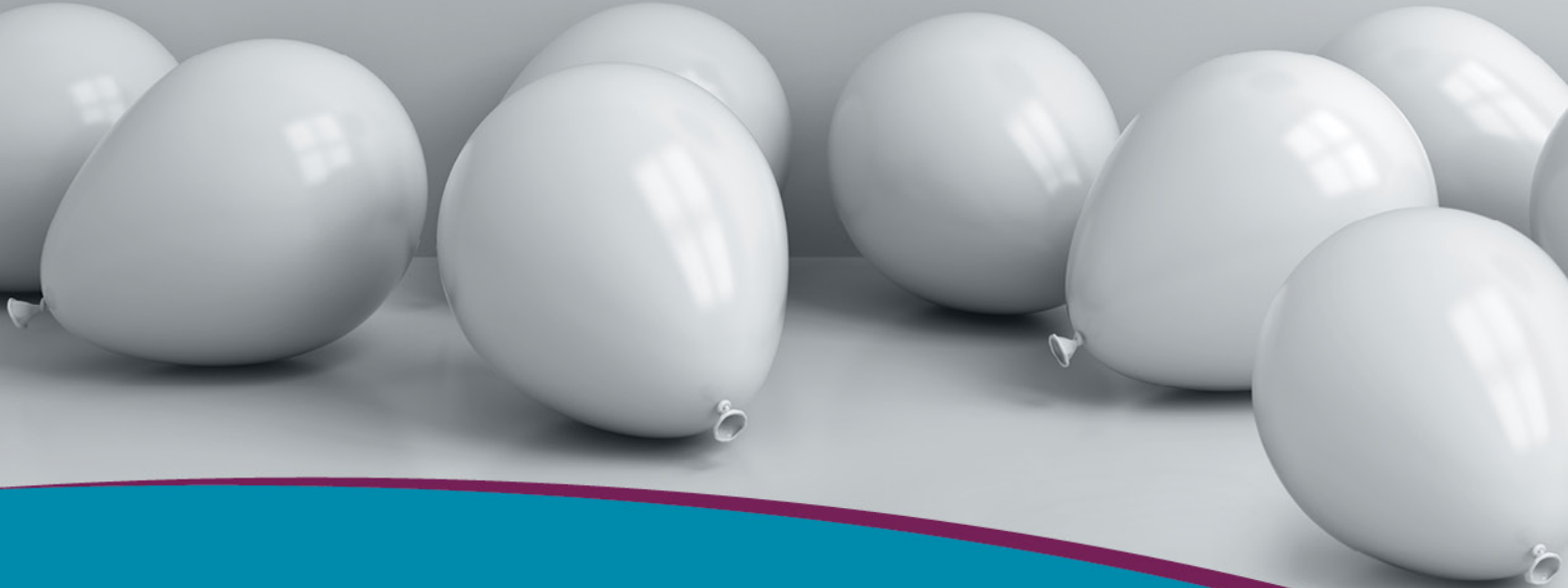


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Longitudinal examination of urine pH, specific gravity, protein, culture, and antimicrobial resistance profiles in healthy dogs

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Abstract

Background: Urine is routinely evaluated in dogs to assess health. Reference ranges for many urine properties are well established, but the scope of variation in these properties over time within healthy dogs is not well characterized.

Objectives: Longitudinally characterize urine properties in healthy dogs over 3 months.

Animals: Fourteen healthy client-owned dogs.

Methods: In this prospective study, dogs were evaluated for health; then, mid-stream free-catch urine was collected from each dog at 12 timepoints over 3 months. Urine pH, urine specific gravity (USG), protein, cultures, and antimicrobial resistance profiles were assessed at each timepoint.

Results: Urine pH varied within and between dogs over time (Friedman's test: within $P = .03$; between $P < .005$). However, USG, protein, and bacterial diversity of urine were consistent within dogs over time, and only varied between dogs (Kruskal-Wallis: between all $P < .005$). Antimicrobial resistant isolates were identified in 12 out of 14 dogs with 34 of 48 of the isolates demonstrating resistance to amoxicillin.

Conclusions and Clinical Importance: Urine pH should be assessed at multiple timepoints via pH meter before making clinical decisions. Mid-stream free-catch urine with high concentrations of bacteria ($>10^5$ CFU/mL) should not be considered the only indicator of urinary tract infection. Bacterial isolates from dogs in this study had widespread resistance to amoxicillin/oxacillin underscoring the need for antimicrobial stewardship.

KEYWORDS

antimicrobial resistance, canine, urine culture, urine protein, urine specific gravity

Abbreviations: CBC, complete blood count; CFU, colony-forming unit; IACUC, Institutional Animal Care and Use Committee; LDS, lithium dodecyl sulfate; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight mass spectrometry; MES, 2-(N-morpholino) ethanesulfonic acid; MHA, Muller Hinton Agar; TP, timepoint; USG, urine specific gravity; UTI, urinary tract infection.

Andrew McGlynn and Ryan Mrofchak contributed equally as first authors.

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1 | INTRODUCTION

Urine provides many insights into host health and is routinely included in clinical evaluations of dogs. Routinely evaluated urine properties include color, pH, urine specific gravity (USG), protein content, and the presence of chemical compounds such as ketones, bilirubin, and

glucose.¹⁻³ Urine can also be cultured and urine sediments evaluated for red and white blood cells, epithelial cells, and bacteria. Urinalysis aids in screening asymptomatic animals, and provides critical information for diagnostic evaluations of kidney damage, metabolic diseases (eg, diabetes), infection, stone formation, or other health conditions.^{1,3-5} Whereas reference intervals for most of these urine properties are well established, the scope and degree of variation within a healthy dog over time are less well defined.

Urine pH is linked to urinary tract infection (UTI) risk and stone formation risk in dogs and is monitored to assess response to diets designed to prevent stone formation.⁶⁻⁸ USG tracks concentrating ability of the kidneys and can serve as an indicator of diseases such as kidney disease and diabetes.^{1,3,9} The presence and type proteins in urine can also help identify kidney disease, urinary tract inflammation, and distinguish tubular from glomerular damage.^{1,10,11} However, these same urine properties—pH, USG, and urine protein profiles—are also affected by many factors other than disease, including diet, medications, and hydration status.^{1,3,12,13} Characterizing the range of variation in urine properties within a dog over time informs the clinical application and interpretation of these values.

Urine variability can also alter the niches available to commensal bacteria.¹⁴ Urinary tract commensals are thought to play a role in host health through immune stimulation, colonization resistance, and pathogen clearance.¹⁵⁻¹⁹ However, few studies have examined the urinary microbiota of dogs via sequencing or culture, and even fewer have evaluated change over time.^{15,18,20-24} Multiple studies report that dogs and humans share microbes, including urinary tract pathogens.²⁵⁻³³ Thus, assessing the urinary microbiota of healthy dogs and the associated resistance profiles over time is valuable both for evaluating dog health and for assessing implications for human health.

In this study, we evaluated urine pH, USG, urine protein profiles, and urine culture in 14 healthy dogs over 12 timepoints ranging from a few hours to a few months apart. We also compared pH as measured by dipstick to pH measured via pH meter. Finally, we phenotypically assessed antimicrobial resistance of isolates cultured from the urine of these healthy dogs against amoxicillin, ciprofloxacin, oxacillin, and nalidixic acid.

2 | MATERIALS AND METHODS

2.1 | Recruiting and enrollment

All dogs were recruited through the Ohio State University Veterinary Medical Center (IACUC: 2020A00000050). Each dog underwent a physical exam, serum chemistry, complete blood count (CBC), urinalysis, and urine culture prior to enrollment to assess health (Figure 1). All dogs were required to be between 1 and 10 years of age, have a body weight of at least 20 lbs (9 kg), be able to produce ≥ 10 mL of urine in a single urination, have a body condition score of 4 or 5, and be spayed or neutered.³⁴ Dogs were excluded if they had any history or signs of urinary tract disease, liver or kidney disease, skin infection, gastrointestinal disease, or urogenital abnormalities. Other exclusion

criteria included antibiotic use, chemotherapy, or radiation within 3 months of enrollment.

2.2 | Sample collection

Mid-stream free-catch urine samples were collected from 14 dogs (7 males, 7 females) over 12 timepoints between September 2020 and September 2021 (Table S1). The 12 timepoints included: Day 1 Morning (TP1), Day 1 Afternoon (TP2), Day 2 Morning (TP3), Day 2 Afternoon (TP4), Day 3 Morning (TP5), Day 3 Afternoon (TP6), End of Week 1 (TP7), End of Week 2 (TP8), End of Week 3 (TP9), End of Week 4 (TP10), End of Month 2 (TP11), and End of Month 3 (TP12). First-morning urine was collected for all timepoints except the 3 timepoints that were specifically aimed at collecting “afternoon” urine on Days 1, 2, and 3 (TP2, TP4, TP6). All urine samples were immediately placed on ice following collection and transported to the lab for aliquoting and processing within 6 hours of urination. Urine aliquots designated for pH and USG analysis were brought to room temperature before assessment.

2.3 | pH (meter vs dipstick)

Urine pH was assessed via pH meter (SevenEasy S20, Mettler Toledo, Columbus, Ohio) and dipstick (Chemstrip 9, Roche Diagnostics, Rotkreuz, Switzerland). The pH meter was calibrated before measurement using calibration buffer solutions with pH values of 4.00, 7.00, and 10.00. Following calibration, the pH meter probe was submerged in a urine sample until a stable pH reading could be obtained. To measure urine pH via dipstick, 1 drop (~ 50 μ L) of urine was placed on the dipstick square that evaluates pH. After 1 minute at room temperature, (per manufacturer instructions) the color of the square was matched to a manufacturer guide to assign a pH value to the sample.

2.4 | Urine specific gravity

USG was assessed using a refractometer (Reichert VeT 360, Depew, New York). The refractometer was calibrated before usage by placing 1 to 2 drops (~ 50 -100 μ L) of deionized, ultrapure water on the refractometer and adjusting to a specific gravity of 1.000 as necessary. Following calibration, 1 to 2 drops (~ 50 -100 μ L) of urine were placed on the refractometer and USG was recorded.

2.5 | Urine protein profiles

Urine protein was measured by dipstick (Chemstrip 9, Roche Diagnostics, Rotkreuz, Switzerland), and urine protein profiles were generated via gel as described previously in Hokamp et al. with a few modifications¹⁰. The gel apparatus used in this study was a Mini Gel Tank (Thermo Fisher Scientific, Waltham, Massachusetts), which was loaded with precast 4% to 12% Bis-Tris gels (Bolt, Thermo Fisher Scientific,

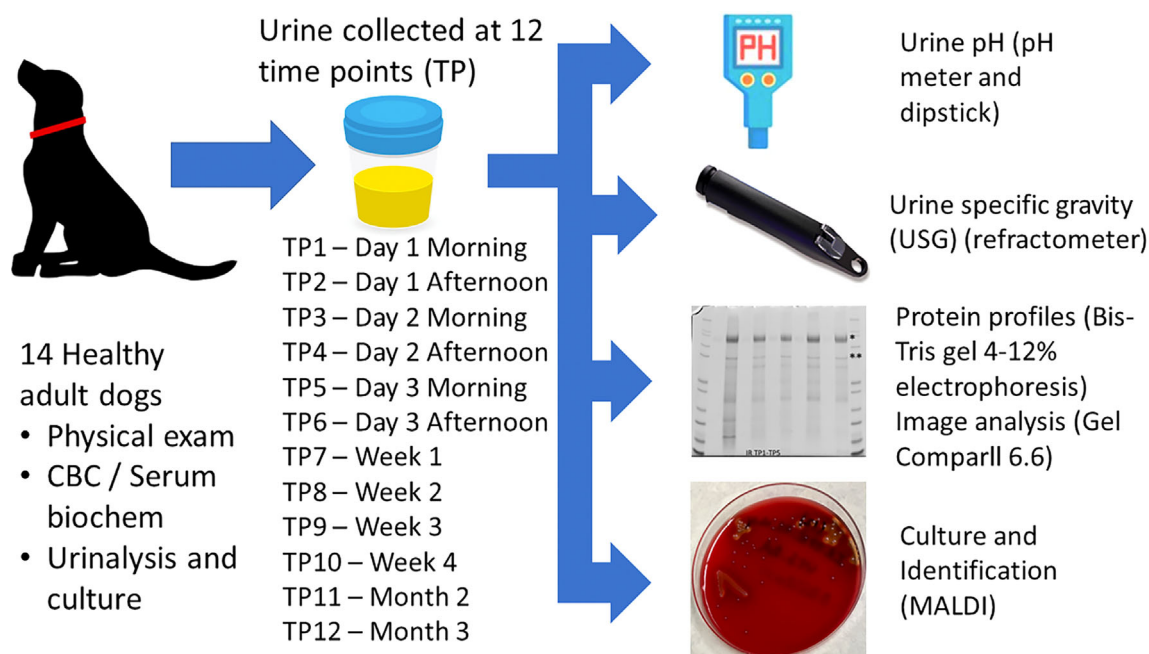


FIGURE 1 Experimental design.

Waltham, Massachusetts) and 2-(N-morpholino)ethanesulfonic acid (MES) running buffer solution (Bolt, Thermo Fisher Scientific, Waltham, Massachusetts). A standard ladder (Mark12, 2.5-200 kDa, Thermo Fisher Scientific, Waltham, Massachusetts) was run in lanes 1 and 12 of each gel. Each urine sample was run in 2 lanes. The first lane contained the USG-normalized urine samples, and in the second lane, referred to as the “MAX loaded” lane, urine was undiluted (not normalized based on USG). Electrophoresis was performed at 200 V for 30 to 32 minutes. Gels were subsequently stained, destained, and imaged on an Amersham Typhoon (GE Healthcare, Chicago, Illinois). Images of each gel were then analyzed based on densitometric curves (GelComparII 6.6, Applied Maths NV, Sint-Martens-Latem, Belgium) generated from protein bands in USG-normalized lanes. MAX-loaded lanes were used for confirmation of band location.

2.6 | Urine culture

Fifty microliters of each urine sample were vortexed and then aliquoted into a sterile 2.0 mL microcentrifuge tube. Samples were then vortexed briefly and centrifuged for 1 min. Ten microliters of each urine sample was then plated onto blood agar and MacConkey agar. All plates were then incubated aerobically at 37°C and checked for growth at 24 and 48 hours. Total viable colonies were counted, and all colonies with unique morphologies were picked and individually stored in a 75% glycerol solution at –80°C. Stored samples were later replated onto blood agar for 24 hours and then subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF; Bruker Corporation, Billerica, Massachusetts) for bacterial identification. Culture plates with mixed bacterial species were subsequently replated to establish pure cultures, before MALDI-TOF identification.

2.7 | Antimicrobial resistance profiles

A subset of isolates cultured across all dogs and timepoints were selected and grown on blood agar plates and incubated for 18 to 24 hours at 37°C. Colonies from each isolate were then picked and inoculated into 3 mL of double distilled water and normalized to a 0.5 McFarland standard at 625 nm with a turbidity range of 0.08 to 0.1. The normalized bacterial solution was then streaked on Muller Hinton Agar (MHA) supplemented with antimicrobials at breakpoint concentrations reported in dogs or specific to the urinary tract of dogs. Gram-negative isolates were cultured on 3 different MHA plates: 1 containing nalidixic acid (32 µg/mL), 1 containing ciprofloxacin (4 µg/mL), and 1 containing amoxicillin (8 µg/mL).³⁵⁻³⁸ Gram-positive isolates were cultured on 2 different MHA plates: 1 containing oxacillin (0.5 µg/mL), and 1 containing amoxicillin (8 µg/mL).³⁹ All plates were incubated for 18 to 24 hours at 37°C then checked for bacterial growth. Amoxicillin, a beta-lactam, was selected because it is a first-line antimicrobial for lower UTIs.⁴⁰ Oxacillin was selected to evaluate resistance against a second beta-lactam antibiotic. Ciprofloxacin, a fluoroquinolone, was selected because fluoroquinolones are the recommended alternatives to beta-lactams for UTIs in the case of beta-lactam resistance.⁴⁰ Nalidixic acid, a first-generation quinolone, was selected to parse potential quinolone versus fluoroquinolone resistance.

2.8 | Statistical analyses

All analyses were performed in R Studio version 4.1.0 and statistical significance was assessed at a *P*-value of .05. Data on urine pH, specific gravity, and protein profiles were tested for normality using a Shapiro-Wilks Test. To test for differences by dog and time, we employed Kruskal-Wallis, Pairwise Wilcoxon Rank Sum, and Friedman's test. To test

for differences by sex in normally distributed data, we employed linear mixed models (LMM) (R package: lme4). For non-normally distributed data, we used a penalized quasilielihood (PQL) model which is a generalized linear mixed model (GLMM; R package: MASS, function: glmmPQL). Sex was included as a fixed effect and dog as a random effect.

3 | RESULTS

3.1 | Study subjects

We collected mid-stream free-catch urine from 14 adult dogs including 7 neutered males and 7 spayed females (Table S1). There was no significant difference in age between males and females (Males: median = 4, range 1-7; females: 4.92, range 1-8; $P = .5$).

3.2 | pH within and between dogs over time

Urine pH was highly variable both within and between dogs. The median pH across all dogs and all timepoints as measured by pH meter was 6.44 (range: 5.32-8.93), and as measured by dipstick was 6 (range:

5.0-9.0). The largest pH ranges recorded in single dogs over time (12-time points) were 5.45 to 8.31 by pH meter in dog HF and 5 to 9 by dipstick in 3 dogs—ArB, FC, HF. The smallest pH ranges recorded in single dogs over time were 5.56 to 6.98 by pH meter in dog AbB, and by dipstick, 5 to 7 in 3 dogs (AbB, IR, LM), and 7 to 9 in 1 dog (GC). pH values from both meter and dipstick were not normally distributed (Shapiro-Wilk Normality Test, $P < .001$) and varied significantly between dogs (pH meter: Kruskal-Wallis, $P < .001$; dipstick: Kruskal-Wallis, $P < .001$, Figure 2A,B) and over time within dogs (pH meter: Friedman's, $P = .03$; dipstick: Friedman's, $P = .01$, Figure 2A,B). Dog GC had a significantly higher pH as measured by meter and dipstick than almost all other dogs (all Wilcoxon pairwise, $P < .05$; Tables S2 and S3). There was no significant difference in pH between males and females (meter: median female pH = 6.26, range 5.32-8.31, median male pH = 6.49, range 5.34-8.93, GLMM $P = .32$, dipstick: median female pH = 6, range 5-9, median male pH = 6, range 5-9, GLMM: $P = .42$).

3.3 | pH meter vs dipstick

Overall, urine pH measured via meter differed significantly from pH measured via dipstick (Kruskal-Wallis, $P = .04$). The median difference

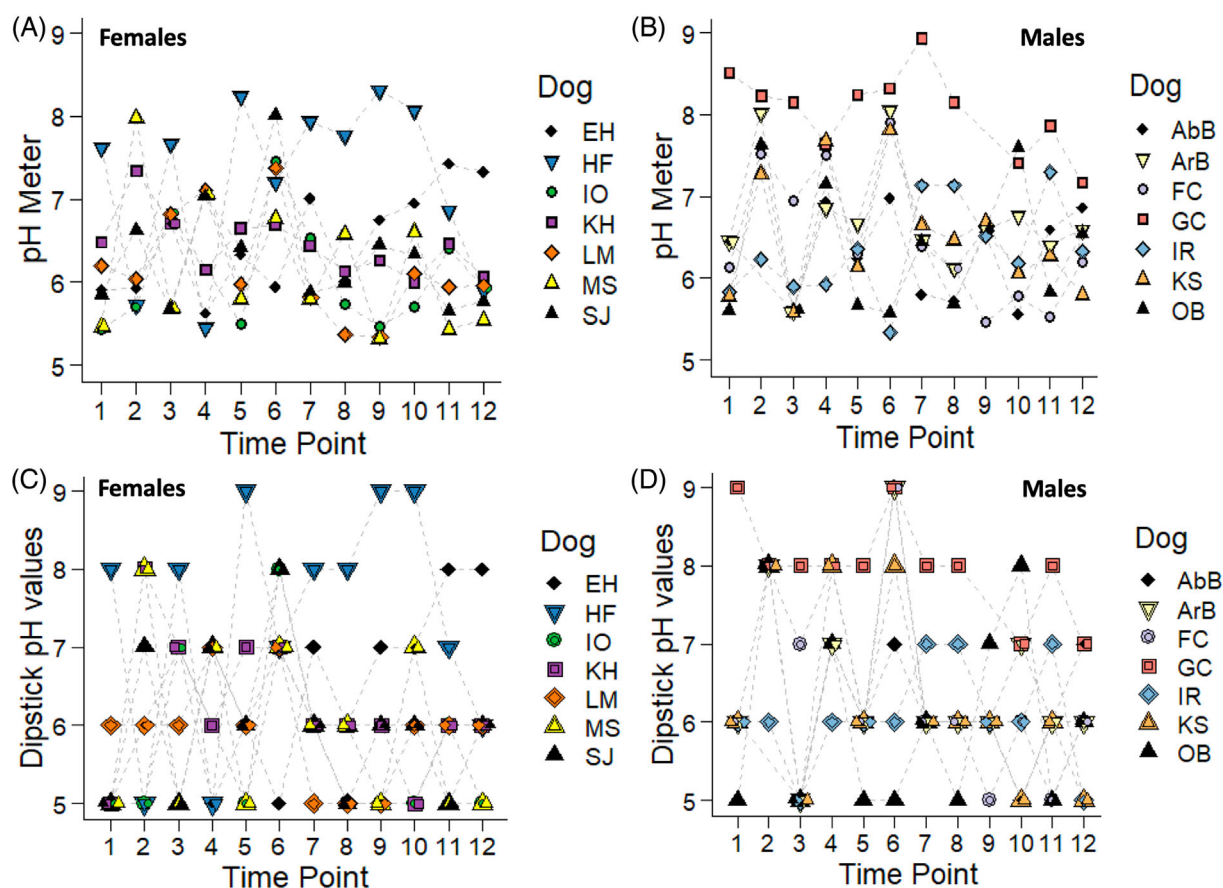


FIGURE 2 Urine pH of dogs over 12 timepoints. Urine pH as measured by pH meter in (A) female dogs ($n = 7$) and (B) male dogs ($n = 7$). Urine pH was measured by dipstick in (C) female dogs ($n = 7$) and (D) male dogs ($n = 7$). TP = Timepoint. TP1 = Day 1 Morning, TP2 = Day 1 Afternoon, TP3 = Day 2 Morning, TP4 = Day 2 Afternoon, TP5 = Day 3 Morning, TP6 = Day 3 Afternoon, TP7 = End of Week 1, TP8 = End of Week 2, TP9 = End of Week 3, TP10 = End of Week 4, TP11 = End of Month 2, TP12 = End of Month 3.

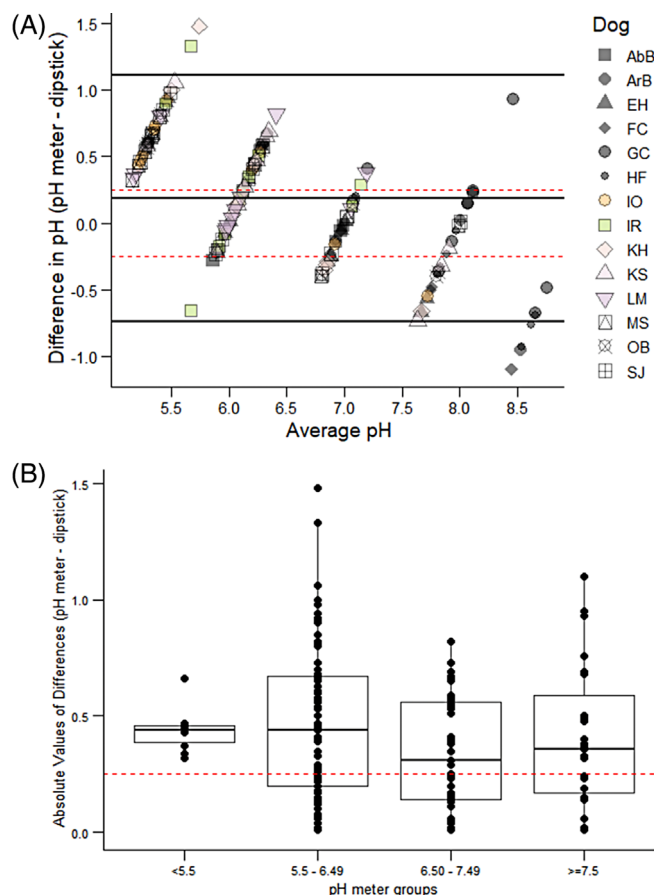


FIGURE 3 Comparison of urine pH as measured by pH meter vs dipstick. (A) Bland-Altman plot showing calculated differences in pH (pH meter pH – dipstick pH) of 14 dogs over 12 timepoints. Bold black lines represent the mean difference (0.187) and 95% CI interval. Dashed red lines represent the clinically significant threshold for differences between methods (0.25 and –0.25). (B) Box-and-Whiskers plot showing absolute differences in pH by group: pH <5.5, pH 5.5–6.49, pH 6.50–7.49, and pH ≥7.5. Dashed red line indicates the clinically relevant threshold for differences between methods (0.25).

(pH meter – dipstick pH = difference) across all samples was 0.2 (Figure 3) whereas the median absolute difference (absolute value of the difference) across all samples was 0.39 (mean absolute difference = 0.419). Outside of a neutral pH range (6.5–7.5), dipsticks were less accurate than the pH meter, and at a basic pH (pH > 7.5), dipsticks consistently overestimated pH. When pH was acidic (pH < 6.5), dipsticks consistently underestimated pH. To determine if specific pH ranges resulted in greater differences between pH meter and dipstick readings, we grouped samples into 4 categories based on pH meter: pH < 5.5 (n = 10 samples), pH 5.5–6.49 (n = 81 samples), pH 6.50–7.49 (n = 47 samples), and pH ≥ 7.5 (n = 27 samples). We then compared the absolute value of differences between pH measurement methods across these 4 groups. The lowest difference (most similarity) between pH meter and dipstick values was in the neutral range group (pH 6.50–7.49; median difference = 0.31, range 0.01–0.82). At pH values below 6.5 and above 7.5, the absolute differences between

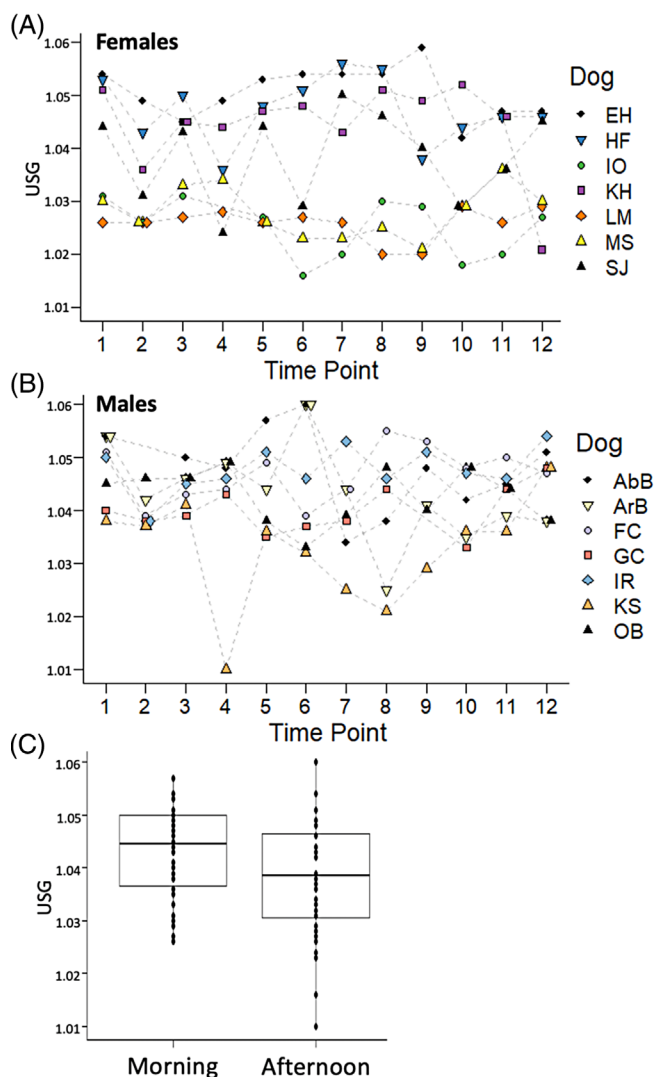


FIGURE 4 Urine specific gravity (USG) of dogs over time. USG of (A) female dogs (n = 7) and (B) male dogs (n = 7) over 12 timepoints. (C) USG values compared between first-morning urine and afternoon urine. TP = timepoint. TP1 = Day 1 Morning, TP2 = Day 1 Afternoon, TP3 = Day 2 Morning, TP4 = Day 2 Afternoon, TP5 = Day 3 Morning, TP6 = Day 3 Afternoon, TP7 = End of Week 1, TP8 = End of Week 2, TP9 = End of Week 3, TP10 = End of Week 4, TP11 = End of Month 2, TP12 = End of Month 3.

methods were greater (Figure 3B); although, average differences did not differ significantly between groups (Kruskal-Wallis, $P = .32$).

3.4 | USG differences between morning and afternoon

Healthy dogs generally exhibited limited variation in USG over time. The median USG across all dogs and all timepoints was 1.043 (range: 1.010–1.060). USG values were not normally distributed (Shapiro-Wilk Normality Test, $P < .001$) and varied significantly between dogs (Kruskal-Wallis, $P < .001$, Figure 4A,B), but not within dogs over time

(Friedman's, $P = .38$, Figure 4A,B). The median difference between minimum and maximum USG within dogs was 0.0165 (range: 0.009–0.038). Males generally exhibited higher USG although this difference was not significant (median female USG = 1.036, range

1.016–1.059; median male USG = 1.044, range 1.01–1.06, LMM $P = 0.38$). In pairwise comparisons, dogs LM, IO, and MS (all females) had significantly lower USGs than most other dogs (Wilcoxon pairwise, most $P < .05$, Table S4). To evaluate if USG values varied significantly between first-morning and afternoon urine, we analyzed a subset of samples from timepoints 1 to 6 for all 14 dogs. Morning USG values were higher (median = 1.042, range = 1.026–1.057) than afternoon USG values (median = 1.039, range = 1.01–1.06); although, this difference was not statistically significant (Friedman's, $P = .17$, Figure 4C), likely because of a relatively small sample size. These results suggest that within-day variation in USG was not as strong as the variation observed between different dogs.

3.5 | Urine protein profiles

Urine proteins were assessed via dipstick and 4% to 12% Bis-Tris gels. Dipstick values were semi-quantitative and included urine protein levels identified as negative, negative-trace, trace (<30 mg/dL), or positive (30 mg/dL). Average dipstick urine protein levels did not differ significantly by sex (t -test, $P = .85$, Table S4). For gels, total band number and relative surface area of protein bands were quantified. Total band number estimates protein richness or the number of different types of proteins present while relative surface area is a proxy for protein concentration.^{10,41} Total band number ranged from 0 to 6 across all samples, and differed significantly between dogs (Kruskal-Wallis test, $P < .001$) and by sex (Kruskal-Wallis, $P = .06$) but not within dogs over time (Friedman test, $P = .19$; Figure 5A,B; Table S6). The 2 most commonly detected protein bands displayed apparent molecular weights consistent with albumin (54.86–58.95 kDa) and Tamm-Horsfall protein (82.74–86.34 kDa).^{10,41} A few protein bands with apparent molecular weights between 9.10 and 47.31 kDa were also observed occasionally. The nature of these proteins is currently unknown, and they occurred infrequently and at low concentrations compared to albumin and Tamm-Horsfall. The relative surface area of protein bands consistent with albumin and Tamm-Horsfall differed significantly between dogs (Kruskal-Wallis test: albumin $P < .001$; Tamm-Horsfall $P < .001$; Tables S7 and S8), but did not differ significantly within dogs over time (Friedman's: albumin $P = .35$; Tamm-Horsfall $P = .61$). Average albumin and

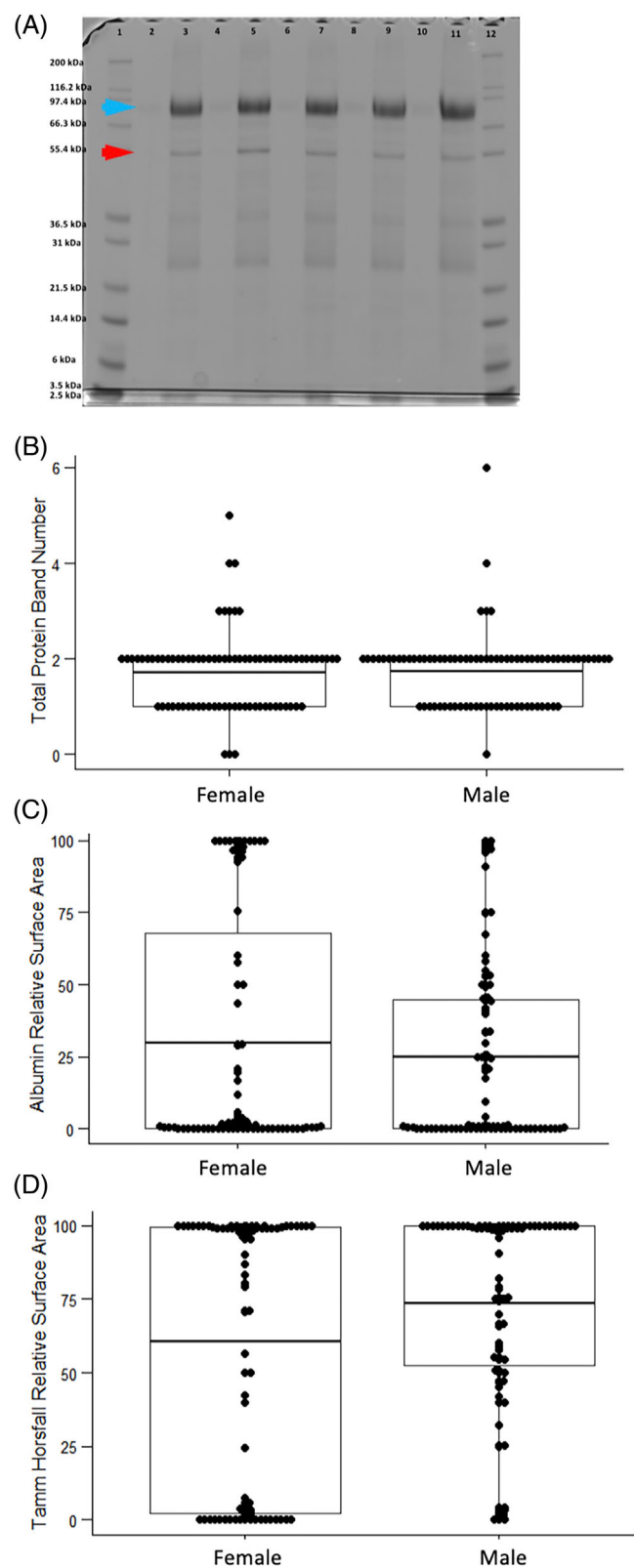


FIGURE 5 Urine protein profiles. (A) An example gel from dog KH. Lanes are numbered and ladders are included in lanes 1 and 12. Lanes 2 and 3 contain urine samples from TP6, Lanes 4 and 5 are samples from TP7, Lanes 6 and 7 are samples from TP8, Lanes 8 and 9 are samples from TP9, and Lanes 10 and 11 are samples from TP10. Lanes 2, 4, 6, 8, and 10 contain USG-normalized urine while lanes 3, 5, 7, 9, and 11 are “MAX Loaded” loaded lanes that contain urine not normalized by USG. Red arrow denotes protein bands ~58.95 to 54.86 kDa, which is consistent with albumin. Blue arrow denotes bands around ~86.34 to 82.74 kDa, which is consistent with Tamm-Horsfall protein. (B) Total band number, (C) Albumin concentration, and (D) Tamm-Horsfall concentration by sex ($n = 7$ females, 7 males).

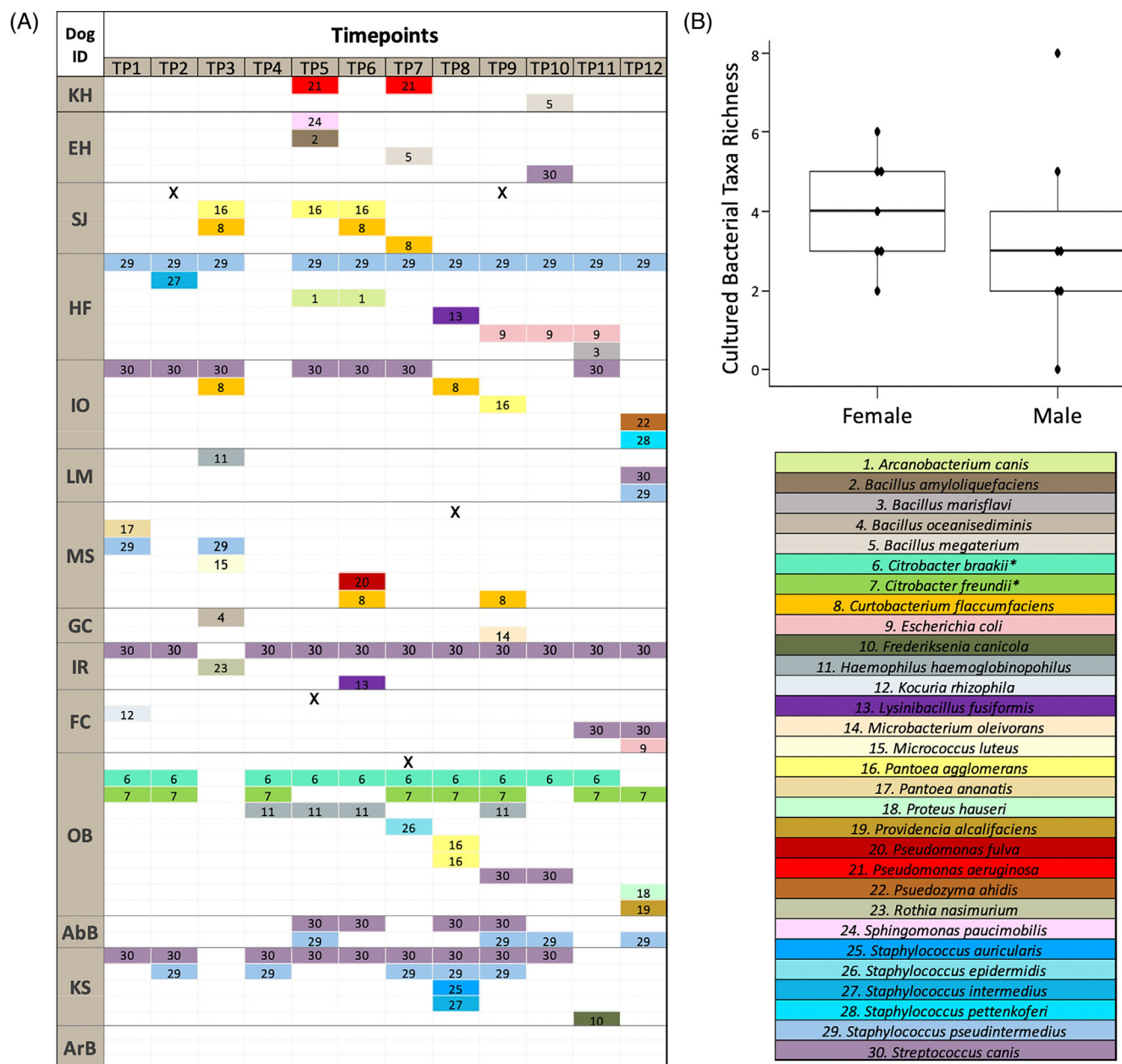


FIGURE 6 Urine bacterial culture results. (A) Bacterial species cultured by dog ($n = 14$) and over time. (B) Bacterial taxa richness by sex. X indicates that an organism was cultured but not able to be identified by MALDI-TOF. *Per MALDI: Citrobacter species are challenging to speciate and these taxonomic assignments are probable but not definite. TP = timepoint. TP1 = Day 1 Morning, TP2 = Day 1 Afternoon, TP3 = Day 2 Morning, TP4 = Day 2 Afternoon, TP5 = Day 3 Morning, TP6 = Day 3 Afternoon, TP7 = End of Week 1, TP8 = End of Week 2, TP9 = End of Week 3, TP10 = End of Week 4, TP11 = End of Month 2, TP12 = End of Month 3.

Tamm-Horsfall concentrations did not differ significantly by sex (albumin: median female = 31.6, range = 0.25-93.5; median male = 7.06, range = 0.6-73.7; GLMM $P = 0.83$; Tamm-Horsfall: median female = 59.8, range 4.7-00.7; median male = 84.6, range 26.8-99.4; LMM $P = .44$; Figure 5C,D). There was no significant correlation found between pH and total protein band number, albumin concentration, or Tamm-Horsfall concentration (total protein band number: $R = .057$, $P = .36$; albumin: $R = .067$, $P = .22$; Tamm-Horsfall: $R = -.0099$, $P = .86$; Figure S1).

3.6 | Urine cultures

Bacteria were cultured in 50% (85/168) of the urine samples collected over 12 timepoints in 14 dogs. The most commonly cultured bacteria were *Streptococcus canis* and *Staphylococcus pseudintermedius* (Figure 6A). Three out of 14 healthy dogs (IR, KS, HF) exhibited urine cultures with $>10^5$ CFU/mL, and 2 of these dogs (IR, HF) cultured $>10^5$ CFU/mL at more than 1 timepoint. In all but 1 case, these cultures were exclusively composed of *S. canis* or *S. pseudintermedius*. In dog HF,

1 time point (TP11) included a mixed culture of *S. pseudintermedius*, *E. coli*, and *Bacillus marisflavi*. There was no significant difference in the number of colonies observed at 24 or 48 hours on blood agar or MacConkey agar (Kruskal-Wallis: blood agar $P = .5$; MacConkey $P = .77$; Table S9). The presence or absence of aerobic culturable bacteria did not differ by sex (Fisher exact test, $P = 1$). Furthermore, the number of total bacterial taxa (richness) cultured aerobically in each dog did not differ significantly by sex (t-test, $P = .53$, Figure 6B) or timepoint (Friedman test, $P = .32$), but did differ significantly by dog (Friedman test, $P < .001$). Specifically, dog OB's cultures exhibited significantly greater bacterial diversity than most other dogs in this study, and *Citrobacter* spp. were consistently cultured at 11/12 (91.7%) timepoints in OB. The 8 most common taxa cultured at multiple timepoints and in multiple dogs were *Streptococcus canis*, *Staphylococcus pseudintermedius*, *Curtobacterium flaccumfaciens*, *Pantoea agglomerans*, *Haemophilus haemoglobinophilus*, *Escherichia coli*, *Lysinibacillus fusiformes*, and *Staphylococcus intermedius* (Table S10). However, most taxa were cultured intermittently at fewer than 5 timepoints, and not found as consistently as *Staphylococcus* or *Streptococcus* spp., except for *Citrobacter* spp. in dog OB.

3.7 | Antimicrobial resistance profiles

A total of 220 isolates were cultured across all dogs and timepoints. A subset of these isolates ($n = 48$) was then selected for antimicrobial resistance evaluation including every *Escherichia coli* ($n = 4$) and *Pseudomonas aeruginosa* ($n = 2$) isolate based on the association of these bacterial species with UTIs; 1 isolate from all other bacterial species identified in each dog; and, in some dogs from which we cultured the same bacterial species repeatedly, we selected the first and last isolate of that bacterial species cultured per dog.³⁵ Of the 48 selected isolates, 17 were gram-negative and 31 were gram-positive (Table S11). The 17 gram-negative isolates were cultured from 7 dogs (4 females, 3 males). Urine from OB grew the greatest number of unique gram-negative taxa ($n = 6$). All 17 (100%) gram-negative isolates were resistant to amoxicillin (at 8 $\mu\text{g/mL}$), and 3 of 17 (17.6%) were resistant to nalidixic acid (at 32 $\mu\text{g/mL}$). None of the gram-negative taxa were resistant to ciprofloxacin (at 4 $\mu\text{g/mL}$). The 31 gram-positive isolates were cultured from 13 dogs (6 males, 7 females). Seventeen out of 31 (54.8%) of the gram-positive isolates were resistant to amoxicillin (at 8 $\mu\text{g/mL}$), whereas 14 out of 31 (45.2%) were resistant to oxacillin (0.5 $\mu\text{g/mL}$). Eight gram-positive isolates were resistant to both oxacillin and amoxicillin whereas 15 isolates were resistant to either oxacillin or amoxicillin but not both. Seven gram-positive isolates were susceptible to both oxacillin and amoxicillin. In a few cases, the same taxa from the same dog had differing resistance profiles over time. For example, in dog AbB, a *Staphylococcus pseudintermedius* at timepoint 5 (Day 3 Morning) displayed resistance to amoxicillin, but at timepoint 12 (End of Month 3), *S. pseudintermedius* from AbB was not resistant to amoxicillin. Differing taxa within the same dog at the same timepoint also displayed differing resistance profiles. For example, in dog KS at timepoint 8, a *Staphylococcus intermedius* demonstrated

resistance to both oxacillin and amoxicillin whereas *S. auricularis* present in the same dog at the same timepoint, was only resistant to amoxicillin. In total, we observed resistance against at least 1 antibiotic in 40 of 48 (83%) tested isolates, representing 12 of 14 healthy dogs (86%).

4 | DISCUSSION

In this study, we longitudinally evaluated urine pH, specific gravity, protein (via dipstick and gel), culture, and antimicrobial resistance profiles in 14 healthy dogs over a 3-month period. Urine pH varied significantly within and between dogs over time. However, USG, urine protein, and the number of taxa cultured from urine were consistent within dogs over time, and only varied significantly between dogs. Only 1 dog consistently cultured bacterial species other than *Staphylococcus* and *Streptococcus* spp. suggesting a urinary bacterial signature unique to this individual dog. The scope of this study was limited to routine urine culture techniques. However, further research using Enhanced Quantitative Urinary Culture (EQUC) might provide additional insights into viable urobiome taxa and the stability of these taxa within an individual over time.⁴² Evidence for antimicrobial resistance was identified in 12 out of 14 healthy dogs with the majority of isolates (34 of 48, 71%) demonstrating resistance to amoxicillin.

Urine pH was highly variable within and between dogs. This was not unexpected as urine pH is influenced by multiple factors including diet, disease, age of urine specimen, drug therapies, and bacterial types present in the urine/bladder.⁴³ Notably, while we specified "first morning urine" for all samples except Days 1, 2, and 3 afternoon samples, we did not specify or have owners record whether dogs were fed before sampling, which might also contribute to the intra-dog variability we observed in pH. While we did not control for diet in this study, all dogs were confirmed to be healthy based on physical exam, blood work, and urinalysis, and owners of dogs reported no signs of urinary tract disease in the 18 months after enrollment in this study (Table S12). We also processed all urine samples within 6 hours of urination, limiting the potential for pH changes because of specimen handling. Eight dogs (26 total samples accounting for multiple timepoints) exhibited a urine pH outside of, and specifically higher than, the urine pH range (5.0–7.5) for healthy dogs established by Chew et al.¹ This indicates that urine from healthy dogs can vary outside this range. Urine from 11 dogs also exhibited variation from acidic to basic—ranging from pH at or below 6 to pH at or above 7.25, indicating that urine pH was not consistently acidic or consistently basic in most dogs. In comparing pH meter vs dipstick, pH values differed significantly, with the meter producing higher values on average than the dipsticks; although, dipsticks tended to overestimate pH at basic pH values. Additionally, the average absolute difference between pH meter and dipstick was 0.419 (range 0.01–1.48), which exceeds a previously established clinically acceptable difference of 0.25 between pH measurement methods.^{5,44} Our results support previous findings indicating a poor concordance between pH meter and pH dipstick values.^{6,44,45} Because of the dynamic nature of urine pH

observed in healthy dogs, pH readings at multiple timepoints via pH meter are recommended before making clinical decisions (including differential diagnoses and treatment) involving urine pH management.

The USG values observed in this study (1.01–1.060) fall within an established USG reference range (1.010–1.070) for healthy dogs.⁴⁶ USG values were also relatively consistent within a dog over time, with a mean difference of 0.021 ± 0.009 , similar to a previous study that reported a mean difference of 0.015 ± 0.007 within dogs over a week.⁴⁷ Although USG can fluctuate based on factors like hydration status, our results indicate that a single USG measurement from a healthy dog will generally be representative of that dog's USG.⁴⁷ Unlike van Vonderen et al., we did not observe a significant difference in USG between first morning and afternoon urine; however, average USG was lower in the afternoon in our study and our sample size ($n = 14$ dogs) was small compared to the van Vonderen et al.'s study ($n = 89$ dogs) suggesting that temporal differences in USG are small but consistent.¹³ As such, first morning urine is still recommended for USG measurements. USG was higher (although not significantly) in males as compared to females. Higher USG in males has been reported in other species but not dogs.^{48–51} Notably, males in this study were, on average, but not significantly, younger than females (mean \pm SD: males 3.6 ± 2.5 , median = 4; females 4.5 ± 2.1 ; median = 4.92). There is a 0.001 unit decline in USG in the urine of dogs for each increasing year of age; thus, age might be contributing to the sex difference observed in USG here.⁴⁷

Like USG, urine protein profiles differed significantly between dogs but not within dogs over time. The 2 most commonly detected protein bands were consistent with Tamm-Horsfall protein and albumin. Tamm-Horsfall, a tubular protein involved in immune defense, and small amounts of albumin (≤ 30 mg/dL), a plasma protein, are considered normal findings in the urine of dogs.^{1,52–54} Differences in protein profiles between dogs might be driven by age or breed. Increased protein loss through urine is typically observed as dogs age and renal filtration function declines through irreversible nephron loss or glomerulosclerosis, which is more common in older animals.⁵⁵ Although unconfirmed, the number of nephrons per kidney might also vary between breeds, affecting renal filtration.⁵⁵ Whereas gel electrophoresis has been used in previous studies to investigate urine protein profiles in dogs with diseases including chronic kidney disease, pyometra, and leptospirosis, this is the first study, to the best of our knowledge that characterizes urine protein profiles in healthy dogs over time.^{10,56,57}

Urine culture results were, dominated by skin-associated microbes (*Staphylococcus* and *Streptococcus* spp.), as urine was collected midstream free-catch.⁵⁸ In general, cystocentesis or catheterization is recommended for culturing to avoid skin and genital contaminants. In addition to skin microbes, we also observed several potential urinary tract pathogens in culture including *E. coli* and *P. aeruginosa*. However, these taxa, at low abundances, can be part of the normal urinary tract microbiota in dogs, and the presence of these taxa in asymptomatic individuals does not warrant treatment.^{15,23,24,59} Notably, 3 dogs cultured high concentrations bacteria ($>10^5$ CFU/mL)

at least once. This bacterial concentration in midstream free-catch urine (10^5 CFU/mL) has been considered suggestive of UTI, but the bacteria we observed at these concentrations were generally skin commensals (*S. pseudintermedius*, *S. canis*).⁶⁰ Additionally, all of these dogs remained healthy, asymptomatic, and never exhibited pyuria during and after the study (Table S11). These results demonstrate the potential for high-level contamination in free-catch urine from healthy dogs, as noted previously.³⁵

In relation to antibiotic resistance profiles, 86% of the dogs (12 out of 14) and 83% of the isolates demonstrated resistance to beta-lactams (amoxicillin, oxacillin, or both). Only 3 gram-negative isolates—all *Pseudomonas* spp.—displayed resistance to quinolones (nalidixic acid), and no gram-negative isolates demonstrated resistance to fluoroquinolones (ciprofloxacin). *Pseudomonas* species are commonly resistant to quinolones and fluoroquinolones.^{61,62} The absence of resistance to fluoroquinolones was considered positive as these drugs are typically reserved for beta-lactam-resistant infections.^{35,40} However, the overwhelming resistance to beta-lactams was concerning considering the source: Healthy dogs that had not received any antibiotics in at least 3 months, with most not having received antibiotics for over a year or more. Other studies have reported similarly widespread resistance to beta-lactams in bacterial isolates from dogs, including isolates from healthy dogs.^{63–65} Amoxicillin is one of the most commonly used antibiotics in veterinary medicine and is the most frequently prescribed antibiotic for UTIs in dogs in the United States.^{66,67} The common presence of amoxicillin-resistant bacteria isolated in the urine of healthy dogs raises several questions: Is resistance being promoted through prior exposure to a beta-lactam given frequency of use? Is resistance being acquired or transferred from the dog's environment (eg, soil, water, diet) or from other hosts (eg, other pets or humans within a household) as it has already been established that dogs and humans can share urinary tract microbes and pathogens?^{25–33} Given that amoxicillin/beta-lactams are also used to treat a variety of human infections, what are the public health implications of high resistance burdens in healthy dogs that share our households?

5 | CONCLUSIONS

This study is a comprehensive examination of urine from healthy dogs including pH, USG, protein, culture, and resistance profiles. Key takeaways from this study on healthy dogs include (1) Urine pH varied widely over time indicating that pH should be assessed at more than 1 timepoint via pH meter before making clinical decisions based on pH. (2) USG and protein results were relatively stable over time, suggesting that measurement of these properties at a single timepoint can portray an accurate representation of that dog's true values. (3) Mid-stream free-catch urine from multiple healthy dogs yielded high concentrations of bacteria in culture ($>10^5$ CFU/mL) confirming that free-catch urine can be highly contaminated and such concentrations of skin bacteria in asymptomatic dogs should not be considered an indicator of a UTI. (4) Urine bacterial isolates demonstrated

widespread resistance to amoxicillin and oxacillin underscoring the critical need for antimicrobial stewardship in practice.

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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

Approved by The Ohio State University College of Veterinary Medicine IACUC, #2020A00000050.

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.

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