Received: 21 May 2018 | Accepted: 16 November 2018

DOI: 10.1111/jvim.15389

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

STANDARD ARTICLE

The fecal microbiome and serum concentrations of indoxyl sulfate and p-cresol sulfate in cats with chronic kidney disease

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Funding information
Buttons Fund For Feline Chronic Kidney Disease Research, Grant/Award Number: N/A; University of Colorado Cancer Center Shared Resource Support Grant, Grant/Award Number: P30CA046934

Background: Intestinal dysbiosis has been documented in humans with chronic kidney disease (CKD) and is thought to contribute to production of the uremic toxins indoxyl sulfate (IS) and p-cresol sulfate (pCS). Characteristics of the fecal microbiome in cats with CKD and correlation to serum concentrations of uremic toxins are unknown.

Objectives: To characterize the fecal microbiome and measure serum IS and pCS concentrations of cats with CKD in comparison to healthy older cats.

Animals: Thirty client-owned cats with CKD (International Renal Interest Society stages 2-4) and 11 older (≥8 years) healthy control cats.

Methods: Prospective, cross-sectional study. Fecal samples were analyzed by sequencing of 16S rRNA genes and *Escherichia coli* quantitative PCR (qPCR). Serum concentrations of IS and pCS measured using liquid chromatography tandem mass spectrometry.

Results: Cats with CKD had significantly decreased fecal bacterial diversity and richness. *Escherichia coli* qPCR showed no significant difference in bacteria count between control and CKD cats. Cats with stage 2 (P = .01) and stages 3 and 4 (P = .0006) CKD had significantly higher serum IS concentrations compared to control cats. No significant difference found between stage 2 and stages 3 and 4 CKD. The pCS concentrations were not significantly different between CKD cats and control cats.

Conclusions and Clinical Importance: Decreased fecal microbiome diversity and richness is associated with CKD in cats. Indoxyl sulfate concentration is significantly increased with CKD, and cats with stage 2 CKD may suffer from a similar uremic toxin burden as do cats with later stage disease.

KEYWORDS

chronic renal disease, dysbiosis, feline, microbiota, uremic toxin

1 | INTRODUCTION

The wide array of microorganisms that reside in the intestinal tract (gut microbiome) play an important role in maintaining host health, and alteration of the gut microbiome has been associated with many illnesses in humans including chronic kidney disease (CKD). The uremia associated with CKD has been shown to negatively impact the gut microbiome in humans and rats causing intestinal dysbiosis.[1,2] In

Abbreviations: ANOSIM, analysis of similarity; BUN, blood urea nitrogen; CKD, chronic kidney disease; EPO, erythropoietin; IRIS, International Renal Interest Society; IS, indoxyl sulfate; LEfSe, linear discriminant analysis effect size; OTUs, operational taxonomic units; PCoA, principal coordinate analysis; pCS, p-cresol sulfate; QA/QC, quality assurance/quality control; QIIME, Quantitative Insights Into Microbial Ecology; qPCR, quantitative PCR; SDMA, symmetric dimethylarginine; USG, urine specific gravity.

humans and rats with CKD, these changes shift the microbiota to a less diverse community that is dominated by certain bacterial families.[1] Other instigators of intestinal dysbiosis in CKD patients include frequent use of antibiotics,[3] dietary changes such as decreased fiber intake, and use of phosphate binders.[2,4–6]

The intestinal dysbiosis associated with CKD has been shown to exacerbate the accumulation of colonic-derived uremic toxins such as indoxyl sulfate (IS) and p-cresol sulfate (pCS) because toxin-producing bacterial families may be more prevalent in dysbiosis.[5,7–9] p-Cresol is generated from the partial breakdown of tyrosine and phenylalanine by many intestinal obligate or facultative anaerobes including the genera *Bacteroides*, *Lactobacillus*, *Enterobacter*, *Bifidobacterium*, and *Clostridium*.[5,7,10] Indoles are produced by the metabolism of dietary tryptophan by tryptophanase in intestinal bacteria such as *Escherichia coli* (*E. coli*), *Proteus vulgaris*, and *Bacteroides* spp.[5,7,11] Indole and p-cresol are absorbed into the blood, metabolized to IS and pCS in the liver, and excreted in the urine.[5,7] In people, the accumulation of IS and pCS in CKD has been associated with progression of the disease by inducing inflammation and damaging renal tubular cells,[12,13] promoting renal fibrosis,[14,15] and by stimulating progression of glomerular sclerosis.[16] These uremic toxins also contribute to morbidity and mortality by impairing the nervous system,[17] lowering erythropoietin (EPO) production,[18] altering bone turnover,[19,20] and increasing the risk of cardiovascular disease.[21–23]

In veterinary medicine, IS concentrations have been correlated to the severity of CKD in cats and dogs,[24] and were found to be an independent predictor for disease progression.[25] However, the role of pCS has not been explored in veterinary medicine. Various studies have evaluated the gastrointestinal microbiome of cats in health and in disease,[26–33] but the fecal microbiome in cats with CKD and its association with IS and pCS have not been characterized. The primary aim of our study was to characterize the fecal microbiome of cats with CKD in comparison to healthy control cats. A secondary aim was to measure serum IS and pCS concentrations in the same population of cats. We hypothesized that cats with CKD would have an altered fecal microbiome and higher serum concentrations of IS and pCS when compared to healthy control cats.

2 | MATERIALS AND METHODS

2.1 | Study design and selection of animals

This prospective cross-sectional study was performed at the Colorado State University Veterinary Teaching Hospital. All client-owned cats with CKD that presented between August 2016 and August 2017 were eligible for enrollment into the study. Cats were considered to have CKD based on a serum creatinine concentration >1.6 mg/dL and urine specific gravity (USG) <1.035 or an increased serum creatinine concentration on at least 2 time points (over at least 3 months) in addition to an increased symmetric dimethylarginine (SDMA) concentration (>14 ug/dL).[34] To be eligible for inclusion, CKD and control cats underwent a thorough evaluation that included a review of past medical record, complete physical examination (including body condition score), minimum database consisting of CBC, serum biochemistry panel, urinalysis, serum total thyroxine concentration, blood pressure, fecal flotation, SDMA (when USG > 1.035 and persistent serum creatinine concentration > 1.6 mg/dL), and urine protein:creatinine ratio (if urine dipstick testing detected at least 1+ protein). Physical examination and Doppler blood pressure were performed by a single trained individual for consistency. The CKD cats were staged based on the International Renal Interest Society (IRIS) guidelines.[34] A control population of older (≥8 years) clinically healthy cats was recruited from employees, students, and staff of the veterinary teaching hospital. Cats were considered healthy based on unremarkable client history and past medical record, physical examination, and normal laboratory test results including serum creatinine concentration <1.6 mg/dL and USG >1.035.

Exclusion criteria included a history of antibiotic, probiotic, or antacid administration (eg, omeprazole, famotidine) <6 weeks before enrollment. Other medications or supplements including mirtazapine, maropitant, PO potassium supplementation, glucosamine/chondroitin joint supplements, and transdermal methimazole were allowed. In addition, cats were excluded if they had a history of uncontrolled hyperthyroidism and known or suspected gastrointestinal disease including intestinal parasitism and food-responsive chronic enteropathy. A client questionnaire was provided to the owner and the following information was obtained: diet, current medications or supplements, medications or supplements administered in previous 3 months, appetite and fecal score at the time of enrollment, and frequency of vomiting. A table with descriptions of appetite, fecal, and vomiting scores is provided in Supporting Information (Table S1). The project was approved by the Institutional Animal Care and Use Committee at Colorado State University and all owners gave written informed consent before participation.

2.2 | Fecal microbiome analysis

A fresh fecal sample was collected by the owner and placed on ice until frozen within 24 hours of collection. The samples were stored at −80°C until analysis of the fecal microbiome by sequencing of the 16S ribosomal RNA (16S rRNA) gene as previously described.[35] Briefly, DNA was extracted following the manufacturer's instructions (Mo Bio Power soil DNA isolation kit, Mo Bio Laboratories, Carlsbad, California). The DNA then was amplified and sequenced (Illumina, Inc., San Diego, California) using primers 515 F (5-GTGC CAGCMGCCGCGGTAA-3) to 806 R (5-GGACTACVSGGGTATC-TAAT-3) at MR DNA Laboratory (Shallowater, Texas). Sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) v 1.9.[36] Sequences were filtered for chimeras with USEARCH. The remaining sequences were clustered into operational taxonomic units (OTUs) by using an open reference approach in QIIME against the 97% clustered representative sequences from the Greengenes v 13.8 database.[37] Before downstream analysis, sequences that were assigned to chloroplast, mitochondria, unassigned, and low abundance OTUs, containing <0.01% of the total reads in the dataset were removed. The samples were rarefied to an equal depth of 80 875 sequences per sample to account for unequal sequencing depth. The rarefaction depth was chosen based on the lowest sequence depth of samples. The sequences were deposited in

the National Institutes of Health Sequence Read Archive under accession number SRP117611.

To evaluate bacterial species diversity within the fecal samples, alpha diversity was measured with the Chao1 and Shannon diversity indices, and observed OTUs metrics. Chao1 and Shannon indices take into account the number of observed OTUs and the evenness of bacterial species distribution within the fecal sample. Species richness can be defined as the number of unique OTUs and can be used as a proxy for bacterial species within the fecal sample.

To compare the bacterial communities among samples, beta diversity was evaluated with the phylogeny-based weighted and unweighted UniFrac[38] distance matrix and visualized using Principal Coordinate Analysis (PCoA) plots. Weighted UniFrac gives importance to the abundance of a particular OTU present in the fecal sample and unweighted does not. Linear discriminant analysis effect size (LEfSe) was used to elucidate bacterial taxa and genes that were associated with healthy control cats or CKD cats. The LEfSe was used in the Galaxy workflow framework (http://huttenhower.sph.harvard.edu/galaxy/) with the parameters set at $\alpha = 0.01$, linear discriminant analysis score = 3.0. Quantitative PCR (qPCR) was used as described previously[39] to evaluate *E. coli* bacterial counts between healthy control cats and CKD cats.

2.3 | Assays for serum indoxyl sulfate and p-cresol sulfate concentrations

Blood was collected in sterile non-heparinized tubes and centrifuged at 5000 rpm for 5 minutes. Serum was harvested and samples were frozen and stored at −80°C until analysis by the Pharmacology Core at Colorado State University. Total IS and pCS serum concentrations were determined by liquid chromatography tandem mass spectrometry modified from a published method[40] using a Waters Sunfire C8 5 μm, 4.6 × 50 mm column with a Phenomenex C18 Filter Frit Guard Cartridge on the Agilent 1200 Series Binary Pump SL high-performance liquid chromatography system coupled to the 3200 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems Inc., Foster City, California).

The calibration curves were constructed by spiking standard IS (Alpha Asear, Haverhill, Massachusetts) and pCS (Tokyo Chemical Industries, Tokyo, Japan) solutions into normal serum collected from a clinically healthy cat that had normal kidney function. Narageninine (1000 ng/mL) was used as the internal standard (Sigma-Aldrich, St. Louis, Missouri). Assay performance for each batch was assessed utilizing at least 10% quality assurance. Quality assurance/quality control (QA/QC) samples were dispersed among unknown samples at concentrations representing low, mid, and high regions of the calibration curves, with acceptable batches showing <25% of QA/QC samples outside of the accepted level of 85% accuracy. The accuracy plus/minus coefficient of variation of QA/QC samples among the batches analyzed for this study were 93.7 ± 4.8% for IS and 92.5 ± 4.0% for pCS. Standard curves in spiked cat serum were linear over the range of 100-50 000 ng/mL for both IS and pCS. The linearity of the curves was greater than $r^2 = 0.99$ using $1/x^2$ weighting. As measured in cat serum, the lower limit of quantitation for this assay was based on the level of detection with >85% accuracy and a coefficient of variation <15%, and was determined to be 250 ng/mL for IS and 100 ng/mL for pCS.

2.4 | Statistical analysis

For statistical analysis between the stages of CKD, stages 3 and 4 CKD cats were combined given the few stage 4 cats that were enrolled in the study. For all analyses, a value of $P < .05$ was considered significant. Normality was assessed by the Shapiro-Wilk test.

Analysis of similarity (ANOSIM) test within PRIMER 6 software package (PRIMER-E Ltd., Luton, UK) was used to analyze significant differences in microbial communities between healthy control cats and CKD cats. R values (range −1.0 to 1.0) were computed to evaluate the strength of the factors on the fecal samples. R values closer to 1.0 indicate a difference between the study groups. The remainder of the microbiome data was analyzed using commercial software (JMP Pro 11, SAS Software Inc., Cary, North Carolina). Because most datasets did not meet the assumptions of normal distribution, comparisons between healthy and disease groups were determined using nonparametric Kruskal-Wallis tests (healthy cats and stage of CKD [stage 2, stages 3 and 4]) or a Mann-Whitney *U* test (healthy control cats versus CKD [all stages combined]) in Prism software (Prism 7, Prism Graphpad Inc., La Jolla, California). The resulting *P* values of the Kruskal-Wallis tests or Mann-Whitney *U* test were adjusted for multiple comparison using Benjamini and Hochberg's false discovery rate[41] at each taxonomic level. Post hoc Dunn's multiple comparison test was used to determine the bacterial taxa that were different between the groups. The OTU tables generated also were uploaded into Calypso,[42] a web-based application to evaluate the correlations between bacterial taxa and IS and pCS serum concentrations.

For IS and pCS concentrations, data were analyzed with Prism. Median IS and pCS concentrations between healthy control cats and CKD cats were compared using the Mann-Whitney *U* test or Student *t* test depending on normality. Healthy control cats and the stage of CKD (stage 2, stages 3 and 4) were compared using Kruskal-Wallis testing with Dunn's post hoc analysis. Spearman correlation coefficient (rho) was computed to evaluate the association between IS and pCS serum concentrations and the following variables: appetite score, fecal score, frequency of vomiting, body condition score, presence of proteinuria, exclusive feeding of prescription renal diet, number of unique OTUs, hematocrit, and serum creatinine concentration, blood urea nitrogen (BUN) concentration, and serum calcium, phosphorus, sodium, and potassium concentrations. Mann-Whitney *U* test was used to compare IS and pCS concentrations between cats that ate ≥75% of their food and cats that ate <75% of their food.

3 | RESULTS

3.1 | Animals

Thirty cats with CKD (17 cats had IRIS stage 2 CKD, 11 cats had IRIS stage 3 CKD, and 2 cats had IRIS stage 4 CKD) and 11 healthy older control cats were enrolled in the study. Fecal microbiome analysis was performed on healthy control cats (n = 11) and CKD cats (n = 30).

TABLE 1 Characteristics of study groups including healthy control cats, International Renal Interest Society (IRIS) stage 2 chronic kidney disease (CKD) cats and IRIS stages 3 and 4 CKD cats

	Healthy controls cats (n = 11)	CKD stage 2 (n = 17)	CKD stages 3 and 4 (n = 13)
Variable (reference interval)	Mean ± SD	Mean ± SD	Mean ± SD
Age (yr)	10.5 ± 1.3[a]	14.5 ± 3.2[b]	13.4 ± 3.9[b]
Hematocrit (32-47%)	41 ± 4[a]	36.5 ± 4[b]	34.5 ± 5[b]
Sodium (149-157 mEq/L)	153 ± 2.1	151 ± 1.2	154 ± 1.5
	Median (range)	Median (range)	Median (range)
BCS (1-9)	5 (4-8)	5 (2–8)	5 (3-7)
Fecal score (1-7)	2 (2)	2 (1-3)	2 (2-3)
Appetite score (0-4)	0 (0-1)[a]	1 (0-3)[b]	1 (0-3)[b]
Vomiting score (0-3)	0 (0-2)[a]	2 (0-3)[b]	1.5 (0-3)[c]
Creatinine (0.8-2.4 mg/dL)	1.2 (0.7-1.6)[a]	2 (1.6-2.6)[b]	3.2 (2.9-6.9)[b]
BUN (18-35 mg/dL)	24 (20-38)[a]	43 (20-60)[b]	52 (33-98[b]
Total calcium (9.2-11.1 mg/dL)	9.8 (9.1-11.3)[a]	10.1 (9.1-10.7)[b]	10.6 (10-14)[b]
Phosphorus (3.0-6.0 mg/dL)	4.3 (2.9-5.0)	3.7 (2.6-5.6)	4.4 (3.3-8.2)
Potassium (3.7-5.4 mEq/L)	4.6 (4.2-5.54)	4.5 (3.89-5.31)	4.5 (4-5.1)
Indoxyl sulfate (ng/mL)	1201 (202-2860)[a]	3370 (746-10 300)[b]	6385 (1020-27 600)[b]
p-Cresol sulfate (ng/mL)	2905 (901-7220)	6940 (34-30 600)	5300 (189-35 300)
Observed OTUs	2575 (1274-3208)[a]	2198 (1541-3092)[b]	2032 (1427-2511)[b]

Rows bearing a different superscript letter are significantly different from one another.
Abbreviations: BCS, body condition score; BUN, blood urea nitrogen; OUT, operational taxonomic unit.

Serum IS and pCS concentrations were measured in healthy control cats (n = 10) and compared to CKD cats (n = 28). An inadequate amount of serum was available to perform IS and pCS concentrations on 3 cats.

Descriptions of age, body condition score, clinical scores, hematologic, and biochemical variables of cats in each group (healthy cats, stage 2 CKD cats, and stages 3 and 4 CKD cats) are presented in Table 1. The healthy control cats were significantly younger than the CKD cats ($P < .001$). As expected, compared with the healthy control group, CKD cats had a significant increase in serum concentrations of creatinine ($P < .001$) and BUN ($P < .001$). Additionally, the CKD cats had significantly lower hematocrit ($P = .003$) and appetite scores ($P = .004$), and higher vomiting scores ($P = .009$) and serum total calcium concentration ($P = .04$) compared with healthy control cats. Stage 2 ($P = .008$) and stages 3 and 4 ($P = .007$) CKD cats had lower appetite scores compared to healthy control cats. Stage 2 CKD cats had higher vomiting scores compared to healthy control cats ($P = .005$). No difference was found in fecal scores, presence of proteinuria, and serum phosphorus, sodium, and potassium concentrations between groups. Healthy control cats were being fed variable commercial maintenance diets. Nine of 30 CKD cats ate only a commercial renal diet, 3of 30 CKD cats ate a combination of commercial renal diet and maintenance diet, and 18 of 30 ate variable commercial maintenance diets. Current medications at the time of enrollment for the CKD cats included transdermal mirtazapine gel (4 of 30 cats), amlodipine (4 of 30 cats), PO potassium supplementation (3 of 30 cats), and transdermal methimazole, alendronate, levothyroxine, maropitant, psyllium powder, aluminum hydroxide, glucosamine/chondroitin joint supplement, transmucosal buprenorphine, and polyethylene glycol (1 cat each). None of the healthy control cats were on medications at the time of enrollment.

3.2 | Fecal microbiome analysis

The sequence analysis yielded 5 085 660 quality sequences for all analyzed samples (n = 41) (mean, 124 040; range, 80 890-369 577). The samples were rarefied to a depth of 80 875 sequences per sample to account for unequal sequencing depth. When alpha diversity, as described by Chao1 and Shannon diversity indices, was compared between CKD cats and healthy control cats, the Chao1 diversity index was significantly lower in CKD cats (median, 4286; range, 2729-6171) when compared to healthy control cats (median, 5099; range, 2258-6633; $P = .03$). The Shannon diversity index was lower in CKD cats (median, 5.9; range, 4.1-7.3) compared to healthy control cats (median, 6.4; range, 3.8-7.4) but this finding was not statistically significant ($P = .06$). Species richness, as described by the number of unique OTUs, was significantly decreased in CKD cats (median, 2152; range, 1427-3092) when compared to healthy control cats (median, 2575; range, 1274-3208; $P = .03$; Figure 1). When the CKD cats were analyzed by IRIS stage and compared to healthy control cats, the Chao1 diversity index was significantly lower in stage 2 CKD cats and in stages 3 and 4 CKD cats compared to healthy control cats ($P = .03$). No significant differences were found in Shannon diversity index or species richness (number of unique OTUs) among stage 2 CKD cats, stages 3 and 4 CKD cats, and healthy control cats.

For evaluation of beta diversity, no significant difference was found in microbial communities between CKD cats and healthy control cats based on ANOSIM of weighted ($R = -0.08$) and unweighted ($R = 0.07$) UniFrac distances. Also, no significant differences in microbial communities were observed in CKD stages 2-4 compared to healthy control cats (weighted $R = -0.04$; unweighted $R = 0.06$). The PCoA plots are provided in Supporting Information (Figure S1).

When individual bacterial groups were analyzed based on LEfSe, several bacterial taxa were identified as being significantly different

among the groups. When comparing healthy control cats to CKD cats, CKD cats had significantly lower relative abundances of the genera *Holdemania*, *Adlercreutzia*, *Eubacterium*, *Slackia*, and *Mogibacterium*. When comparing healthy control cats to stage 2 CKD cats, the genera *Eubacterium* and *Adlercreutzia* were less abundant and the genus *Prevotella* was more abundant in stage 2 CKD cats. When healthy control cats were compared to stages 3 and 4 CKD cats, the genera *Adlercreutzia* and *Eubacterium* were less abundant and the genus *Prevotella* was enriched in stages 3 and 4 CKD cats. Based on univariate statistics, genus *Prevotella* within class Prevotellaceae was less abundant in cats with CKD, whereas *Adlercreutzia* and [*Eubacterium*] were less abundant in the stages 3 and 4 CKD cats. However, these changes were not significant after correcting for multiple comparisons. Quantitative PCR was performed for *E. coli* and no significant difference in bacterial count was found between healthy control cats and CKD cats.

3.3 | Serum indoxyl sulfate and p-cresol sulfate analysis

Serum concentrations of IS and pCS are shown in Table 1. The IS concentrations were found to be significantly higher in CKD cats compared to healthy control cats ($P < .001$). Significantly higher IS concentrations were seen in stage 2 CKD cats ($P = .01$) and stages 3 and 4 CKD cats ($P < .001$) in comparison to healthy control cats (Figure 2). No significant difference was found in IS concentrations between stage 2 CKD cats and stages 3 and 4 CKD cats. The pCS concentrations were not significantly different between CKD cats and healthy control cats.

Serum concentrations of IS were found to be correlated to hematocrit (rho −0.68; $P < .001$), BUN (rho 0.71; $P < .001$), creatinine (rho 0.64; $P < .001$), and pCS (rho 0.41; $P = .01$). No correlation of either IS or pCS concentrations to appetite score, vomiting score, fecal score, body condition score, exclusive feeding of a prescription renal diet, number of unique OTUs, and presence of proteinuria, or serum phosphorus, calcium, sodium, and potassium concentrations were found. When IS and pCS concentrations were compared between cats that ate ≥75% of their food and cats that ate <75% of their food based on reported appetite scores at enrollment, serum concentrations of IS tended to be higher in cats that had a worse appetite ($P = .07$), but no significant difference in pCS concentrations was seen between the cats. The genera *Bifidobacterium* (rho −0.41; $P = .01$) and *Eubacterium* (rho −0.40; $P = .01$) were significantly correlated with pCS serum concentrations, whereas the genus *Mogibacterium* (rho −0.32; $P = .05$) was significantly correlated with serum IS concentrations.

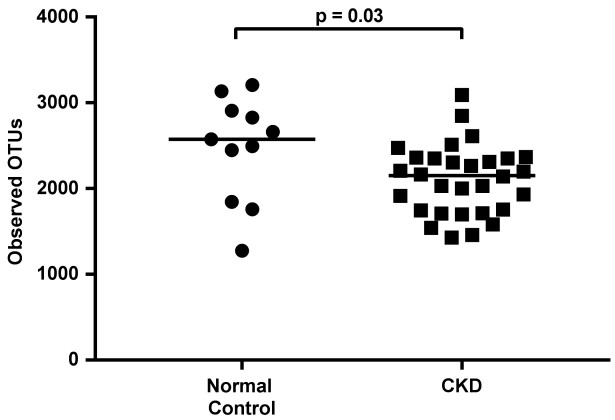

FIGURE 1 Scatter plot of the number of observed unique operational taxonomic units (OTUs) in chronic kidney disease (CKD) and healthy control cats. Species richness was significantly decreased in CKD cats when compared to healthy control cats ($P = .03$)

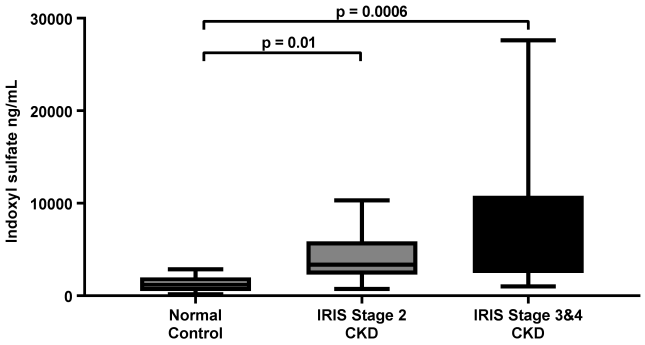

FIGURE 2 Serum indoxyl sulfate (IS) concentrations in healthy control cats, chronic kidney disease (CKD) stage 2 cats, and CKD stages 3 and 4 cats. Significantly higher IS concentrations were seen in stage 2 CKD cats ($P = .01$) and stages 3 and 4 CKD cats ($P = .0006$) in comparison to healthy control cats. There was no statistical difference in IS concentrations between stage 2 CKD cats and stages 3 and 4 CKD cats

4 | DISCUSSION

Decreased fecal bacterial species diversity and richness in CKD cats compared to healthy control cats is consistent with previous assessment of the intestinal microbiome in humans with CKD and rat CKD models.[1] The effect of uremia on the composition of the gut microbiome and its association with IS and pCS are highly variable among studies in rats and humans.[1,8,43–45] in vitro studies in rats have shown that several bacterial families generate toxic compounds such as p-cresol and indole.[9,46] However, our study did not show a correlation between the types of bacteria known to produce indole and p-cresol in humans and serum IS and pCS concentrations in cats. Although *E. coli* is a documented tryptophanase-producing bacteria in humans,[10,11] qPCR did not show quantifiable differences between CKD cats and healthy control cats, although the serum IS concentrations were significantly different between the 2 groups. In humans, bifidobacteria have been found to generate p-cresol,[10,47] but in our study a negative correlation was found between the genus *Bifidobacterium* and serum pCS concentrations.

The reasons for differences between this study and studies in humans is likely multifactorial. First, species differences exist in the commonly isolated bacterial groups from the gastrointestinal tract between humans and cats, making it difficult to make a direct comparison.[30] Second, although all dogs and cats harbor similar bacterial groups when analyzed at the level of the family or genus, much variability exists in the gut microbiome at the species level with generally only 5%-20% overlap of species among individuals.[48] This variability

makes it difficult to incriminate a single bacterial species or genus as the culprit for increased serum IS and pCS serum concentrations in CKD patients. In addition, knowledge of the intestinal microbial composition alone does not lead to a complete understanding of its metabolic activity because of functional redundancy among different microbial species.[49] Third, historical antibiotic use, variable diets, and prolonged intestinal transit times are well-known factors that promote uremic toxin generation in people. Although these factors were highly variable among study cats, they were similarly variable for both the control and CKD cats. Further investigation is warranted before the composition of the responsible bacterial species contributing to the increased serum IS and pCS concentrations in CKD cats can be determined.

Serum IS concentrations were correlated with severity of CKD in cats in a previous study in which CKD was defined based on persistent azotemia or azotemia with smaller kidneys on imaging, without urinalysis.[24] More specifically, this study demonstrated that all stages of CKD cats had significantly higher IS concentrations compared to non-azotemic cats, and that stage 4 CKD cats had significantly higher IS concentrations compared to stages 2 and 3 cats. However, no significant difference in IS concentrations between CKD stage 2 and CKD stage 3 cats was seen, similar to our study. Serum IS concentrations were found to be significantly correlated with serum phosphorous concentrations in this previous study, and in both people and cats with CKD, IS and serum phosphorous concentration are reported to be important progression factors.[24,25,50–52] These findings were not repeated in our study, which may have been because of lower numbers of cats with hyperphosphatemia or CKD IRIS stage 4 that were enrolled. Although there are similarities between the 2 studies, caution should be taken in directly comparing results because the enrolled populations are different. In our study, more stringent exclusion and inclusion criteria were used that centered on the primary aim of characterizing the fecal microbiome of cats with CKD. For example, stage 4 cats enrolled in the previous study might have recently received antibiotics, potentially altering their fecal microbiome and influencing uremic toxin concentrations.[53]

A negative correlation between hematocrit and IS concentration was observed in our study. This association may be coincidental because non-regenerative anemia is common in CKD cats.[24,54] However, IS has been documented in humans to decrease EPO production by dysregulation of oxygen metabolism in tubular cells and impairing oxygen sensing in EPO-producing cells in the kidney.[18] Further investigation is required to determine if IS has a similar effect on EPO production in CKD cats.

In humans, serum pCS concentrations are higher in hemodialysis and CKD patients than in controls.[43,55] Although we did not show a significant difference in serum pCS concentrations between CKD cats and healthy control cats, the highest serum pCS concentrations in the healthy control group was 7220 ng/mL and 13 of 28 (46%) CKD cats had serum pCS concentrations higher than the healthy control maximum with the maximum serum pCS concentration in CKD cats (35 300 ng/mL) documented in a stage 4 CKD cat. Based on a post hoc sample size calculation, a sample size of 42 cats per group would be necessary to obtain 80% statistical power. Thus, it is possible that the inability to detect significance in pCS concentrations between the 2 groups was because of small sample size.

Indoxyl sulfate and pCS lead to progression of CKD,[12–16] contribute to multi-organ dysfunction,[17,18,21,56] and are associated with increased mortality in human CKD patients.[17,18,21] In addition, because removal by hemodialysis in human patients is markedly lower for protein-bound IS and pCS than for urea and creatinine,[57] human medicine has focused on strategies to decrease production of IS and pCS including modulation of bacterial growth in the colon by dietary management,[58] prebiotics, probiotics, and target adsorption of uremic toxins by the use of adsorbents.[2,5,46] The generation of IS and pCS can be modulated by selectively increasing saccharolytic and decreasing proteolytic bacteria in the colon and by increasing intestinal transit time. Prebiotics and probiotics have been shown to influence the composition of the colonic microbiota and have been successfully used to decrease IS and pCS concentrations in human CKD patients.[43,59] In addition, increasing carbohydrate and fiber and decreasing protein intake have been shown to decrease IS and pCS concentrations.[47,58,60] Adsorbents such as sevelamer hydrochloride[61,62] (Renalgel, Genzyme, Cambridge, Massachusetts) and AST-120[61,62] (Kremezin, Kurecha Chemical Industry, Tokyo, Japan) also are used to limit intestinal absorption of IS and pCS. In veterinary medicine, there has been no published investigation into strategies to decrease gut-derived uremic toxins in CKD patients and, based on a growing body of literature,[24,25] further exploration as a potential therapeutic target seems warranted.

Our study had several limitations. The control group could not be age-matched to the CKD group. Because of the high prevalence of CKD in the geriatric cat population and the study exclusion criteria, an age-matched healthy control population (ie, without CKD or concurrent illness) could not be identified in the referral hospital feline population. Another limitation of the study was the low number of enrolled CKD stage 4 cats. Because of the exclusion criteria (in particular, no antibiotics or antacid therapy for 6 weeks before enrollment), only 2 stage 4 CKD cats could be enrolled. Because of this, stage 3 and stage 4 CKD cats were combined into a single group. Also, 2 of 30 cats with CKD maintained a normal USG (>1.035) and were diagnosed based on persistently increased serum creatinine concentration >1.6 mg/dL over a 3-month period and an increased SDMA serum concentration. According to IRIS guidelines, USG > 1.035 still can occur in some cats with azotemic CKD.[34] A glomerular filtration rate study (iohexol or nuclear scintigraphy) to document decreased renal function would have been required to confirm renal dysfunction in these cats but was not feasible. Lastly, accurate determination of dietary protein intake was not possible because of the common practice of feeding a mixture or rotation of diets. Thus, analysis of a possible relationship between percent dietary protein and uremic toxin concentrations was not possible.

In summary, we demonstrated that CKD is associated with a decreased fecal bacterial species diversity and richness in cats. Indoxyl sulfate is significantly increased in cats with CKD and IRIS stage 2 CKD cats may suffer from a similar uremic toxin burden as do cats with later stage disease. Future studies are needed to further understand the interplay between the fecal microbiome and serum concentrations of uremic toxins.

ACKNOWLEDGMENT

This study was presented in abstract form at the 2018 ACVIM Forum, Seattle, WA.

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

The project was approved by the IACUC at Colorado State University. All owners gave written informed consent before participation.

HUMAN ETHICS APPROVAL DECLARATION

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

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REFERENCES

1. Vaziri ND, Wong J, Pahl M, et al. Chronic kidney disease alters intestinal microbial flora. *Kidney Int*. 2013;83:308-315.
2. Ramezani A, Raj DS. The gut microbiome, kidney disease, and targeted interventions. *J Am Soc Nephrol*. 2014;25:657-670.
3. Jernberg C, Lofmark S, Edlund C, Jansson JK. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology*. 2010;156:3216-3223.
4. Anders HJ, Andersen K, Stecher B. The intestinal microbiota, a leaky gut, and abnormal immunity in kidney disease. *Kidney Int*. 2013;83:1010-1016.
5. Evenepoel P, Meijers BK, Bammens BR, et al. Uremic toxins originating from colonic microbial metabolism. *Kidney Int Suppl*. 2009;76:S12-S19.
6. Lau WL, Kalantar-Zadeh K, Vaziri ND. The gut as a source of inflammation in chronic kidney disease. *Nephron*. 2015;130:92-98.
7. Nallu A, Sharma S, Ramezani A, Muralidharan J, Raj D. Gut microbiome in chronic kidney disease: challenges and opportunities. *Transl Res*. 2017;179:24-37.
8. Wong J, Piceno YM, DeSantis TZ, et al. Expansion of urease- and uricase-containing, indole- and p-cresol-forming and contraction of short-chain fatty acid-producing intestinal microbiota in ESRD. *Am J Nephrol*. 2014;39:230-237.
9. Kikuchi M, Ueno M, Itoh Y, Suda W, Hattori M. Uremic toxin-producing gut microbiota in rats with chronic kidney disease. *Nephron*. 2017;135:51-60.
10. Cummings JH. Fermentation in the human large intestine: evidence and implications for health. *Lancet*. 1983;1:1206-1209.
11. DeMoss RD, Moser K. Tryptophanase in diverse bacterial species. *J Bacteriol*. 1969;98:167-171.
12. Shimizu H, Bolati D, Adijiang A, et al. NF-kappaB plays an important role in indoxyl sulfate-induced cellular senescence, fibrotic gene expression, and inhibition of proliferation in proximal tubular cells. *Am J Physiol Cell Physiol*. 2011;301:C1201-C1212.
13. Sun CY, Hsu HH, Wu MS. p-Cresol sulfate and indoxyl sulfate induce similar cellular inflammatory gene expressions in cultured proximal renal tubular cells. *Nephrol Dial Transplant*. 2013;28:70-78.
14. Watanabe H, Miyamoto Y, Honda D, et al. p-Cresyl sulfate causes renal tubular cell damage by inducing oxidative stress by activation of NADPH oxidase. *Kidney Int*. 2013;83:582-592.
15. Miyazaki T, Ise M, Seo H, et al. Indoxyl sulfate increases the gene expressions of TGF-beta 1, TIMP-1 and pro-alpha 1(I) collagen in uremic rat kidneys. *Kidney Int Suppl*. 1997;62:S15-S22.
16. Niwa T, Ise M. Indoxyl sulfate, a circulating uremic toxin, stimulates the progression of glomerular sclerosis. *J Lab Clin Med*. 1994;124:96-104.
17. Watanabe K, Watanabe T, Nakayama M. Cerebro-renal interactions: impact of uremic toxins on cognitive function. *Neurotoxicology*. 2014;44:184-193.
18. Chiang CK, Tanaka T, Inagi R, Fujita T, Nangaku M. Indoxyl sulfate, a representative uremic toxin, suppresses erythropoietin production in a HIF-dependent manner. *Lab Invest*. 2011;91:1564-1571.
19. Mozar A, Louvet L, Godin C, et al. Indoxyl sulphate inhibits osteoclast differentiation and function. *Nephrol Dial Transplant*. 2012;27:2176-2181.
20. Iwasaki Y, Kazama JJ, Yamato H, Shimoda H, Fukagawa M. Accumulated uremic toxins attenuate bone mechanical properties in rats with chronic kidney disease. *Bone*. 2013;57:477-483.
21. Barreto FC, Barreto DV, Liabeuf S, et al. Serum indoxyl sulfate is associated with vascular disease and mortality in chronic kidney disease patients. *Clin J Am Soc Nephrol*. 2009;4:1551-1558.
22. Lin CJ, Wu V, Wu PC, Wu CJ. Meta-analysis of the associations of p-cresyl sulfate and indoxyl sulfate with cardiovascular events and all-cause mortality in patients with chronic renal failure. *PLoS One*. 2015;10:e0132589.
23. Wu IW, Hsu KH, Hsu HJ, et al. Serum free p-cresyl sulfate levels predict cardiovascular and all-cause mortality in elderly hemodialysis patients--a prospective cohort study. *Nephrol Dial Transplant*. 2012;27:1169-1175.
24. Cheng FP, Hsieh MJ, Chou CC, Hsu WL, Lee YJ. Detection of indoxyl sulfate levels in dogs and cats suffering from naturally occurring kidney diseases. *Vet J*. 2015;205:399-403.
25. Chen CN, Chou CC, Tsai PSJ, Lee YJ. Plasma indoxyl sulfate concentration predicts progression of chronic kidney disease in dogs and cats. *Vet J*. 2018;232:33-39.
26. Bell ET, Suchodolski JS, Isaiah A, et al. Faecal microbiota of cats with insulin-treated diabetes mellitus. *PLoS One*. 2014;9:e108729.
27. Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS. Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. *FEMS Microbiol Ecol*. 2011;76:301-310.
28. Honneffer JB, Minamoto Y, Suchodolski JS. Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. *World J Gastroenterol*. 2014;20:16489-16497.
29. Masuoka H, Shimada K, Kiyosue-Yasuda T, et al. Transition of the intestinal microbiota of cats with age. *PLoS One*. 2017;12:e0181739.
30. Minamoto Y, Hooda S, Swanson KS, Suchodolski JS. Feline gastrointestinal microbiota. *Anim Health Res Rev*. 2012;13:64-77.
31. Ramadan Z, Xu H, Laflamme D, et al. Fecal microbiota of cats with naturally occurring chronic diarrhea assessed using 16S rRNA gene 454-pyrosequencing before and after dietary treatment. *J Vet Intern Med*. 2014;28:59-65.
32. Ritchie LE, Steiner JM, Suchodolski JS. Assessment of microbial diversity along the feline intestinal tract using 16S rRNA gene analysis. *FEMS Microbiol Ecol*. 2008;66:590-598.
33. Suchodolski JS, Foster ML, Sohail MU, et al. The fecal microbiome in cats with diarrhea. *PLoS One*. 2015;10:e0127378.
34. International Renal Interest Society. Algorithm for staging of chronic kidney disease in cats. http://www.iris-kidney.com/pdf/003-5559.001-iris-website-staging-of-ckd-pdf_220116-final.pdf#page=7. Accessed February 7, 2018.
35. Isaiah A, Parambeth JC, Steiner JM, Lidbury JA, Suchodolski JS. The fecal microbiome of dogs with exocrine pancreatic insufficiency. *Anaerobe*. 2017;45:50-58.
36. Caporaso JG, Kuczynski J, Stombaugh J, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. 2010;7:335-336.

37. DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol*. 2006;72:5069-5072.
38. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol*. 2005;71:8228-8235.
39. Minamoto Y, Dhanani N, Markel ME, Steiner JM, Suchodolski JS. Prevalence of *Clostridium perfringens*, *Clostridium perfringens* enterotoxin and dysbiosis in fecal samples of dogs with diarrhea. *Vet Microbiol*. 2014;174:463-473.
40. Shu C, Chen X, Xia T, Zhang F, Gao S, Chen W. LC-MS/MS method for simultaneous determination of serum p-cresyl sulfate and indoxyl sulfate in patients undergoing peritoneal dialysis. *Biomed Chromatogr*. 2016;30:1782-1788.
41. Benjamini Y, Hochberg P. Controlling the false discovery rate - a practical and powerful approach to multiple testing. *J R Statist Soc*. 1995;57:289-300.
42. Zakrzewski M, Proietti C, Ellis JJ, et al. Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions. *Bioinformatics*. 2017;33:782-783.
43. Hida M, Aiba Y, Sawamura S, Suzuki N, Satoh T, Koga Y. Inhibition of the accumulation of uremic toxins in the blood and their precursors in the feces after oral administration of Lebenin, a lactic acid bacteria preparation, to uremic patients undergoing hemodialysis. *Nephron*. 1996;74:349-355.
44. Wang F, Zhang P, Jiang H, Cheng S. Gut bacterial translocation contributes to microinflammation in experimental uremia. *Dig Dis Sci*. 2012;57:2856-2862.
45. Yoshifuji A, Wakino S, Irie J, et al. Gut lactobacillus protects against the progression of renal damage by modulating the gut environment in rats. *Nephrol Dial Transplant*. 2016;31:401-412.
46. Gryp T, Vanholder R, Vaneechoutte M, Glorieux G. p-Cresyl sulfate. *Toxins (Basel)*. 2017;9:52. doi:10.3390/toxins9020052.
47. Smith EA, Macfarlane GT. Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. *J Appl Bacteriol*. 1996;81:288-302.
48. Suchodolski JS. Intestinal microbiota of dogs and cats: a bigger world than we thought. *Vet Clin North Am Small Anim Pract*. 2011;41:261-272.
49. Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. *Nature*. 2012;489:220-230.
50. Wu IW, Hsu KH, Lee CC, et al. p-Cresyl sulphate and indoxyl sulphate predict progression of chronic kidney disease. *Nephrol Dial Transplant*. 2011;26:938-947.
51. Da J, Xie X, Wolf M, et al. Serum phosphorus and progression of CKD and mortality: a meta-analysis of cohort studies. *Am J Kidney Dis*. 2015;66:258-265.
52. Chakrabarti S, Syme HM, Elliott J. Clinicopathological variables predicting progression of azotemia in cats with chronic kidney disease. *J Vet Intern Med*. 2012;26:275-281.
53. Nazzal L, Roberts J, Singh P, et al. Microbiome perturbation by oral vancomycin reduces plasma concentration of two gut-derived uremic solutes, indoxyl sulfate and p-cresyl sulfate, in end-stage renal disease. *Nephrol Dial Transplant*. 2017;32:1809-1817.
54. Boyd LM, Langston C, Thompson K, Zivin K, Imanishi M. Survival in cats with naturally occurring chronic kidney disease (2000-2002). *J Vet Intern Med*. 2008;22:1111-1117.
55. Fukuuchi F, Hida M, Aiba Y. Intestinal bacteria-derived putrefactants in chronic renal failure. *Clin Exp Nephrol*. 2002;6:99-104.
56. Nii-Kono T, Iwasaki Y, Uchida M, et al. Indoxyl sulfate induces skeletal resistance to parathyroid hormone in cultured osteoblastic cells. *Kidney Int*. 2007;71:738-743.
57. Dhondt A, Vanholder R, Van Biesen W, et al. The removal of uremic toxins. *Kidney Int Suppl*. 2000;76:S47-S59.
58. Ling WH, Hanninen O. Shifting from a conventional diet to an uncooked vegan diet reversibly alters fecal hydrolytic activities in humans. *J Nutr*. 1992;122:924-930.
59. Meijers BK, De Preter V, Verbeke K, et al. p-Cresyl sulfate serum concentrations in haemodialysis patients are reduced by the prebiotic oligofructose-enriched inulin. *Nephrol Dial Transplant*. 2010;25:219-224.
60. Sirich TL, Plummer NS, Gardner CD, Hostetter TH, Meyer TW. Effect of increasing dietary fiber on plasma levels of colon-derived solutes in hemodialysis patients. *Clin J Am Soc Nephrol*. 2014;9:1603-1610.
61. Lin CJ, Pan CF, Chuang CK, et al. Effects of sevelamer hydrochloride on uremic toxins serum indoxyl sulfate and p-cresyl sulfate in hemodialysis patients. *J Clin Med Res*. 2017;9:765-770.
62. Yamamoto S, Kazama JJ, Omori K, et al. Continuous reduction of protein-bound uraemic toxins with improved oxidative stress by using the oral charcoal adsorbent AST-120 in haemodialysis patients. *Sci Rep*. 2015;5:14381.

SUPPORTING INFORMATION

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

How to cite this article: Summers SC, Quimby JM, Isaiah A, Suchodolski JS, Lunghofer PJ, Gustafson DL. The fecal microbiome and serum concentrations of indoxyl sulfate and p-cresol sulfate in cats with chronic kidney disease. *J Vet Intern Med*. 2019;33:662–669. https://doi.org/10.1111/jvim.15389