or unrelated to urolith formation remains uncertain. Overall, these results provide important foundational data for delineating the role of the urogenital microbiome in CaOx urolithiasis in dogs. Two distinct microbiome clusters also were observed, which appear unrelated to processing of urine specimens, clinical factors, or urolith status. The clinical implication of this finding requires additional exploration.

Taxonomic profiles in the study urine samples were dominated by members of the Proteobacteria phylum, which is consistent with previous findings in both dogs and cats.[19,20,60] In contrast, the genera *Lactobacillus* and *Gardnerella* commonly dominate urotypes of women.[25,61,62] These differences might be a result of variations in urine collection method, anatomic or hormonal differences between species, or other biological or environmental factors. Several bacterial genera with uropathogenic potential were present across study samples, even in urine from healthy dogs. Because of inherent limitations of short amplicon sequencing, ASVs for these genera rarely achieved species-level taxonomic resolution. Thus, whether true pathogenic species or strains were present remains unclear. However, this finding is consistent with previous reports in dogs[19-21] and highlights the complexity of defining infectious pathogens given emerging awareness of commensal urinary microbes.

In our study, overall alpha and beta diversity in the urogenital microbiome did not significantly differ by urolith status. One potential explanation is that the current sample size was insufficient to detect bacterial community distinctions at large, which still could be biologically relevant. Importantly, only abdominal radiography was performed to screen for uroliths in control dogs. Computed tomography or ultrasonographic assessment would have provided a more sensitive detection method, particularly for small uroliths. If latent urolith formers were present in the control group, it would impede our ability to detect significant differences between groups. We attempted to decrease the risk of including latent urolith formers in the control group through the exclusion of dogs with hypercalciuria. Additionally, urine sample volumes were variable, collected by different individuals, and the heterogenous study population contained dogs with different diets, medications, and comorbidities. Complete diagnostic assessments, such as advanced urinary tract imaging, were not performed in all dogs, and consequently, relevant comorbidities may have gone undetected. Finally, to minimize confounders related to urogenital health, dogs with pyuria or bacteriuria were excluded. Only 3 dogs had urine culture performed at the time of sample collection, but the absence of bacteriuria and pyuria has a high negative predictive value for bacterial growth.[63,64] However, 2 dogs also lacked a urinalysis. Thus, another limitation of our study is the potential for inclusion of dogs with urogenital inflammation or culturable bacterial infections. These types of limitations could have introduced variables that confounded results, creating challenges in detecting factors driving larger microbiome differences between groups. Finally, CaOx urolithiasis is a complex disease, likely initiated by the interaction of multiple risk factors, and the microbiome might influence CaOx formation differently in certain subsets of dogs. For example, urogenital dysbiosis may not be a common feature in the Miniature Schnauzer breed, but might occur with CaOx urolithiasis in other breeds.

Despite similarities in overall microbial composition, the abundance of 5 bacterial taxa differed between case and control groups. Four taxa were over-represented in urolith formers, *Acinetobacter*, 2 unique ASVs of *Geobacillus*, *Hydrogenophaga*, and 1 taxon was overrepresented in urolith-free control dogs, *Sphingopyxis*. Interestingly, both *Acinetobacter* and *Geobacillus* also have been reported to be significantly overrepresented in the urine of human urolith formers,[9] and in 1 study, *Acinetobacter* was the single most discriminatory taxon in the urine of men with calcium-based uroliths.[59] This genus is also overrepresented in the urine of humans with obstructive urinary retention secondary to both uroliths and uroepithelial tumors, and even has been directly isolated from nephroliths of humans.[65,66] The independent association of specific organisms with urolithiasis across species supports a potential role of core taxa, such as *Acinetobacter*, in disease pathogenesis. *Acinetobacter* secretes oxalic acid

during experimental lecithin degradation and can contribute to mineralization of organic compounds in soil, but whether these properties translate into lithogenic risk in the urine remains unclear.[67,68] *Acinetobacter* also serves as an opportunistic uropathogen with a complex array of virulence factors.[69] This genus has been reported in the urine of febrile humans with upper urinary tract uroliths and as a cause of urinary tract infections in both humans and dogs,[50,70] but no clinical indicators of infection were observed in these study dogs. Additional mechanistic studies are necessary to explore whether certain microbes, including *Acinetobacter*, mediate the lithogenic potential of urinary microenvironments, particularly microbes associated with urolithiasis in multiple studies and across species. In addition, culture-based and metagenomic approaches could further elucidate the true uropathogenic potential of *Acinetobacter* at the strain level. Expanded quantitative urine culture (EQUC) is a sensitive urinary culture technique that can be used as a complement to amplicon sequencing.[71,72]

The presence of differentially abundant microbes does not prove a causal relationship with disease phenotype. Although these organisms might promote or protect against urolith formation, several additional explanations could be proposed. For instance, the abundance of specific microbes might shift in response to an altered urinary environment induced by uroliths or urolith management strategies. In the study samples, mean USG was lower in the case group. This finding has been reported previously,[73] and a lower USG in urolith formers is expected given that increased water consumption is a standard recommendation in the management of CaOx urolithiasis in dogs.[74] Thus, urolith management can modify the urinary environment in a way that might alter the microbiome. This issue highlights the difficulty in discriminating which microbiome alterations occurred before urolith formation and might truly serve as risk factors for urolithiasis. Furthermore, our study utilized 16S rRNA short amplicon sequencing, which is a cost effective and straightforward method for taxonomic characterization of microbial communities. However, shotgun metagenomics, urinary metabolomics, and other multi-omics approaches would provide a more comprehensive overview of the behavior and functional capacity of these microbes, which would help expose potential mechanistic explanations for disease causation. The viability of detected organisms also cannot be verified using amplicon sequencing alone. Pairing microbial DNA detection with EQUC or other culture-based methods would provide more insight into the viability of these microbial populations in future studies.

An unexpected finding of our study was the presence of 2 microbiome subtypes that were independent of urolith status. Examination of several technical variables related to sample storage, handling, and processing, did not identify any significant differences to explain this clustering. Additionally, none of the individual dog variables that were investigated differed between these clusters. Thus, we propose 3 potential explanations for this discovery. First, this difference could be driven by a variable that was not investigated because of missing data. For instance, specific dietary formulations and certain urinary biochemical properties, such as urinary citrate and oxalate concentrations, were often unavailable. Additionally, short-term medication histories were required for all dogs, but long-term antimicrobial histories were often unavailable. This deficiency constitutes an important limitation of our study. The long-term implication of antimicrobials on the urogenital microbiome remains unclear, raising the possibility that antimicrobials administered before the month leading up to urine collection could have lasting effects on the microbiome. Therefore, despite screening for recent antimicrobial exposure, these medications still represent a potential driver of microbiome subtypes in this population. The second proposed explanation for these groupings is related to the small number of dogs included in our study. The patient population was subject to strict inclusion and exclusion criteria to minimize confounders, which resulted in small sample size. Therefore, our study may have been underpowered to detect significant differences in the variables we examined (Table 2) that could be driving the formation of these 2 groups. The final explanation for these subgroups is that distinct urinary subtypes (ie, urotypes) may exist in Miniature Schnauzers unrelated to urolith status, similar to the enterotypes previously detected in the gut microbiome.[75] Various urotypes have been identified in the urine of healthy men and women,[61,62,76,77] and more recently, in cats.[60] Because the study population was restricted to Miniature Schnauzers, it is also possible that such urotypes are unique to the breed. Given that these clusters were identified in a small population comprised only of a single breed, such urotypes should be validated in larger and more diverse datasets.

Another limitation of the study is that low microbial biomass of urine specimens renders them particularly vulnerable to contamination and technical biases, and such issues are even more challenging to control retrospectively. Recent consensus recommends using nucleic acid preservatives in urine samples to maximize microbial yield.[28] A preservative was not used in our study. Although other urine and urogenital microbiome studies in dogs and cats have been performed without such preservatives,[19,20,60] it is unknown how the lack of preservative affected the results of our study. Careful sample selection, uniform processing of the specimens, and stringent bioinformatic removal of putative contaminants were employed to mitigate technical biases.

In conclusion, midstream voided urine samples from Miniature Schnauzers with CaOx urolithiasis in our study had a global microbial composition similar to that of samples from urolith-free, breed-matched controls. However, several bacterial taxa varied in abundance according to urolith status. The association of these taxa with disease is concordant with findings in humans with calcium-based uroliths and might indicate a role of these microbes in the pathogenesis of urolithiasis. The discovery of 2 distinct subsets of the urogenital microbiome, independent of urolith status, was unexpected. No clear driver of these groups was identified, and it is possible that different urotypes exist in Miniature Schnauzers. Larger prospective studies will help determine the clinical utility of these results.

ACKNOWLEDGMENT

The work in this study was funded by the University of Minnesota's College of Veterinary Medicine Resident and Graduate Student Research Grant. Dr Coffey was supported by an institutional NIH T32 Comparative Medicine and Pathology Training Grant (University of

Minnesota, T32 OD010993-15). Partial support for Dr. Furrow was provided by an NIH ORIP K01 Mentored Research Scientist Development Award (K01-OD019912).

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Samples used in this study had been previously collected with IACUC approval (protocol #1207A17243, 1509-33019A, 1807-36213A).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Coffey EL, Gomez AM, Burton EN, Granick JL, Lulich JP, Furrow E. Characterization of the urogenital microbiome in Miniature Schnauzers with and without calcium oxalate urolithiasis. *J Vet Intern Med*. 2022; 36(4):1341-1352. doi:10.1111/jvim.16482