DISSERTATION

ASSESSMENT OF NOVEL CAUSES AND INVESTIGATION INTO THE GUT MICROBIOME IN CATS WITH CHRONIC KIDNEY DISEASE

Submitted by

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ABSTRACT

ASSESSMENT OF NOVEL CAUSES AND INVESTIGATION INTO THE FECAL MICROBIOME IN CATS WITH CHRONIC KIDNEY DISEASE

Chronic kidney disease (CKD) is the most commonly diagnosed acquired disease in cats, especially senior cats (≥ 8 years). The disease is a common cause of death in cats and causes significant clinical signs that negatively impacts quality of life. Despite the commonality of the disease, often at the time of diagnosis the underlying etiology is not identified, and renal histopathology of idiopathic CKD cases shows non-specific findings of interstitial nephritis with fibrosis and tubular atrophy. This raises questions about potential etiologies for idiopathic CKD. In addition, there is no cure for the disease except renal transplantation and the only therapy documented to slow progression of the disease is dietary intervention supporting the gut to be a site of therapeutic intervention.

The first part of this project (Chapter 3 and Chapter 4) describes studies that investigated possible novel etiologies of the disease. Chapter 3 described a retrospective study that compared the estimated prevalence rates of *Bartonella henselae* IgG serum antibody and nucleic acids in the blood of cats ≥ 5 years of age from the United States with and without evidence of kidney dysfunction. Using an IgG ELISA and *Bartonella* spp. PCR, it was found that *B. henselae* IgG antibodies were not associated with kidney dysfunction, proteinuria (urine dipstick ≥ 1+), hematuria (> 5 RBC/HPF), or the presence of WBC in the urine (> 2-5 WBC/HPF). *Bartonella* spp. DNA was amplified from the blood of one cat with kidney dysfunction (1/106; 0.01%) but none of the urine samples. It was concluded that routine testing for *B. henselae* is not warranted

as the infection does not appear to be a cause of kidney dysfunction or urinary disease in older cats in the United States.

Another possible etiology of CKD in cats is frequent vaccination with vaccines containing the immunodominant Crandell-Rees feline kidney (CRFK) cell antigen alpha-enolase, a glycolytic enzyme found predominantly in the kidney. The project outlined in Chapter 4 evaluated whether hyperinoculation of healthy adult cats with a market leading core vaccine over a 16-week period induces renal changes consistent with interstitial nephritis. Hyperinoculation over a 16-week period with a FVRCP core vaccine induced changes in the immunoreactivity of alpha-enolase within the kidney based on an immunohistochemical stain, induced an antibody and cell-mediated immune response towards alpha-enolase, and increased serum concentrations of select inflammatory cytokines and chemokines. However, hyperinoculation over this short time period did not affect functional renal clinicopathologic variables or induce renal inflammatory disease detectable by light microscopy. This study showed that cats after vaccination with a FVRCP core vaccine mount an immune response towards antigens contained within the vaccine and the immunoreactivity of alpha-enolase is altered within the kidney. However, this 16-week vaccine hyperinoculation model cannot be used to study interstitial nephritis in cats.

The second part of this PhD (Chapters 5-7) focuses on the significant need for therapeutic biomarkers and additional therapies for the management of CKD in cats. The scientific literature supports that restricted protein and low phosphorus prescription renal diets reduce disease progression and signs of uremia in cats but the mechanisms by which it does so has not been fully elucidated. Therefore, investigation into the interplay between the gut microbiome and CKD in cats is warranted. Chapter 5 describes a study that characterized the fecal microbiome

and measured colonic microbial metabolites in the serum (i.e. major gut-derived uremic toxins) and feces (i.e. short-chain fatty acids) from CKD cats and compared findings to healthy senior control cats. Using 16S rRNA gene sequencing, the study found that CKD cats have intestinal dysbiosis characterized by decreased bacterial richness and diversity. The serum concentrations of the gut-derived uremic toxins indoxyl sulfate (IS) and p-cresol sulfate (pCS) were measured, and IS was significantly elevated in cats with CKD, especially cats with late-stage disease (International Renal Interest Society CKD stages 3-4). The branched-chain fatty acid isovaleric acid was increased in cats with late-stage disease compared to healthy senior cats. Both microbial metabolites (i.e. IS and isovaleric acid) are products of protein fermentation by colonic bacteria and are correlated positively to serum creatinine, blood urea nitrogen. Serum pCS and IS concentrations were higher in cats with clinical evidence of muscle wasting. The study demonstrated that CKD is associated with a functional dysbiosis in cats and findings support protein malassimilation in cats with CKD. The gut microbiome is a potential therapeutic target to reduce production of deleterious gut-derived uremic toxins and minimize kidney disease cachexia.

Chapter 6 describes a study that evaluated the effect a probiotic has on the gut microbiome and on clinical and renal clinicopathologic variables in cats with CKD. In a prospective, randomized, placebo-controlled study, cats with CKD were fed a commercial product containing a probiotic *Enterococcus faecium* strain SF68 (SF68) and a palatability enhancer for 8 weeks and were compared to CKD cats fed only the palatability enhancer and to CKD cats fed a commercial diet for CKD with no oral supplement. The probiotic SF68 did not significantly change renal clinicopathologic variables, fecal microbial richness, diversity, or community structure, or serum concentrations of IS, pCS, or trimethylamine-n-oxide [TMAO]).

In conclusion, the palatability enhancer may augment appetite in some cats but in this pilot study the probiotic SF68 did not significantly change the fecal microbial community structure or serum concentrations of IS, pCS, and TMAO in cats with CKD.

The previous studies described in Chapter 5 and Chapter 6 showed substantial variability in serum concentrations within the same cat and between the stages of CKD. This raised the question of the impact recent feeding has on serum concentrations and the clinical utility of using serum concentrations as therapeutic markers. Chapter 7 describes the short- and medium-term biological variation estimates of IS, pCS, and TMAO in healthy adult research cats and the effect of recent feeding on serum concentrations. The study determined that the index of individuality was intermediate using both short-term and medium-term biological variation estimates for serum pCS concentrations (0.98 and 1.17, respectively) and TMAO concentrations (1.47 and 0.83, respectively). For serum IS concentrations, the short-term biological variation estimates corresponded to a high IOI (1.96) and the medium-term biological variation estimates corresponded to a low IOI (0.65). The RCV for IS, pCS, and TMAO based on the medium-term biological variation estimates suggest that serum concentrations would have to decrease by 21.9%, 28.9%, and 52.2%, respectively, between serial measurements to suggest a significant change. In addition, feeding may reduce serum concentrations of pCS, IS, and TMAO over a 12-hour period in cats. To compare serial measurements, the study showed that it would be prudent to collect samples at the same time of day and consistently in either a fasted or non-fasted state. These findings provide guidance for researchers and veterinarians when determining the significance of a change in the concentration of serial measurements and describes the importance of standardizing sample collection.

In conclusion, the work described in this dissertation is aimed to assist veterinarians in the diagnosis and management of CKD in cats. Much of the work described translates to other mammalian species, notably dogs and humans, and will lay the groundwork for future exploration into the disease etiology and the role of gut dysbiosis in disease pathogenesis.

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DEDICATION

To my husband, Andy, and my parents, Jack and Karen

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1.1.1 Prevalence of Chronic Kidney Disease

Chronic kidney disease (CKD) is a common acquired disease in cats and is a major cause of morbidity and mortality, particularly in senior cats. The International Renal Interest Society (IRIS) has developed well-established guidelines to diagnose and stage CKD (stages 1-4) based on severity of azotemia, presence of proteinuria, and blood pressure assessment.[1] Based on a European study evaluating mortality of cats attending primary care veterinary practices, a renal disorder (12.2%) was the most common cause of mortality in cats of all ages, a higher prevalence than neoplasia (10.8%).[2] The overall prevalence of renal disease in cats examined at private veterinary practices in the United States was reported to be 1.9%, and renal disease was a common reason for examination in cats along with cystitis, feline urologic syndrome, and inappetence.[3] However, this study used azotemia to make the diagnosis of kidney disease and therefore missed non-azotemic CKD cases, particularly IRIS CKD stage 1 and early stage 2. When a prevalence study considered results of serum chemistry, urinalysis, and radiographic determination of degenerative changes and kidney size, the overall prevalence was strikingly higher. The prevalence of CKD increases with age as follows: 0-5 years 37.5%, 5-10 years 40.9%, 10-15 years 42%, and >15 years 80.9%.[4] In addition, pure bred cats appear to have an increased likelihood of developing the disease.[4]

1.1.2 Etiology and Pathogenesis of Chronic Kidney Disease Progression

Chronic kidney disease is an umbrella term to describe any renal disease that leads to progressive loss of kidney function over time.[5] Although primary renal diseases have been

implicated, in most cases an underlying cause of the CKD is not identified at the time of diagnosis and the disease is deemed idiopathic. On histopathology, non-specific features are found including mononuclear cell interstitial inflammation, tubular atrophy, and fibrosis with secondary glomerulosclerosis. These changes are present in the early stages of disease and are more severe in end-stage disease.[6] Lymphoplasmacytic inflammation is the most common type of inflammation identified and often surrounds atrophic tubules. Granulomatous inflammation is most common in IRIS stage 2-4 and thought to be secondary to tubular ischemia.[6] Renal interstitial lipid accumulation is a feature unique to CKD cats and is associated with tubular basement membrane fragmentation and epithelial degeneration and lysis.[7] Similar renal changes are found in senior (10-14 years) and geriatric cats (>15 years) without kidney disease,[8] thus these findings are not specific to CKD. Primary glomerulopathies are less common in cats compared to dogs, however recently it has been discovered that 70% of renal biopsies from cats obtained for evaluation of proteinuria had glomerular lesions, of which 72% had immune-complex glomerulonephritis.[9]

Causes of primary renal disease in cats include congenital disease (juvenile renal dysplasia),[10] genetic disease (amyloidosis, polycystic kidney disease),[11-13] neoplasia (renal lymphoma),[14] infection (bacterial pyelonephritis, retroviruses, feline infectious peritonitis),[14-16] upper urinary tract obstructions,[17,18] immune-complex glomerulonephritis,[9] hypertension,[19] and consumption of high phosphorus diets.[20-22]

Acute or chronic renal hypoxia of any cause can also initiate renal damage.[23] Causes of renal hypoxia include anemia, hypotension, sympathetic nervous system activation, renin-angiotensin-aldosterone system (RAAS) activation, and non-steroidal anti-inflammatory drugs.[6] Renal hypoxia ultimately leads to nephron loss by multiple mechanisms. Hypoxia causes a

degradation of intracellular adenosine triphosphate (ATP) to adenosine diphosphate and adenosine monophosphate. The ATP depletion within renal tubular cells increases intracellular calcium which subsequently causes cellular damage and decreased activity of Na^+K^+-ATPase. This results in cell swelling from water movement into the cell. The cell swelling can cause tubular obstruction and further renal damage. In addition, renal hypoxia changes the cellular cytoskeleton of tubular cells resulting in loss of microvilli and of cellular polarity. The Na^+K^+-ATPase and glycoproteins that mediate cell-to-cell adhesion dissociate from the basolateral plasma membrane and move to the apical cell membrane. This changes sodium handling allowing more sodium to reach the macula densa resulting in afferent arteriolar constriction and decreased glomerular filtration rate (GFR). Without glycoproteins, the tubular cells detach from the basolateral membrane and cells slough into the tubular lumen.[23,131] Renal regeneration can occur in mild cases. If there is continued nephron loss from repeated or sustained injury then maladaptive repair responses occur that perpetuate the injury leading to chronic, irreversible disease.[24]

In response to injury, proximal tubular epithelial cells generate the pro-inflammatory and chemotactic cytokines tumor necrosis factor-α, monocyte chemoattractant protein 1, tumor growth factor-β, and interleukin-6.[25] These inflammatory mediators recruit and activate inflammatory cells and transform fibroblasts to myofibroblasts leading to extracellular matrix production. Renal fibrosis occurs starting in areas of inflammation and expanding into surrounding tissue, which destroys normal renal architecture.[6] The loss of peritubular capillaries and separation of tubules from peritubular capillaries by fibrosis leads to chronic tubular hypoxia which worsens fibrosis, thus creating a maladaptive positive feedback loop.[25,26] Other mediators of progression of CKD in cats includes RAAS activation and oxidative injury. The generation of

angiotensin II with RAAS activation leads to systemic hypertension and glomerular hypertension. Glomerular hypertension leads to development of glomerulosclerosis and proteinuria, an independent predictor for survival in cats with CKD.[27] Damaged renal tubular cells increase production of reactive oxygen species (ROS) leading to oxidative stress, renal cell apoptosis, cellular senescence, reduced regenerative capacity, and fibrosis. This is supported by the finding of altered antioxidant status in cats with CKD, notably in early stage disease.[28-30]

Although the underlying etiology may differ, the maladaptive repair responses leading to irreversible damage is a consistent finding among cats with CKD. Further exploration of possible causes of CKD in cats is warranted to better understand the disease and to define targeted therapies to institute early in the disease process before irreversible renal changes occur.

1.1.3 Prognosis for Chronic Kidney Disease

Although the underlying etiology of CKD in most cases is obscure, the disease is always progressive and irreversible. Prognosis depends on severity of the disease; however, it can be quite variable among CKD cats. Several studies have evaluated prognosis based on IRIS stage. For cats with IRIS stage 2, stage 3, and stage 4 CKD, the median reported survival time is 490-1151 days, 154-778 days, and 20-103 days, respectively.[27,31-33] Negative prognostic indicators of survival and disease progression in cats with CKD include fibroblast growth factor-23 (FGF-23),[34,35] hyperphosphatemia,[32,36] hypomagnesemia,[37] the gut-derived uremic toxin indoxyl sulfate (IS),[35,38] magnitude of proteinuria,[27,32,36] and lower hematocrit.[32,36]

The risk factors for CKD disease progression and survival are all interconnected. Phosphorus retention occurs secondary to reduction in GFR.[39] Osteocytes and osteoblasts secrete FGF-23 in response to hyperphosphatemia and increased plasma calcitriol concentrations.[40,41] Fibroblast growth factor-23 works to reduce plasma phosphorus by reducing vitamin D

synthesis, increasing phosphaturia, and decreasing parathyroid production and secretion. Eventually with CKD, these mechanisms cannot control phosphorus accumulation from reduced GFR and the cat develops CKD-mineral and bone disorders (CKD-MBD).[33,42] A high plasma FGF-23 concentration is thus an indicator of phosphorus dysregulation and CKD-MBD. Both hypomagnesemia[37] and high plasma IS concentrations[35] in cats with CKD are associated with high plasma concentrations of FGF-23. Indoxyl sulfate is a gut-derived uremic toxin produced in the colon by bacteria during fermentation of tryptophan. Indoxyl sulfate has been documented to accumulate in the blood of CKD cats and is associated with disease severity.[43,44] Non-regenerative anemia is common in cats with CKD and is secondary to reduced erythropoietin production in the kidney, gastrointestinal hemorrhage or other sources of blood loss, shortened red blood cell survival from uremic toxins, and systemic inflammation.[45,46] The anemia causes chronic renal tissue hypoxia and contributes to disease progression and to poor quality of life. Proteinuria, whether tubular or glomerular in origin, contributes to disease progression by promoting tubular inflammation and renal fibrosis.[47]

1.2 Novel Causes of Chronic Kidney Disease in Cats

1.2.1 Role of *Bartonella henselae* in Renal Disease

Bartonella species are fastidious, gram-negative, intracellular bacteria that have tropisms towards erythrocytes and endothelial cells. Domestic cats are the primary reservoir hosts for *Bartonella henselae* which is the causative agent of Cat Scratch Disease in people. The flea *Ctenocephalides felis* is the disease vector. Cats are usually subclinical carriers, however *B. henselae* has been associated with disease in cats including fever, lymphadenopathy, anterior uveitis, and endocarditis.[48] Current diagnostic testing include microbiological culture techniques,

polymerase chain reaction, immunohistochemistry, and serology. In cats, the clinical diagnosis is based on concurrent clinical signs, positive serology, and demonstration of the organism by culture or molecular methods.

There is supporting evidence that *B. henselae* may be associated with urinary disease in people and cats. In people, bartonellosis most commonly causes a subacute and self-limited lymphadenopathy. Infection can be secondary to immunosuppression which often leads to severe clinical signs of disease, multi-organ involvement, and formation of micro-abscesses. In a recent meta-analysis, it was found that persistent fever and lymphadenopathy was often caused by bartonellosis in CKD and renal transplantation patients.[49] In addition, necrotizing glomerulonephritis has been documented to be a complication of *B. henselae* infections.[50,51]

While human *Bartonella* spp. infections can vary in disease presentation and severity, cats appear to tolerate chronic bacteremia without developing overt signs of disease in most cases. No overt signs of disease were appreciated when specific-pathogen-free cats were inoculated with *B. henselae* and/or *B. clarridgeiae* despite documentation of persistent infection. Histopathology of multiple organs in inoculated cats revealed lymphocytic inflammation in the biliary tree, liver, heart, and kidney (interstitial lymphocytic nephritis; 4/13 cats). In addition, *Bartonella* DNA was amplified from multiple organs including the kidney (9/13 cats) in both blood-culture positive cats and blood-culture negative cats.[52] Similarly, in a study of experimentally-infected SPF young cats, after inoculation serum creatinine significantly increased over time and *Bartonella* DNA was isolated from the urine in one cat which was associated with hematuria.[53] Cats with *B. henselae* positive titer were more likely to have hematuria noted on urinalysis in a retrospective study of 436 sick client-owned cats.[54] In a study evaluating seroprevalence of *B. henselae* in 728 cats from Switzerland and Germany, an increase

in the frequency of various diseases of the kidneys and urinary tract was found in sick seropositive cats; unfortunately, the urinary diseases were not further differentiated in this study.[55] Despite these findings, no strong association between bacteremia or seropositivity to *Bartonella* spp. and CKD has been documented. In fact a retrospective study in 298 cats from a tertiary referral hospital showed no association between CKD and infection or seropositivity to *Bartonella* spp.[56]

1.2.2 Potential Link of Frequent Vaccination to Chronic Kidney Disease in Cats

Many client-owned cats are routinely inoculated with a core feline herpesvirus-1, calicivirus, and panleukopenia virus-containing vaccine (FVRCP). The American Association of Feline Practitioners (AAFP) Advisory Panel categorize the FVRCP vaccine as a core vaccine recommended for all cats. After a primary series in kittens or in un-vaccinated adults, the AAFP Advisory Panel recommended revaccination 1 year after the primary series and then every 3 years lifelong.[57] Historically, FVRCP vaccines were given yearly and some veterinarians may continue to give the vaccine yearly despite these recommendations to encourage yearly follow-up for physical examination. Both intranasal and parenteral FVRCP vaccines are available to veterinarians.

The Crandell-Rees feline kidney (CRFK) cell line is used to propagate the viruses in many of the manufactured FVRCP vaccines. Although a direct link between FVRCP vaccines and interstitial nephritis in cats has not been identified, a previous study showed that healthy purpose-bred cats inoculated with a parenteral FVRCP vaccine grown on CRFK cells (4 times over 50-week period) or inoculated with CRFK cell lysates (12 times over 50-week period) developed antibodies against CRFK cell and feline renal cell lysates. However, none of the cats developed evidence of renal disease on histology. Interestingly, only the cats that received

parenteral vaccination (and not the intranasal vaccine) had detectable antibodies.[58] One year later, a subsequent study documented lymphoplasmacytic interstitial nephritis in 50% of the cats previously sensitized to CRFK lysates, boosted with CRFK lysates, and then biopsied 2 weeks later.[59]

In a subsequent study,[60] kittens were either vaccinated with FVRCP vaccines or received CRFK cell lysate injections. Serum from cats were used to determine the immunodominant antigens inducing antibodies against the CRFK lysates which were then identified by protein sequencing. Three CRFK antigens were identified and the most immunodominant was α-enolase, a glycolytic enzyme that is widely distributed in the body and found in greatest concentration in the kidney and thymus.[60] Alpha-enolase autoantibodies are generated by uptake of enolase by antigen-presenting cells and subsequent B cell activation. Formation of these autoantibodies has been reported in apparently healthy subjects ranging from 0% to 11.7%, however the incidence is significantly higher in people with a variety of autoimmune disorders, especially in disorders with active renal involvement.[61] Excessive production of α-enolase autoantibodies potentially induces tissue injury by immune-complex deposition, direct cytopathic effect or by interfering with membrane fibrinolytic activity.[62] In people, α-enolase autoantibodies are nephrogenic by inducing endothelial cell injury and cell death through an apoptotic process.[63] Anti-α-enolase antibodies are detected in 67-80% of patients with autoimmune nephritis associated with systemic lupus erythematous (SLE) compared to 6% in healthy controls.[64,65] Therefore, it is reasonable to consider that inoculation with FVRCP vaccine sensitizes cats to the self-antigen leading to autoimmune attack of kidney cells, especially in cases of frequent vaccination. This is further supported by a retrospective study that showed

annual or frequent vaccination predicted development of azotemic CKD in cats (P = 0.003; hazard ratio, 5.68; 95% confidence interval, 1.83-17.64).[66]

In humans, renal α-enolase is found primarily in the epithelial cells of the tubules and nearly undetectable in the glomeruli.[67] The enzyme is found in the cytoplasm and cell membrane of kidney cells.[62] On renal biopsies in patients with SLE α-enolase expression is increased in the tubules, but also expressed in variable regions of the glomeruli including mesangium, in glomerular and parietal epithelium, and in crescents.[68] Recently, the distribution of α-enolase in cats with and without kidney disease has been described using a α-enolase immunohistochemical stain. In young cats (< 2 years), α-enolase staining was found in the tubules and absent in the glomeruli (Figure 1.1). In senior cats (> 10 years), α-enolase staining was found in both the tubules and glomeruli. In cats with CKD, α-enolase staining was decreased in atrophic tubules, similar to healthy cats in normal tubules, and increased in the glomeruli (Figure 1.2). The data suggested that α-enolase changes distribution in the kidney prior to development of CKD in cats.[69]

Currently, studies evaluating CKD utilize cats with naturally occurring disease, often client-owned pets. This introduces significant variability in studies because the underlying etiologies, environmental factors, genetics, and comorbidities differ significantly among cats. Because of this, many rodent models have been developed to enable mechanistic understanding of CKD progression and to identify potential therapeutic targets. The most used experimental rodent model of CKD is the 5/6 subtotal nephrectomy approach. This model mimics progressive renal failure. After resection of one kidney, ½ of the other kidney is resected 2 weeks later. After eight weeks, glomerular hypertension (secondary to renin-angiotensin system activation), oxidative stress, and inflammation results in glomerulosclerosis, tubulointerstitial injury, renal

atrophy, and proteinuria. The disadvantages of the model are that it best represents end-stage renal disease, post-surgery mortality is high by week 12 post-surgery, and the majority of renal tissue is removed limiting the study.[70] Other commonly used models to study tubulointerstitial fibrosis is unilateral ureteral obstruction, folic acid nephropathy, or cyclosporine A nephropathy.[71] At this time, no non-lethal research model is available to researchers to study CKD in cats. A non-lethal model to evaluate early interstitial nephritis in cats is sorely needed to identify biomarkers of disease before advanced disease occurs and to understand pathogenesis of disease progression.

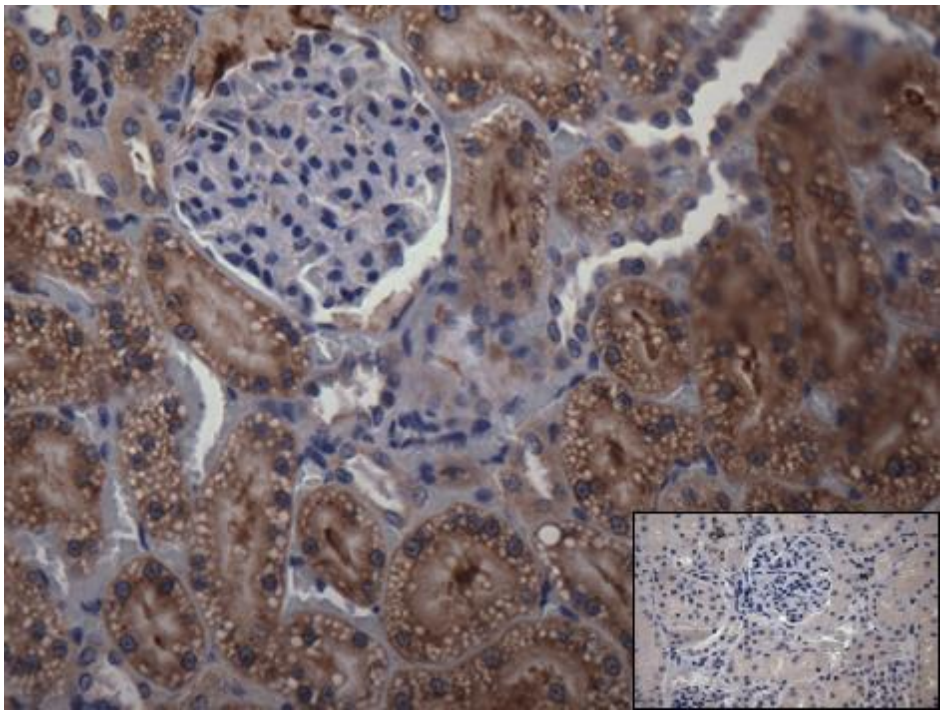

Figure 1.1 Alpha-enolase immunohistochemistry of the kidney from a young (<2 years) control cat without kidney disease (McLeland et al. 2019). Inset: negative control.

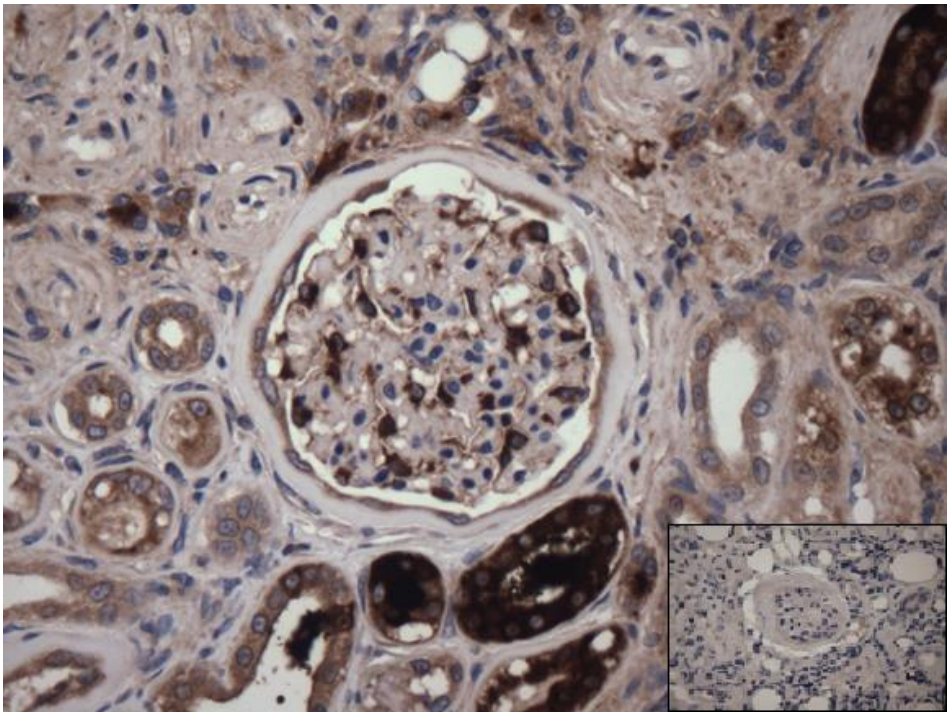

Figure 1.2 Alpha-enolase immunohistochemical stain of the kidney from a cat with chronic kidney disease (McLeland et al. 2019). Inset: negative control.

1.3 The Gut-Kidney Axis

1.3.1 Role of Nutrition in Chronic Kidney Disease Management

Chronic kidney disease in cats is commonly associated with clinical signs of disease including cachexia, weight loss, vomiting, and poor appetite, which negatively impacts the quality of life for the cat and is a source of stress for the owners.[72,73] Interestingly, many of the clinical signs that CKD cats suffer suggest that uremia has an negative impact on the gastrointestinal tract. Nutrition is a vitally important center of focus of treatment in CKD cats and the mainstay treatment is centered on diet and phosphorus restriction. Renal prescription diets are restricted in protein and low in phosphorus and often have a high caloric density. Feeding of a renal prescription diet to CKD cats has been shown to drastically increase survival and reduce clinical signs of uremia.[74-76] In a hallmark paper by Ross et al., a renal prescription

diet containing restricted protein (≤ 67.4 g/Mcal) and phosphate (≤ 1.2 g/Mcal) reduced the incidence of uremic crisis and renal-related deaths in IRIS stage 2 and 3 CKD cats when compared to a maintenance diet containing protein > 92.0 g/Mcal and phosphate ≥ 1.8 g/Mcal.[76] With renal diets, there is an obvious benefit to dietary phosphorus restriction to slow progression of renal-secondary hyperparathyroidism and mineral-bone disorder, both of which negatively impact survival in cats with CKD.[39] The exact mechanism behind reduction in the incidence of uremic crisis has not been fully elucidated, but assumed to be the benefit of protein restriction.[76]

Chronic kidney disease is associated with cachexia. Freeman et al.[73] documented that weight loss can be detected before diagnosis, accelerates after diagnosis, and is associated with reduced survival in cats with CKD. Anecdotally, CKD cats commonly can develop paraspinal muscle atrophy, even with adequate caloric intake. Previous literature suggests that weight loss and muscle wasting in people with end-stage renal disease patients is due, at least in part, to impaired small intestinal protein digestion and absorption, alterations in protein metabolism and inadequate protein intake.[77,78] Renal diets generally have 6-7 grams of protein per 100 kcal. In comparison, adult maintenance diets have 9-10 grams of protein per 100 kcal or greater. Studies suggest that protein requirements are higher for senior cats than for young cats due to reduced protein digestion.[79] This observation questions the benefit of protein restriction in cats with CKD, most of which are older cats, considering the potential negative impact protein restriction has on body weight and muscle mass, especially in cats with dysrexia.

1.3.2 The Gut Microbiome in Health and Chronic Kidney Disease

The intestinal microbiome is defined as the collection of microorganisms that reside in the intestine and consists of primarily bacteria. These microorganisms form an ecosystem with complex interactions with each other and the host. In cats, there are thousands of bacterial

phylotypes that reside in the gut amounting to trillions of cells with an extensive functional capacity.[80,81] Thus, this wide array of microorganisms play an important role in maintaining host health via products of bacterial metabolism and by influencing gene expression in the gut. A healthy gut microbiome is vital for the development and maintenance of a healthy immune system, assimilation of nutrients from the diet, nutrient synthesis (i.e. short-chain fatty acids [SCFA], vitamin B12), and protection against invading enteric pathogens.[82]

Dysbiosis is defined as an imbalanced intestinal microbial community with alteration in microbial composition and metabolic activities. In many diseases, dysbiosis is not just a marker of disease, but also actively contributes to pathology.[83] In people with CKD and rat models, intestinal dysbiosis has been extensively documented. In both cases, CKD shifts the intestinal microbiota from a more evenly distributed and complex community to one that is simpler and dominated by certain bacterial families.[84] Proposed reasons for intestinal dysbiosis in CKD patients include a direct effect of urea and subsequent increased production of ammonia by gut bacteria, increased excretion of uric acid and oxalate, and formation of uremic enterocolitis. Other causes of dysbiosis in people with CKD include reduced fiber intake and frequent use of antibiotics.[85]

1.3.3 Diagnostic Evaluation of the Gut Microbiome

To better understand the pathogenesis of the morbidity and mortality in CKD patients, researchers are evaluating the intricate and complex relationship between the gut microbiome and host health. Using techniques to characterize the genes contained within the gut microbiome (metagenomics) with concurrent evaluation of the small metabolites produced by the gut microbiota (metabolomics) researchers can determine the functional potential of the intestinal microbiome in disease states, including CKD.

Amplicon sequencing of the 16S ribosomal RNA (16S rRNA) gene in fecal samples is the most common (and least expensive) method to identify the bacterial groups confidently to genus level within the fecal microbiome. The method uses a primer to identify a 16S rRNA gene found ubiquitous in bacteria. The gene also has variable regions that differ between bacterial taxa which allows researchers to identify bacteria phylogeny present within a sample. However, 16S rRNA gene sequencing does not confidently provide information on the function of the intestinal microbiota. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) is a computational approach designed to predict metagenome functional content using a 16S rRNA data set and a database of reference genomes. However, PICRUSt can only provide rough estimates of the functional repertoire and thus only theories on the functional capacity of the gut microbiome can be acquired from this method. Because it is only moderately accurate at predicting function, the findings from PICRUSt should then later be proven by the gold standard (i.e. deep whole-genome shotgun metagenomics).[86] Also, 16S rRNA gene sequencing can only identify bacteria and no other microbes (fungus, protozoa, viruses) within the ecosystem.[87] An additional disadvantage of 16S rRNA gene sequencing is the unequal amplification among 16S rRNA genes of different bacterial species, thereby overestimating or not detecting bacterial taxa. 16S rRNA gene sequencing also only gives relative abundances of bacterial taxa, not true bacterial counts.

The preferred method to evaluate the microbial composition and functional gene potential of the fecal microbiome is deep whole-genome shotgun metagenomics. Deep whole-genome shotgun metagenomics sequencing generates accurate taxonomic and functional profiles of the microbiome with species-level resolution.[88] Shotgun sequencing also allows for the detection of low abundance microbial communities. Therefore, shotgun metagenomic analysis is the

preferred method to thoroughly evaluate the composition of the gut microbiota and to describe the genetic potential of the microbiome. Shallow-sequence shotgun metagenomics is a middle ground option between 16S rRNA gene sequencing and deep whole-genome shotgun metagenomics. In comparison to 16S rRNA gene sequencing, shallow shotgun sequencing recovered more accurate species-level taxonomic and function profiles of the human microbiome. Research into the fecal metagenome in healthy cats[88] and humans[89] has identified an immense array of bacterial functions contained in the intestinal microbiome beyond metabolism of nutrients including RNA and DNA metabolism, regulation and cell signaling, and membrane transport to name a few.

Only a subset of genes are expressed at a given time, thus metagenome sequencing can only tell us the genetic potential of a microbial ecosystem. In order to determine function within a disease state, application of other methods is required to elucidate mechanisms by which microbiome perturbation can cause a phenotypic change in the host. Options include metatranscriptomics, metaproteomics, and metabolomics. Metabolomics aims to identify small metabolites (< 1000 Daltons) within a sample. Metabolomics is the most common method utilized because it is thought to better represent the disease phenotype.[90] Untargeted metabolomics allows for the unbiased identification of hundreds of metabolites within a sample produced by the bacterial microbiota and host cells. Using the untargeted approach, researchers can analyze the metabolic interactions between the microbiome and host in dysbiosis, discover pathophysiologic pathways, and identify new biomarkers in disease states. Metabolites identified in untargeted approaches can then be used in quantitative targeted studies to define their clinical significance.[82] Untargeted metabolomics on urine, serum, and fecal samples has been performed extensively in human medicine and the technique has been successful in identifying metabolite

profiles that correspond to early CKD diagnosis, disease prediction, and risk of disease progression independent of estimated GFR.[91-94] Some metabolite biomarkers have better performances in CKD stratification than creatinine, the main marker used in veterinary medicine to determine renal function.[94,95] Although untargeted metabolomics has not been used in cats or dogs with CKD, there are examples of veterinary researchers successfully using untargeted metabolomics in dogs[96] and cats[97] with chronic enteropathy to identify unique metabolic pathways.

1.3.4 Role of Dysbiosis and Gut Microbial Metabolites in Chronic Kidney Disease

A growing body of evidence in human medicine implicates the microbiome and its metabolites directly in CKD pathogenesis. Metagenomics and untargeted metabolomic profiling in people have identified gut-derived uremic toxins as biomarkers of CKD progression and mortality and as therapeutic targets.[92,95,98,99]

People with CKD have an increased number of bacteria that produce the major uremic toxins IS, p-cresol sulfate (pCS), and trimethylamine-N-oxide (TMAO).[100-102] Intestinal dysbiosis impairs intestinal barrier function allowing absorption of these toxins and translocation of bacteria.[103,104] In people, the accumulation of IS and pCS in systemic circulation has been associated with progression of CKD[105,106] by inducing renal inflammation, damaging renal tubular cells, promoting renal fibrosis, and stimulating the progression of glomerular sclerosis.[107-109] TMAO is also elevated in CKD patients and contributes to progressive renal fibrosis and mortality risk.[110] The systemic absorption of uremic toxins also worsens cachexia associated with uremia.[111] Using metagenomics and untargeted metabolomics in people with end-stage renal failure[95] and rat models,[112] researchers have shown that renal failure is associated with increased abundances of proteolytic bacteria, increased amounts of undigested

amino acids in the colon, and increased concentrations of serum (including IS, pCS, TMAO) and fecal metabolites that are by-products of gut microbial protein digestion. This supports that CKD leads to prominent perturbations of the pathways of amino acid metabolism and protein malassimilation[113] in the intestines which explains the increased production of gut-derived uremic toxins IS, pCS, and TMAO.

Additionally, people with CKD have a reduced number of beneficial colonic microbiota that produce straight-chain short-chain fatty acids (SCFA).[100-102] The SCFAs produced by the colonic microbiota consist of the straight-chain SCFAs acetic acid, propionic acid, butyric acid, valeric acid, and the branched-chain (BCFA) SCFAs isovaleric acid and isobutyric acid. The straight-chain SCFAs are the most abundant SCFAs in the human intestinal tract, representing 90-95% of the SCFA present in the colon.[114] Straight-chain SCFAs are major end-products of saccharolytic fermentation of complex polysaccharides (including non-digestible dietary fibers) and epithelial-derived mucus. Straight-chain SCFAs are essential nutrients vital for both intestinal and host-health.[115] They have several beneficial local and systemic effects including promotion of colonic motility, lipid and glucose metabolism, blood pressure regulation, and anti-inflammatory properties.[116-121]

In contrast, BCFAs consist of only a small portion (5%) of total SCFA production. Similar to the major gut-derived uremic toxins, BCFAs are produced when protein passes through the small intestine unabsorbed and protein-derived branched chain amino acids are fermented by microbiota in the colon.[115,122,123] Branched-chain SCFAs and other products of protein fermentation in the colon are considered deleterious to the gut, and may serve as an instigator of inflammation as well as have negative effects on motility.[122-124]

1.4 Manipulation of the Gut Microbiome in Chronic Kidney Disease

1.4.1 The Use of Probiotics in Chronic Kidney Disease

Probiotic supplementation as an adjuvant therapy in people with CKD has emerged in the recent years because of the relatively low cost of probiotics, increased general interest in gut health, and the thought that probiotics could potentially alter the gut microbial composition and accumulation of gut-derived uremic toxins in systemic circulation. Several studies evaluated the effect of probiotics in people with CKD; some showed benefit and some showed no remarkable changes. It is difficult to compare between studies because of differences in study duration, dose, diversity of probiotic strains, and samples sizes. A recent meta-analysis of randomized controlled trials[125] was performed on studies that lasted at least 4 weeks in people with CKD; eight studies were included in the meta-analysis. The major finding was that probiotics could reduce the concentrations of pCS and increase concentrations of IL-6. Probiotics were found to have no effect on serum creatinine, blood urea nitrogen, and hemoglobin concentrations. Although only 50% of the studies reported adverse events, only one study reported a case of vomiting and nausea during probiotic supplementation.[126]

Limited information is available regarding the use of probiotics in dogs and cats with CKD. In a double-blinded, controlled clinical trial in 10 cats with CKD, daily administration of a 3 strain probiotic-prebiotic (psyllium husk) combination (synbiotic; Azodyl®, Vetoquinol, USA) did not improve azotemia over a 2-month period compared to CKD cats that were fed only psyllium husk powder.[127] In another study,[128] dogs were fed a renal diet and the high-dose, multi-strain (4 strains) probiotic VSL#3 (n=30) for 2 months and were compared to CKD dogs fed a renal diet (n=30). The study showed that GFR evaluated through plasma clearance of iohexol significantly increased in the dogs that were fed VSL#3 (median: 37 ml/min/m^2 at

enrollment vs 30 ml/min/m^2 at week 8; P = 0.0002) and decreased in the control group (median: 40 ml/min/m^2 at enrollment vs 48 ml/min/m^2 at week 8; P = 0.001) over the 2-month period. Interestingly, the serum creatinine and UPC increased in the VSL#3 group, yet the creatinine was unchanged and the UPC decreased in the control group over the 2-month study period. Neither study evaluated clinical parameters, markers of uremia (such as gut-derived uremic toxins), or fecal microbial composition. In addition, neither study reported adverse effects. The probiotic *E. faecium* SF68 is known to be an immune modulator in cats and dogs but whether those effects are beneficial when fed to dogs and cats with CKD is unknown.[129,130]

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CHAPTER 2: RESEARCH OVERVIEW AND SPECIFIC AIMS

2.1 Research Overview

Chronic kidney disease (CKD) is a common cause of illness and death in senior cats affecting up to 80.9% of cats above the age of 10 years.[1,2] Despite the high prevalence of CKD in cats, the underlying etiology in the majority of cases is not identified at the time of diagnosis, and treatment options are limited to dietary manipulation and supportive care.[3] The goals of the research described in this dissertation are to explore novel etiologies of kidney disease in cats, assess a research model to study the disease in cats, and explore the gut microbiome as a treatment target. Chapter 3 evaluates *Bartonella henselae* as a novel cause for kidney and urinary disease in adult cats. Chapter 4 evaluates the potential link between parenteral vaccination and development of interstitial nephritis in healthy adult cats and assesses a potential research model to study interstitial nephritis. Chapter 5 characterizes the fecal microbiome and specific bacterial metabolites in the serum and feces of cats with CKD. Chapter 6 describes a study that fed a commercially available probiotic to CKD cats. Finally, Chapter 7 describes the biological variation estimates for the main gut-derived uremic toxins indoxyl sulfate (IS), p-cresol sulfate (pCS), and trimethylamine n-oxide (TMAO) in healthy adult research cats.

2.2 Specific Aim 1 (Chapter 3: *Bartonella henselae* in Cats with Kidney Disease)

Bartonella henselae is a fastidious, gram-negative, intracellular bacteria, and domestic cats are the main reservoir hosts. The prevalence of seropositivity and bacteremia in the United States is up to 67% and 33%, respectively, depending on geographic location.[4] Cats are usually subclinical carriers, however evidence suggests that *B. henselae* may be associated with urinary

disease in cats including lymphocytic interstitial nephritis in experimentally infected cats and various undifferentiated diseases of the urinary tract in client-owned cats with chronic infection.[5-9] However, the evidence that *B. henselae* could be an infectious cause of kidney disease in cats varies between studies. Therefore, the primary aim was to determine the estimated prevalence rates of *B. henselae* IgG in serum and *Bartonella* spp. DNA in blood and urine of client-owned cats with evidence of kidney dysfunction and compare to age-group matched cats (≥ 5 years) with normal kidney function within the same geographic location. The secondary aim was to evaluate for an association between *B. henselae* seropositivity and evidence of proteinuria, hematuria, and pyuria. **The hypothesis was that cats with evidence of kidney dysfunction or cats with evidence of urinary tract disease on urinalysis will be more likely be positive in a *B. henselae* test than control cats.** Demonstrating an association between urinary disease in cats, specifically CKD, will provide veterinarians with another diagnostic and treatment option for cats with newly diagnosed disease and potentially prevent progression to end-stage disease if recognized early. In this retrospective case-control study, EDTA whole blood, serum, and urine samples (total sample sets n=456; kidney disease n=117; controls n=339) were obtained from two IDEXX Laboratories located in California and Massachusetts. *Bartonella henselae* IgG serology and *Bartonella* spp. PCR on urine and whole blood were performed.

2.3 Specific Aim 2 (Chapter 4: Evaluation of a Model of Interstitial Nephritis in Cats)

The Crandell-Rees feline kidney (CRFK) cell line is used to propagate the viruses in many of the manufactured feline herpesvirus-1, calicivirus, and panleukopenia virus-containing vaccines (FVRCP).[10] Hyperinoculation with CRFK cell lysates with subsequent booster 1 year

later in healthy adult purpose-bred cats led to the development of interstitial lymphoplasmacytic nephritis in 50% of the cats.[11] Although annual or frequent vaccination was found to be a risk factor for the development of azotemic CKD in cats, no direct link between parenteral vaccination and CKD in cats has been established. The finding that hyperinoculation with CRFK cell lysates lead to histological findings like those found in cats with early CKD makes this method a possible non-lethal model to study the disease. Therefore, the primary aim was to evaluate whether hyperinoculation with a market leading FVRCP parenteral vaccine over a 16-week period would induce renal changes consistent with feline interstitial nephritis and change α-enolase distribution within the kidney. The secondary aims were to assess whether hyperinoculation induces a humoral and cell-mediated immune-response towards α-enolase and changes serum and urine concentrations of inflammatory cytokines and kidney injury markers, respectively. **The hypothesis was that hyperinoculation with a parenteral FVRCP vaccine over a short 16-week period will lead to immune sensitization to ∝-enolase and induce histologic changes within the kidney.** In order to test this hypothesis, renal histopathology, renal α-enolase immunohistochemical stain, serum anti-enolase and anti-CRFK absorbance levels, lymphocyte proliferation assay, serum cytokine/chemokine panel, and a human urinary kidney injury marker panel were compared before (Week 0) and 2 weeks after hyperinoculation (Week 16) with a FVRCP vaccine.

2.4 Specific Aim 3 (Chapter 5: The Fecal Microbiome in Cats with Chronic Kidney Disease)

In people and recently in cats with CKD,[12] gut-derived uremic toxins are documented to accumulate in systemic circulation and are markers of disease progression.[13-15] The production of

the major gut-derived uremic toxins IS and pCS are directly linked to dysbiosis documented in patients with CKD.[16,17] Colonic bacteria produce IS and pCS by fermenting amino acids. The dysbiosis in uremic patients leads to the overproduction and translocation of these molecules.[18] Other products of fiber and protein fermentation (straight-chain [SCFA] and branch-chain short-chain fatty acids [BCFA], respectively) in the colon by bacteria play a role in pathogenesis of CKD and are deranged in CKD patients as a consequence of dysbiosis and protein malassimilation.[17] The role of IS, pCS and short-chain fatty acids play in patients with CKD has not been explored in veterinary medicine. Various studies have evaluated the gastrointestinal microbiome of cats in health and in disease,[19-26] but the fecal microbiome in cats with CKD and its association with IS and pCS have not been characterized. The primary aim was to characterize the fecal microbiome and evaluate fecal SCFAs in cats with CKD and compare to a group of healthy senior control cats. A secondary aim was to correlate finding to serum IS and pCS concentrations. **The hypothesis was that cats with CKD would have an altered fecal microbiome, higher serum concentrations of IS and pCS, and an altered fecal fatty acid profile when compared to healthy senior control cats.** This was investigated by collecting serum and voided fecal samples from client-owned CKD cats and healthy senior cats. Sequencing of the 16S rRNA gene to characterize the fecal microbiome, fecal stable isotope dilution gas chromatography-mass spectrometry to measure fecal fatty acid concentrations, and serum liquid chromatography with tandem mass spectrometry to measure serum IS and pCS concentrations were performed.

2.5 Specific Aim 4 (Chapter 6: Manipulation of the Gut Microbiome with SF68)

Because gut-derived uremic toxins, particularly protein-bound IS and pCS, are inefficiently removed by hemodialysis, there is a focus in human medicine to reduce production of these toxins by the use of probiotics. In people, probiotics have been shown to reduce concentrations of pCS and modulate the immune system.[27] There is weak evidence that probiotics may also increase GFR in dogs with CKD.[28]

To date, the impact of probiotics on the fecal microbiome, serum concentrations of gut-derived uremic toxins, and clinical parameters in CKD cats has not been evaluated. The aim was to evaluate the effect a commercially available probiotic formulated for cats has on the fecal microbiome, serum concentrations of gut-derived uremic toxins (IS, pCS, TMAO), and clinical parameters. **The hypothesis was that dietary manipulation of the gut microbiome with probiotics will alter the fecal microbiome, reduce serum concentrations of gut-derived uremic toxins and improve signs of nausea.** This was evaluated in a prospective, double blinded, placebo-controlled study evaluating the use of the probiotic *Enterococcus faecium* SF68 with a palatability enhancer in client-owned cats with CKD.

2.6 Specific Aim 5 (Chapter 7: Biological Variation of Major Gut-Derived Uremic Toxins)

Liquid chromatography with mass spectrometry is a method to measure the major gut-derived uremic toxins IS, pCS, and TMAO in plasma or serum. Understanding biological variation and knowing how to interpret data is of paramount importance for both research purposes and to assess clinical utility of measuring IS, pCS, and TMAO in samples from client-owned cats with CKD. Analytical measurements all have variability from multiple sources. In addition to expected changes in the physiological status of the patient and analytical imprecision,

biological variation is a source of variability and describes the physiological random fluctuation around a homeostatic set point. Assessment of biological variation for an analyte is necessary to interpret clinical test results, including whether a population-based reference interval is appropriate for the analyte.

The studies from Chapter 5 and 6 showed significant inter- and intra-individual variation of IS and pCS in CKD cats raising the question of the clinical utility of measuring these metabolites, either in a research setting or on the clinical floor. Although an established reference interval and biological variation estimates have been established in healthy humans for serum total IS and pCS,[29] the biological variation for IS, pCS, and TMAO has not been explored in veterinary medicine. In addition, it is unknown to what degree recent feeding affects serum concentrations of these uremic toxins. Therefore, the aim was to measure biological variation estimates and the Index of Individuality for serum total IS, pCS, and TMAO concentrations in healthy adult cats. The secondary aim was to measure the difference in serum concentrations in fasted versus unfasted samples to determine optimal testing conditions to be used in the field. **The hypothesis was that, like humans, the use of subject-based reference intervals should be used for monitoring of changes in serial results and that serum concentrations of uremic toxins will increase shortly after feeding.** This aim was achieved by collecting serum samples on repeat occasions in healthy adult research cats after feeding and after fasting for 12 hours and sampling multiple times over a 3-week period.

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CHAPTER 3: ASSESSMENT OF *BARTONELLA SPP.* AS A NOVEL INFECTIOUS CAUSE OF KIDNEY DISEASE IN ADULT CATS

3.1 Summary

Background: Bartonella spp. are vector-transmitted Gram-negative intracellular bacteria and several species (particularly *B. henselae*) cause chronic subclinical infections in experimentally and naturally infected cats. Chronic kidney disease is common in senior cats and an underlying etiology is often not identified at the time of diagnosis. *Bartonella henselae* DNA has been amplified from the kidney and urine of experimentally infected cats, and there is an association between urinary disease and *B. henselae* antibodies in cats with naturally acquired infection.

Objectives: The primary objective was to measure *B. henselae* IgG in serum and to amplify the DNA of *Bartonella* spp. from blood and urine of cats with evidence of kidney dysfunction and compare the results to those from geographical and age-group matched cats (≥ 5 years) with normal kidney function. The secondary objective was to evaluate for associations amongst *B. henselae* IgG in serum and the presence of proteinuria, hematuria, and white blood cells (2-5/HPF) on urinalysis.

Samples: Serum, EDTA whole blood, and urine samples from cats ≥ 5 years of age were obtained from IDEXX laboratories located in Massachusetts and California USA. Data from CBC, serum creatinine, blood urea nitrogen, symmetric dimethylarginine, urinalysis with urine dipstick and sedimentation was collected.

Methods: Cases were defined as having evidence of kidney dysfunction based on a urine specific gravity ≤ 1.035 and serum symmetric dimethylarginine ≥ 15 μg/dL. Cats were excluded if serum total thyroxine concentration was elevated (> 4.7 μg/dL). Data was collected from 447 cats, of

which 116 cats had evidence of kidney dysfunction and 331 cats had normal kidney function. An IgG ELISA using *B. henselae* as the antigen source was performed on all serum samples and a positive titer was defined as ≥ 128. Estimated *B. henselae* serum antibody prevalence rates were determined and compared between cats with and without kidney dysfunction, proteinuria (urine dipstick ≥ 1+), hematuria (> 5 RBC/HPF), and the presence of white blood cells (> 2-5 WBC/HPF). *Bartonella* spp. PCR was performed on paired whole blood and urine samples from 106 of the 116 cats with kidney dysfunction.

Results: The estimated *B. henselae* IgG seroprevalence rates in cats from the eastern United States (51%) and the western United States (47%) were both high and were not significantly different between cats with normal kidney function and cats with kidney dysfunction. No differences in estimated *B. henselae* IgG seroprevalence rates were found between cats with and without proteinuria, hematuria, and the presence of WBCs on urine sediment. *Bartonella* spp. DNA was amplified from the blood of one of 106 cats with kidney dysfunction but none of the urine samples.

Conclusion: Bartonella henselae IgG antibodies were not associated with kidney dysfunction, bacteriuria, proteinuria, hematuria, or the presence of WBC on urinalysis. *Bartonella* spp. DNA was only amplified from the blood of one cat with kidney dysfunction, and specific DNA was not amplified from any urine sample. Based on these results, routine testing for *B. henselae* is not warranted in the evaluation of kidney dysfunction or urinary disease in older cats.

3.2 Introduction

Bartonella spp. are vector-transmitted, gram-negative, intracellular bacteria that infect cats and multiple other animals.[1,2] Transmission of *Bartonella henselae, B. clarridgeiae*, and *B. koehlerae* is primarily by *Ctenocephalides felis*, exposure to flea feces, or direct blood inoculation. Tick transmission may also occur with some *Bartonella* species.[3] Some cats infected with *B. henselae* or other species exhibit acute clinical signs of disease (fever, lymphadenopathy) and most cats are believed to become chronic subclinical carriers.[4-6] *Bartonella henselae* causes Cat Scratch Disease in people which is characterized by self-limiting regional lymphadenopathy.[1,2] A variety of other inflammatory conditions have also been associated with infection in people.[3] In people with chronic kidney disease (CKD) and in post-renal transplantation patients, *B. henselae* is a cause of persistent fever and lymphadenopathy and uncommonly causes necrotizing glomerulonephritis in cases of culture-negative endocarditis.[7,8]

Most cats that are naturally or experimentally infected with *Bartonella* spp. do not show clinical signs. When bartonellosis is suspected, the diagnosis is based on the presence of compatible clinical signs, exclusion of other compatible diagnoses, and detection of specific serum antibodies, culture proven infection, or amplification of specific DNA. *Bartonella* spp. infection in cats is associated with chronic bacteremia; *B. henselae* infection is most commonly detected.[4,6,9,10] Since bacteremia can be chronic, it has been theorized that *B. henselae* may be associated with chronic clinical conditions, including CKD, in client-owned cats. Experimental inoculation with *Bartonella* spp. in young cats has been documented to cause interstitial lymphocytic nephritis, hematuria, and increases in serum creatinine from baseline. Additionally, *Bartonella* DNA could be amplified from kidney tissue and urine in some cats after experimental

inoculation.[4,11] Retrospective studies in client-owned cats have found associations amongst *B. henselae* serum antibodies and hematuria and other undifferentiated urinary diseases.[12,13] However, a clear correlation between *B. henselae* infection and kidney disease or other urinary tract abnormalities has not been proven.

To date no study has specifically evaluated for associations between *Bartonella* spp. test results and urinary disease in cats. Therefore, the primary aim of this study was to determine the estimated prevalence rates of *B. henselae* IgG in serum and *Bartonella* spp. DNA in blood and urine of client-owned cats with evidence of kidney dysfunction and compare to an age-group matched cats (≥ 5 years) with normal kidney function within the same geographic location. The secondary aim was to evaluate for associations between *B. henselae* IgG antibody prevalence and evidence of protein, red blood cells, and white blood cells in the urine based on microscopic examination of urine sediment.

3.3 Materials and Methods

3.3.1 Sample Selection

In this retrospective case-control study, the records of two commercial diagnostic laboratories (IDEXX Laboratories, Inc., North Grafton, MA 01536; IDEXX Laboratories, Inc., West Sacramento, CA 95605) were searched to find cats ≥ 5 years of age that had remnant serum as well as results of complete blood count (CBC), serum biochemical profile, and urinalysis with urine dipstick and sedimentation. Blood in EDTA and urine were available from some cats. The samples were collected between February and October 2018 and were frozen at -70°C until utilized for this study.

3.3.2 Medical Data Collection

The age, breed, sex, and state of sample collection were obtained from the laboratory submission form. Cat breeds were recorded as domestic shorthair (DSH), domestic longhair (DLH), or a specific purebred. Sex was recorded as intact female, spayed female, intact male, or neutered male. Data from CBC and the serum creatinine, blood urea nitrogen (BUN), symmetric dimethylarginine (SDMA), and urinalysis with sedimentation were available from all cats. Additional values from the serum biochemistry panel and a serum total thyroxine concentration were recorded when available. The following laboratory values were recorded: hematocrit (HCT) and serum BUN, creatinine, SDMA, total calcium, phosphorus, potassium, total thyroxine. Urine specific gravity (USG) was determined by refractometer. The following data was recorded from urine dipstick analysis: pH, protein, glucose, ketones, and bilirubin. With the exception of urine pH, the remaining variables were recorded as trace, 1+, 2+, 3+. The presence, and if positive estimation, of urine bacteria, white blood cells, and red blood cells was determined by direct microscopy of urine sediment.

Cases were determined to have evidence of kidney dysfunction when USG ≤ 1.035 and serum SDMA ≥ 15 μg/dL. Based on these criteria, cats were separated into those with normal kidney function or those with kidney dysfunction. Cats with an elevated total thyroxine, when available, were excluded. Historical and clinical findings of the cases were not available.

3.3.3 *Bartonella henselae ELISA and Bartonella Species PCR* Assay

Samples were shipped frozen overnight to the research laboratory on cold packs and stored at -70°C until assayed. After being thawed at room temperature, sera were assayed with a previously validated IgG ELISA using *B. henselae* as the antigen source that is offered at an accredited commercial laboratory (www.dlab.colostate.edu). After being thawed at room

temperature, both the urine and whole blood samples were processed for DNA extraction. The urine samples were centrifuged at 16,000 g for 30 minutes at 4°C. The supernatant was removed while preserving the urine pellet undisturbed. Five hundred microliters of sterile phosphate buffered solution (PBS) was added to reconstitute and wash the urine pellet by brief vortexing. The PBS-urine pellet mix was centrifuged for 15 minutes at 21,000 g at room temperature. The supernatant was carefully pipetted off the pellet and the pellet was suspended in 200 µL PBS. Total DNA was extracted from each 200 µL washed urine pellet and 200 µl of each blood sample and then assayed for *Bartonella* spp. DNA using a previously validated PCR assay offered at the same commercial laboratory. The estimated sensitivity of this assay is 3.5 ng total DNA.

3.3.4 Statistical Analysis

Titers are reported as the reciprocal of the last positive dilution. Estimated prevalence rates and 95% confidence intervals were determined using an on-line program (http://vassarstats.net/prop1.html). The remainder of the statistics were performed using Prism version 8.3.0. Descriptive statistics were generated for each continuous laboratory value and data were analyzed for normality by the Shapiro-Wilk test. A Mann-Whitney U test was performed to evaluate for difference in age, renal clinicopathologic variables, and urine specific gravity (USG) between cats with kidney dysfunction and cats with normal kidney function. The resulting p-values of the Mann-Whitney U test were adjusted for multiple comparisons using Benjamini and Hochberg's false discovery rate. *Bartonella henselae* IgG titers were log transformed and Mann-Whitney U test was used to compare titers between cats with kidney dysfunction and cats with normal kidney function. The chi-square test was performed to determine differences in proportions of positive *B. henselae* IgG titers in cats with evidence of kidney dysfunction and in

cats with normal kidney function. The analysis was performed using results from all samples and with the samples separated into those from western United States (California, Nevada, Oregon), and those from the eastern United States (Connecticut, Maine, Massachusetts, New Hampshire, New York, Rhode Island). A chi-square test was performed to determine differences in proportions of positive *B. henselae* IgG titers in samples with proteinuria (urine dipstick ≥ 1+ protein), hematuria (>5 RBC/high powered field [HPF]), and presence of white blood cells (2-5 WBC/HPF). The analysis was performed on all samples, samples with evidence of kidney dysfunction, and samples with normal kidney function. A chi-square test was performed to determine the difference in proportions of positive *B. henselae* IgG titers in samples obtained from the western United States and the eastern United States. Spearman correlation coefficient (rho) was computed to evaluate the association between the following variables: age, BUN, creatinine, SDMA, USG, and log transformed positive *B. henselae* IgG titer.

3.4 Results

3.4.1 Description of Cats

A total of 447 cats had remnant sera and results of CBC, serum creatinine, BUN, SDMA, and urinalysis with sedimentation that were available for review. A complete serum biochemistry panel was available for 422 cats. A total serum thyroxine concentration was available for 415 cats.

The majority of samples were obtained from veterinary clinics in California (184/447) followed by Massachusetts (135/447), Connecticut (52/447), New York (25/447), New Hampshire (18/447), Nevada (14/447), Maine (9/447), Rhode Island (8/447), Oregon (1/447) and Hawaii (1/447). Of the 447 total sample sets, 55% (247/447) were obtained from clinics in the

eastern United States and 45% (199/447) were obtained from clinics in the western United States.

Of the 447 cats, 116 cats had evidence of kidney dysfunction (26%) and 331 cats had normal kidney function (74%). The cats with kidney dysfunction (median, 15.0; range, 4.9-20.8 years) were significantly older than the cats with normal kidney function (median, 10.3; range, 6.0-19.0 years; $P < 0.0001$). For the cats with evidence of kidney dysfunction, 46% were reported to be spayed females, 49% were neutered males, and 4% were intact males. Of the cats that had a breed reported (96/116), the majority were DSH cats (62/96) followed by DLH (17/96), Siamese (5/96) and miscellaneous breeds (< 5 cats each of American Shorthair, Balinese, Birman, Maine Coon, Persian, Rag Doll, and Russian Blue). For the cats with normal kidney function, 50% were reported to be neutered males, 45% were spayed females, 3% were intact males, and 3% were intact females. Of the cats that had a reported breed (266/331), the majority were DSH (173/266) followed by DLH (48/266), Siamese (14/266), Persian (6/266) and miscellaneous breeds (<5 cats each of Abyssinian, Bengal, Himalayan, Maine Coon, Norwegian Forest Cat, Rag Doll, Scottish Fold, and Snowshoe).

3.4.2 Clinicopathologic Data

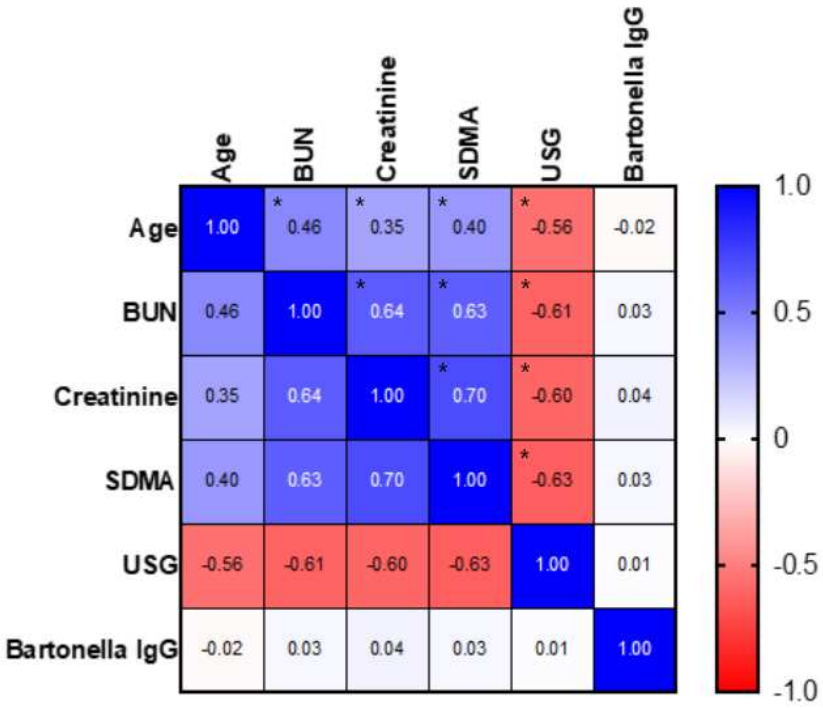
A summary of select continuous values from CBC and serum biochemistry panel and USG for cats with kidney dysfunction and cats with normal kidney function are summarized in Table 3.1. After adjusting for multiple comparisons, several significant differences were found between cats with and without kidney dysfunction. As expected, cats with kidney dysfunction had significantly higher serum creatinine, BUN, SDMA and lower hematocrit, reticulocyte count, and urine specific gravity compared to cats with normal kidney function ($P < 0.0001$). Serum potassium was not different between groups.

Table 3.1 Renal clinicopathologic variables and urine specific gravity (median and range) for cats with evidence of kidney dysfunction and cats with normal kidney function.

Value (Normal Range)	Kidney Dysfunction n=116		Normal Kidney Function n=331	
	Median	Range	Median	Range
Hematocrit (28.2-52.7%)	31.8	16.4-50.3	39.9	13.6-51.2
Reticulocyte (3-50 K/μL)	12	0-140	17	0-185
Blood urea nitrogen (16-37 mg/dL)	59	29-240	25	12-45
Creatinine (0.9-2.5 mg/dL)	3.3	2.6-17.5	1.3	0.5-2.4
SDMA (0-14 μg/dL)	24	15-100	10	3-14
Calcium (8.2-11.2 mg/dL)	10	6.8-12.6	9.5	7.9-12
Phosphorus (2.9-6.3 mg/dL)	5.5	4.6-28.1	4.2	1.5-6.8
Potassium (3.7-5.2 mEq/L)	4.4	2.8-5.8	4.3	3.0-6.0
Urine Specific Gravity	1.014	1.007-1.033	1.046	1.035-1.066

Age, USG, serum BUN, creatinine, and SDMA were significantly correlated with each other ($P < 0.0001$). Log transformed *B. henselae* IgG titers did not significantly correlate to age, USG, serum BUN, creatinine, or SDMA. The Spearman correlation coefficient (rho) for each comparison is reported in Table 3.2.

Table 3.2 Spearman correlation coefficient for age of cats, urine specific gravity (USG), and serum blood urea nitrogen (BUN), creatinine, and symmetric dimethylarginine (SDMA). Asterisk signifies a significant finding (P < 0.0001).



3.4.3 *Bartonella henselae* ELISA and *Bartonella* Species PCR

Bartonella henselae ELISA was performed on all 447 serum samples. No significant difference was found in the log transformed *B. henselae* IgG titers between cats with evidence of kidney dysfunction and cats with normal kidney function (P = 0.390). A chi-square test of independence showed that there was no significant association between kidney function and *B. henselae* IgG titer (≥ 128) for all samples, samples from western United States, and samples from the eastern United States (Table 3.3). No significant association was found between geographic location (eastern United States, 48%; western United States, 46%) and *B. henselae* IgG titer (≥ 128; $\chi^2 = 0.183$; P = 0.669). No significant association was found between

B. henselae titer (≥ 128) and the presence of proteinuria, hematuria, or presence of WBCs for all cats, cats with kidney dysfunction, and cats with normal kidney function (Table 3.4).

Of the 106 paired serum and urine samples from cats with kidney dysfunction, only 1 whole blood sample was positive for *Bartonella* species antigen based on PCR.

Table 3.3 Prevalence of positive *Bartonella henselae* IgG titer (≥ 128) in cats with evidence of kidney dysfunction and cats with normal kidney function. Degrees of freedom is 1 for all analyses.

	All Samples (n=447)		**Eastern United States (n=250)**		**Western United States (n=196)**	
	Kidney Dysfunction	Normal	Kidney Dysfunction	Normal	Kidney Dysfunction	Normal
Number of samples	116	331	63	187	53	143
Seroprevalence (confidence interval)	48% (39-57)	48% (43-53)	43% (31-55)	50% (43-57)	53% (40-66)	45% (37-53)
Chi-square statistic value	0.002		0.964		1.012	
P-value	0.965		0.326		0.314	

Table 3.4 *Bartonella henselae* seroprevalence (≥ 128) in cats with and without hematuria (>5 RBC/HPF), proteinuria (urine dipstick ≥ 1+ protein), and presence of white blood cells (2-5 WBC/HPF).

	All Samples (n=447)					
	Proteinuria	Normal	Hematuria	Normal	WBC (2-5/HPF)	Normal
Number of samples	109	338	128	319	69	378
Seroprevalence % (confidence interval)	52 (43-61)	47 (42-52)	49 (40-57)	48 (43-53)	54 (42-65)	46 (41-51)
Chi-square statistic value	0.911		0.008		1.349	
P-value	0.340		0.927		0.245	
	Kidney Dysfunction (n=116)					
	Proteinuria	Normal	Hematuria	Normal	WBC (2-5/HPF)	Normal
Number of Samples	30	86	36	80	31	85
Seroprevalence % (confidence interval)	47 (30-64)	49 (39-59)	56 (40-70)	45 (35-56)	55 (38-71)	46 (36-56)
Chi-square statistic value	0.838		0.292		0.393	
P-value	0.042		1.108		0.730	
	Normal Kidney Function (n=331)					
	Proteinuria	Normal	Hematuria	Normal	WBC (2-5/HPF)	Normal
Number of Samples	80	251	92	239	38	293
Seroprevalence % (confidence interval)	51 (40-62)	46 (40-52)	47 (37-57)	49 (43-55)	55 (40-70)	47 (41-53)
Chi-square statistic value	0.718		0.207		0.898	
P-value	0.397		0.649		0.343	

3.5 Discussion

We retrospectively evaluated the *B. henselae* IgG antibody prevalence in older (≥ 5 years) client-owned cats in the United States with documented kidney dysfunction based on clinicopathologic parameters. Additionally, we evaluated antibody prevalence in the same population of cats with evidence of urinary tract disease based on finding of proteinuria, hematuria, or the presence of WBCs in the urine on a complete urinalysis with sedimentation. Seropositivity determined by ELISA were similar to those reported in the United States.[14] Because of the high seroprevalence rates in all sample groups, we were unable to make associations between urinary disease and presence of *B. henselae* in our population of cats. Age was likely a cofounder when assessing the relationship between the prevalence of seropositivity and presence of kidney dysfunction since positive serology is associated with an increasing age based on findings of previous studies.[13-15] The median age of the cat population in the present study was 11.4 years which explains the lack of correlation between *B. henselae* IgG titer and age. This finding is consistent with a previous study evaluating *Bartonella* antibody serology in cats.[16]

Despite a high prevalence of positive serum titer in cats with kidney dysfunction, bacteremia was rare, with *Bartonella* spp. DNA being amplified from only 1 of 106 whole blood samples from cats with kidney dysfunction tested by PCR. This supports exposure to *B. henselae* and not an active infection in most cats with kidney dysfunction. The prevalence of bacteremia in a previous study of client-owned cats in California was higher and ranged from 4.4-47.7%.[15] It is important to note that our study included only client-owned cats, and we excluded cats under the age of 5 years to target older cats whom are more likely to have kidney disease.[17] Thus, the demographics of our study population may be a partial explanation for the low level of

bacteremia. Retrospective studies evaluating *Bartonella* antibody and bacteremia prevalence in the United States have shown that young (< 1 year) outdoor or stray cats are more likely to be bacteremic, likely because of increased exposure to fleas.[14,15] Unfortunately, the indoor/outdoor status of the cats included in our study was unknown. Another explanation for the rare bacteremia in our study population are false negative results because cats experimentally infected with *B. henselae* can be intermittently bacteremic for several months after blood inoculation.[15,18] It is also possible that many of the cats in this study were being administered flea control products. If so, new infections would be unlikely, and it has been previously documented that *B. henselae* bacteremia is self-limited.[6]

Bartonella DNA was not amplified from the urine of cats with kidney dysfunction in the present study. In experimentally infected cats with blood inoculation, *Bartonella* DNA has been amplified from urine[11] and from renal tissue.[4] Although *Bartonella* DNA has been amplified from renal tissue and urine in experimentally infected cats, to date *Bartonella* spp. DNA has not been isolated from these sample in client-owned cats.[4] The failure to amplify *Bartonella* spp. DNA from urine in our population of pet cats may be due to a potentially higher bacterial load used to infect research cats allowing evasion of the immune system in comparison to client-owned cats infected by flea dirt and presumably a smaller bacterial load. In addition, a difference in immune function between pet cats and research-bred cats may be a contributing factor. In immunocompromised people, *B. henselae* infections are more severe and long-standing compared to people with normal immunity.[19] Lastly, it may also be that our population of older cats were not truly infected with a *Bartonella* spp. at the time of sample collection.

Antibody-antigen immune complex deposition within the glomerulus is a cause of glomerulonephritis and is associated with infections in both dogs[20] and cats.[21] In people, there

are reports of *B. henselae* causing endocarditis-associated immune-complex glomerulonephritis and acute kidney injury.[8] Recently, a retrospective study found that cats with glomerular disease were young (median age, 3 years) and 72% had immune-complex glomerulonephritis. Of these cats with follow-up, 81.4% became azotemic and the median age of death was 3.6 years.[22] *Bartonella henselae* could potentially be a cause of immune-complex glomerulonephritis considering *B. henselae* infection is most common in young cats[14,15] and prolonged bacteria is a source of chronic immune stimulation.[4] Evidence of chronic immune stimulation in cats with *B. henselae* infection is supported by a positive association with polyclonal hyperglobulinemia[23] and lymphocytosis[12] with *Bartonella* spp. antibodies. Future studies evaluating a possible association between young cats with glomerular disease, particularly immune-complex glomerulonephritis, and *B. henselae* bacteremia may be warranted.

A previous study showed that sick cats from North Carolina with blood present in the urine were 2.31 times more likely to have a positive *B. henselae* IFA titer.[12] In addition, *B. clarridgeiae* DNA was isolated from the urine of an experimentally-infected cat whom also had a concurrent three-fold increase in hematuria. On the contrary, our study did not find an association between hematuria and positive *B. henselae* IgG titer in our population of older cats. The one cat with kidney dysfunction that was positive for *Bartonella* spp. DNA on whole blood had an IgG titer of 64 and significant proteinuria (4+ on urine dipstick) with no hematuria on microscopic evaluation of urine sediment.

The retrospective nature of the study is a limitation. Medical information provided to the laboratory was limited to patient signalment and geographic location of sample collection. Clinical history, including clinical signs of disease, antibiotic usage, flea control product usage, indoor/outdoor status or flea exposure is unknown for this subset of cats. Importantly, it is

unknown whether the kidney dysfunction was due to acute injury or chronic disease and the underlying etiology of the kidney dysfunction is unknown. Absolute pyuria (> 5 WBC/HPF) was not found in any cat in the study population, therefore authors were not able to evaluate the relationship between pyuria (> 5 WBC/HPF) and *B. henselae* IgG titers in the present study. The presence or absence of proteinuria was based on the results of urine dipstick alone which can be non-specific in cats, especially with 1+ result, and sensitivity can vary depending on USG.[24,25] Therefore, it is possible that the number of cats with proteinuria was overestimated in our study. Cross-reactivity amongst other *Bartonella* species is possible for the *B. henselae* IgG ELISA performed in the present study. Future studies with additional clinical context and a larger study population, including young cats, would be beneficial to better elucidate a relationship between *B. henselae* infection and kidney disease. The evaluation of *B. henselae* DNA in kidney tissue of cats with kidney disease, in particular young cats with proteinuria, should be explored in future prospective studies.

In conclusion, *B. henselae* IgG antibodies, bacteriuria, or bacteremia were not associated with kidney dysfunction, proteinuria, hematuria, or pyuria in client-owned cats above the age of 5 years in the United States. Therefore, routine testing for *B. henselae* in not warranted in the evaluation of kidney dysfunction or urinary disease in older cats.

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CHAPTER 4: ASSESSMENT OF A MODEL OF INTERSTITIAL NEPHRITIS AND EVALUATION OF THE POTENTIAL LINK BETWEEN PARENTERAL FVRCP VACCINATION AND INTERSTITIAL NEPHRITIS IN CATS

4.1 Summary

Background: The feline herpesvirus-1, calicivirus, and panleukopenia virus-containing vaccine (FVRCP) is recommended for all cats. Frequent vaccination is a risk factor for chronic kidney disease in cats. Alpha-enolase is an immunodominant antigen contained in all FVRCP vaccines and exposure during vaccination may be a cause of interstitial nephritis.

Objective: Evaluate whether cats hyperinoculated over a 16-week period with a market leading α-enolase containing FVRCP vaccine would develop humoral and cell-mediated immune reactions against Crandell-Rees feline kidney (CRFK) cell lysates or α-enolase and induce renal histological changes consistent with feline interstitial nephritis.

Animals: 6 one-year old, mixed sex (3 males; 3 females) research cats

Methods: Cats were administered a commercial FVRCP parenteral vaccine every 2 weeks for a total of 8 vaccinations (Week 0, 2, 4, 6, 8, 10, 12, 14). Renal cortical biopsies were performed prior to vaccination and 2-weeks after the last vaccination (Week 16). Histopathology and α-enolase immunohistochemistry were performed on renal biopsies. Indirect ELISAs were used to measure the absorbance values of anti-α-enolase and anti-CRFK antibodies prior to vaccination on Week 0 and during the hyperinoculation period (Weeks 4, 8, 12, 16). A lymphocyte proliferation assay was performed using peripheral blood mononuclear cells collected on Week 0 and Week 16. A validated chemokine, cytokine, and growth factor feline serum assay and a human urinary kidney injury marker panel was performed at Week 0 and Week 16.

Results: Serum creatinine concentration and urine specific gravity were unchanged during the 16-week study. Histological evidence of interstitial nephritis was not detected by light microscopy in any of the tissue biopsies. Renal tubular and glomerular α-enolase immunoreactivity scores based on immunohistochemical stain were significantly (P = 0.02) greater at Week 16 when compared to baseline. Serum anti-enolase and anti-CRFK absorbance values were significantly higher at Week 16 when compared to Week 0 (P <0.0001). Serum concentrations of Flt-3L, GM-CSF, CCL5/RANTES were significantly different at Week 16 when compared to Week 0. The kidney injury markers uromodulin, calbindin, and retinol binding protein 4 were detected in the urine of a majority of cats at Week 0 and Week 16. *Conclusions:* Young healthy adult cats that were vaccinated with a parenteral FVRCP vaccine 8 times over 16-week period did not develop histologic evidence of interstitial nephritis, however renal tubular and glomerular α-enolase immunoreactivity scores increased with hyperinoculation. Hyperinoculated cats mounted a humoral and cell-mediated immune response to the α-enolase antigen contained within the vaccine and select inflammatory markers increased in the sera. Uromodulin and calbindin should be evaluated as potential markers of tubular injury in cats.

4.2 Introduction

Chronic kidney disease (CKD) is an irreversible and progressive disease with prevalence up to 80.9% in cats greater than 10 years of age.[1,2] On renal histology, chronic lymphoplasmacytic tubulointerstitial nephritis and renal fibrosis are the most common findings with, in many cases, no underlying etiology identified.[3] In addition to age and severe periodontal

disease,[4] annual or frequent vaccination was found to be a risk factor for the development of azotemic CKD in cats.[5]

Many organizations like the American Association of Feline Practitioners (www.catvets.org) and the World Small Animal Veterinarian Association (www.wsava.org) recommend administration of feline herpesvirus-1 (FHV-1), calicivirus (FCV), and panleukopenia virus (FPV)-containing vaccines (FVRCP) to all cats. The vaccine viruses used to produce the vaccines are all grown on mammalian cell cultures. Since the epidemiological association between frequent vaccination of cats and CKD exists, it is important to provide adequate immunization to protect against important pathogens, but not lead to inadvertent significant side-effects.[5]

Although most excipient proteins used to grow vaccine viruses are removed during production of the vaccines, small concentrations remain. It is possible that these proteins could be associated with the development of CKD in cats. The Crandell-Rees feline kidney (CRFK) cell line was the first to be evaluated in studies that attempted to determine why frequent vaccination could potentially be linked to CKD in cats.[6,7] Parenteral inoculation of healthy purpose-bred cats with a parenteral FVRCP vaccine produced in CRFK cells (4 total vaccinations on weeks 0, 3, 6, 50) or inoculated with CRFK cell lysates (12 total injections on weeks 0, 2, 4, 6, 8, 12, 16, 20, 24, 32, 40, 50) without vaccine viruses, developed antibodies against the CRFK cell lysates and against a lysate of feline renal cells.[7] The 2 cats administered a FVRCP vaccine for intranasal administration in that study did not develop antibodies against CRFK lysates or renal cell lysates, presumably because the excipient antigens are killed and so excluded by the mucosa. None of the cats in this first study had evidence of renal disease on histology of renal biopsies taken 6 weeks after their last injection (week 56).[7]

Cats from the first study that had been administered the FVRCP vaccine for intranasal administration, CRFK lysates at 10 µg per dose, 50 µg per dose, or 50 µg per dose plus an aluminum containing adjuvant were housed for 1 year without other manipulations. At 52 weeks after the last hyperinoculation, the cats (2 per group) were administered a booster of the intranasal FVRCP vaccine or CRFK lysate concentration from the previous study and renal biopsies obtained 2 weeks later. While not present on the renal biopsies collected previously, lymphoplasmacytic interstitial nephritis was seen in 3 of 6 cats hyperinoculated with CRFK lysates but not the cats administered the intranasal FVRCP vaccine.[8]

In a subsequent study, kittens were either vaccinated with FVRCP vaccines or received CRFK cell lysate injections. Serum from cats were used to determine the immunodominant antigens inducing antibodies against the CRFK lysates which were then identified by protein sequencing.[9] The most immunodominant antigen was α-enolase, a glycolytic enzyme that is widely distributed in the body and found in greatest concentration in the kidney and thymus.[9] Cats administered parenteral, but not the intranasal FVRCP vaccine were shown to have rapidly increasing absorbance values to α-enolase.[9] This enzyme is present in all mammalian cells and so all vaccines for cats are likely to have some level of α-enolase contamination. Anti-α-enolase antibodies are detected in 67-80% of patients with autoimmune nephritis associated with systemic lupus erythematous (SLE) compared to 6% in healthy controls.[10,11] In humans, α-enolase autoantibodies are nephrogenic by inducing endothelial cell injury and cell death through an apoptotic process and by immune-complex deposition.[12,13] Based on these findings, α-enolase was chosen for continued study, including in the paper described here.

In humans, renal α-enolase is found primarily in the cytoplasm and cell membrane of the epithelial cells of the tubules and is nearly undetectable in the glomeruli.[14,15] In patients with

SLE, α-enolase expression is increased in the tubules, but also expressed in variable regions of the glomeruli including mesangium, in glomerular and parietal epithelium, and in crescents.[16] Recently, the distribution of α-enolase in cats with and without kidney disease has been described using a α-enolase immunohistochemical stain. In young cats (<2 years), α-enolase was found in the tubules and absent in the glomeruli. In senior cats (>10 years), α-enolase was found in both the tubules and glomeruli. In cats with CKD, α-enolase immunoreactivity was decreased in atrophic tubules, similar to healthy cats in normal tubules, and increased in the glomeruli. Data suggests that α-enolase changes distribution in the kidney prior to development of CKD in cats.[17]

Inflammation and immune system activation are important causal factors in people with kidney disease.[18] During ischemic acute kidney injury, the renal tubular epithelial cells generate pro-inflammatory cytokines that include tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), IL-1 beta (IL-1β), transforming growth factor beta (TGF-β) and the chemokines monocyte chemoattractant protein-1 (MCP) and CCL5 which potentiate inflammation and promote renal immune cell infiltration.[19-21] In both human and veterinary medicine, functional renal serum markers (creatinine, symmetric dimethylarginine [SDMA]) are insensitive[22] and nonspecific[23] for the detection of acute kidney injury. In people, a number of other urinary and serum inflammatory biomarkers have been developed that can detect acute kidney injury much earlier than functional markers.[24-26] A few biomarkers of kidney injury (F2-isoprostanes, kidney injury molecule-1, neutrophil gelatinase-associated lipocalin, cystatin C)[27-29,40] and urinary inflammatory cytokines concentrations (TGF-β, IL-8)[30,31] have been previously evaluated in cats with CKD. A previously validated, commercially available feline-specific bead-based multiplex assay[a] for detection of cytokines, chemokines, and growth factors in cats is commercially available.[32] A commercially available assay titration for humans to assess for urinary biomarkers

of kidney injury is available[b] and several of the molecules are homologs to the domestic cat *Felis catus*.

A model to induce renal inflammation similar to that found in most cats with CKD is greatly needed to study ways to more accurately diagnose, treat, and prevent the syndrome. Since interstitial nephritis was noted in the previous CRFK lysate hyperinoculation study[7] we believed it reasonable to explore derivations of this model further, but within a more compressed time schedule. Thus, the primary aim of the study was to evaluate whether hyperinoculation with a market leading FVRCP vaccine that contained α-enolase over a 16-week period would induce renal histological changes consistent with feline interstitial nephritis and changes to the distribution of α-enolase as determined by immunohistochemical staining. The remaining aims of the study were to determine whether the hyperinoculated cats would:

1. develop humoral and cell-mediated immune reactions against CRFK lysates and α-enolase (Aim 2);
2. develop changes in serum cytokine and chemokine concentrations (Aim 3).
3. develop changes in urine concentrations of acute kidney injury molecules (Aim 4).

4.3 Materials and Methods

4.3.1 Vaccine

A total protein level after reconstitution was performed using a refractometer on five market leading FVRCP parenteral vaccines.[c-g] The vaccine with the highest total protein concentration and the suspected highest α-enolase contamination was used in the study.[d]

4.3.2 Study Design and Selection of Animals

In this prospective cohort study, young (3-month-old), mixed sex cats (3 males; 3 females) were purchased from an FHV-1, FCV, and FPV-free barrier facility (Liberty Research Laboratories). Prior to entering the study at 1 year of age, the kittens were group housed in a barrier facility and the males were neutered. The cats were previously maintained as unvaccinated controls in an FHV-1 inoculation study at 5 months of age. In that study, the cats developed mild signs of FHV-1 infection. All cats were asymptomatic for FHV-1 at enrollment.

The cats were anesthetized, and the three females were ovariectomized. A wedge of renal cortical tissue (5 mm wide x 3 mm deep x 3 mm long) from the left kidney was surgically obtained from each cat either during the ventral celiotomy (in females) or by flank approach (in males). Six weeks after surgery, the cats were administered the parenteral vaccine[d] (Week 0) and then boosted on Weeks 2, 4, 6, 8, 10, 12, and 14. On Week 16, renal biopsies were performed on the right kidney as described via flank approach in all study cats. All cats were administered oral buprenorphine for three days after each surgery for post-operative pain control. Renal tissues were fixed in 10% neutral buffered formalin solution, paraffin embedded and sectioned at 5 microns.

Blood was collected via jugular venipuncture and urine was collected via cystocentesis on Weeks 0, 2, 4, 6, 8, 10, 12, and 16. Blood was immediately placed into a sterile tube without anticoagulant. All samples were stored on ice after collection until processing within 3 hours of collection. Non-heparinized blood was centrifuged at 2,500 rpm for 10 minutes and the serum was removed. The serum was aliquoted and stored at -80°C. At Weeks 0, 8, and 16, serum was submitted for serum biochemical panel and urine was submitted for urinalysis and urine protein: creatinine ratio (UPC) at Weeks 0, 8, and 16.

The cats were housed and cared for in accordance with a protocol that was approved by the Institutional Animal Care and Use Committee at the contract research facility that was used for the study (HQR protocol 170.016). The approval included a rescue clause that allowed for vaccinations to be discontinued in any cat that developed a creatinine concentration of greater than 2.0 mg/dL, inappetance, vomiting, or signs of systemic hypersensitivity reactions. All cats were sedated with ketamine 10 mg IV for blood draws and cystocentesis procedures.

4.3.3 Histologic Evaluation and Enolase immunohistochemistry

The renal biopsy paraffin sections were stained with haematoxylin and eosin (H&E) stain and enolase immunohistochemistry was performed as previously described.[12] Each H&E and enolase immunohistochemical stained tissue section (one from each cat before vaccination and after hyperinoculation on Week 16) was evaluated for histopathological evidence of interstitial nephritis and a α-enolase immunoreactivity score was performed by a board-certified pathologist blinded to the timing of the biopsies. For the α-enolase immunoreactivity score, each tissue section was assigned a score based on signal intensity. Scoring was defined as 0 = no staining; 1 = light brown/tan, 2 = moderate brown staining; and 3 = dark brown which may obscure the nucleus. Ten high powered fields (40X) were selected from a grid for each section of kidney. Twenty renal cortical tubules and twenty glomeruli from each section were scored.

4.3.4 Anti-CRFK and Anti-Enolase ELISA

Two indirect ELISAs as previously described[7] were used to measure the absorbance values of anti-α-enolase (ENO1) and anti-CRFK antibodies. Sera from the 6 cats at Week 0 and during the 16-week hyperinoculation period (Weeks 4, 8, 12, and 16) were used in the assays. Briefly, a CRFK cell lysate and a commercially available Enolase 1 Recombinant protein (ENO1)[h] were each diluted in 0.06M carbonate buffer (pH 9.6) and 100 µL was added to the

appropriate wells on two separate micro-ELISA plates.[i] Both plates were incubated overnight at 4°C. Upon removal from 4°C, the CRFK cell lysate plate was washed three times with phosphate-buffered saline (PBS)-Tween (Tw) 20 solution (PBS-Tw) and then each appropriate well blocked with 100uL 1% casein acid hydrolysate for one hour at approximately 20°C. The ENO1 plate was not blocked following overnight incubation. Both plates were washed three times with PBS-Tw before pipetting 100µL of properly diluted hyperinoculated cat sera in triplicate wells from Weeks 0, 4, 8, 12, and 16. Known CRFK/ENO1 positive and negative cat sera were diluted and run the same as the hyperinoculated samples. Each plate also had duplicate wells of a PBS-TW (reagent only) control. The plates were incubated with the primary antibody (hyperinoculated samples and controls) for 30 minutes at 37°C. The PBS-Tw wash cycle was repeated. One hundred microliters of properly diluted secondary antibody and goat anti-cat IgG (heavy chain only)-horseradish peroxidase[j] in PBS-Tw was pipetted into appropriate wells and incubated for another 30 minutes at 37°C. Each plate was washed as previously described and 100µL of TMB (3,3',5,5'-tetramethylbenzidine) substrate[k] added to all wells. The enzyme reaction was stopped at 10 minutes after incubation at 20°C with a 0.18M H_2SO_4 stop solution. Each plate's optical density absorbance was read with an automated micro-ELISA reader[l] at 450nM. The mean absorbance value of each control and hyperinoculated cat sample was calculated.

4.3.5 Peripheral Blood Mononuclear Cell Isolation and Lymphocyte Proliferation Assay

To evaluate the cell-mediated immune response toward α-enolase and CRFK cell antigen, an lymphocyte proliferation assay was performed using peripheral blood mononuclear cells (PBMC). Eight weeks after the last vaccination, whole blood was collected from 4 of 6 study cats. Whole blood was also collected from 2 young, specific-pathogen free, unvaccinated

control cats. Peripheral blood mononuclear cells were separated from EDTA whole blood via Ficoll centrifugation[m] and washed with PBS. Cells were pelleted by centrifugation at 1200 RPM for 5 min at 8 C. The medium was removed, and the cells were plated in a 96-well plate at a concentration of 5×10^5 cells/well. 5-ethynyl-2'-deoxyuridine[n] (EdU) was added to each well at a final concentration of 1uM. The following experimental groups were used (in duplicate): PBMC control; PBMC and concanavalin A[o] (ConA); PBMC and α-enolase antigen; PBMC and CRFK antigen; PBMC and splenic cells; PBMC and kidney cells. Fresh kidney and spleen samples were collected during necropsy from a young, healthy cat. Cells were incubated for 72 h at 37°C, after which time cells were harvested, washed and then re-suspended in FACS buffer (2% FBS, 0.05% sodium azide, 1 × PBS). The amount of lymphocyte proliferation was analyzed via a Cyan ADP flow cytometer.[p] For the purpose of comparison, lymphocytes stimulated with ConA were set to 100% proliferation.

4.3.6 Serum Inflammatory Cytokine and Chemokine Panel

Sera from study cats at Week 0 and Week 16 were assayed using the commercial feline-specific multiplex bead-based assay[a] that measures the following 19 inflammatory cytokines and chemokines: first apoptosis signal receptor (sFas), fms-like tyrosine kinase-3 ligand (Flt3L), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12 (p40), IL-13, IL-18, CCL5/RANTES, CXCL1, CXCL12, MCP-1, platelet derived growth factor (PDGF)-BB, stem cell factor (SCF), TNF-α. Serum samples were assayed according to manufacturer recommendations and serum analyte concentrations were measured using a multiplexing platform.[q]

4.3.7 Urinary Human Kidney Injury Markers

Urine from study cats obtain at Week 0 and Week 16 were assayed using commercial

human multiplex bead-based assays[b] that measures the following molecules: calbindin, glutathione S-transferase pi (GSTπ), osteoactivin, glutathione S-transferase alpha (GSTα), tissue inhibitor of metalloproteinase 1 (TIMP-1), kidney injury marker-1 (KIM-1), IP-10, renin, fatty acid binding protein-1 (FABP-1), collagen IV, TFF-3, uromodulin, retinol binding protein-4 (RBP4), and beta-2 microglobulin (B2M). Briefly, urine samples were diluted 1:3 for Panel 1 and 1:500 for Panel 3 in Assay Buffer provided in the kits. The paired samples were run in duplicate following manufacturer specifications for each assay and urine analyte concentrations were measured using a multiplexing platform.[q]

4.3.8 Statistical Analysis

Statistical analysis was performed using R studio v8.3.0 (R-Tools Technology, Inc, Boston, MA). Normality of data was assessed by the Shapiro-Wilk normality test. Serum creatinine, blood urea nitrogen (BUN), and phosphorus concentrations, anti-enolase and anti-CRFK serum absorbance levels, serum cytokine and chemokine concentrations, and urinary kidney injury markers were compared between Week 0 and Week 16 using paired Student t test for parametric data and Wilcoxon matched-pairs signed rank test for non-parametric data. The mean tubular and glomerular immunohistochemical stain score were compared between baseline and Week 16 using a Wilcoxon matched-pairs signed rank test (one-tailed).

4.4 Results

4.4.1 Serum Biochemical Kidney Parameters and Urinalysis

The serum creatinine was not significantly different at Week 16 (median, 1.2; range, 0.9-1.4 mg/dL) when compared to Week 0 (median, 1.4; range, 1.0-1.4; P = 0.13) and remained less than 1.4 mg/dl. The serum BUN was significantly lower at Week 16 (median, 26; range, 23-29 mg/dL) when compared to Week 0 (median, 32; range, 25-34 mg/dL; P = 0.03). Serum

phosphorus and globulin were not significantly different at Week 16 when compared to Week 0, P = 0.09 and P = 0.06 respectively. The urine specific gravity remained above 1.045, urine protein:creatinine ratios were normal (< 0.15), and no urine casts suggestive of tubular injury were found on urine microscopy throughout the study period. Other biochemical parameters (including serum sodium and potassium concentrations) remained within the normal range throughout the study period for all 6 cats.

4.4.2 Histological Evaluation

Histological evidence of interstitial nephritis was not detected by light microscopy in any of the tissue biopsies collected on Week 0 and Week 16. Renal tubular and glomerular α-enolase immunoreactivity scores were significantly higher at Week 16 (tubules: median, 1; range, 1-2; glomeruli: median, 2; range, 1-3) when compared to Week 0 (tubules: median, 0; range, 0-1; glomeruli: median, 1; range, 0-2; P=0.02). As an example, Figures 4.1 and 4.2 represent renal biopsy with α-enolase immunochemical stain at baseline and on Week 16 in a research cat.

4.4.3 Anti-CRFK and Anti-Enolase ELISA

Prior to vaccination (Week 0), the mean serum absorbance value for anti-CRFK and α-enolase antibodies was low and similar to the negative control. During the study, the concentration of antibodies against CRFK antigen and α-enolase in sera of all 6 study cats increased after each vaccination in this 16-week model (Table 4.1). Serum anti-enolase and anti-CRFK absorbance values were significantly higher at Week 16 when compared to Week 0 (P <0.0001).

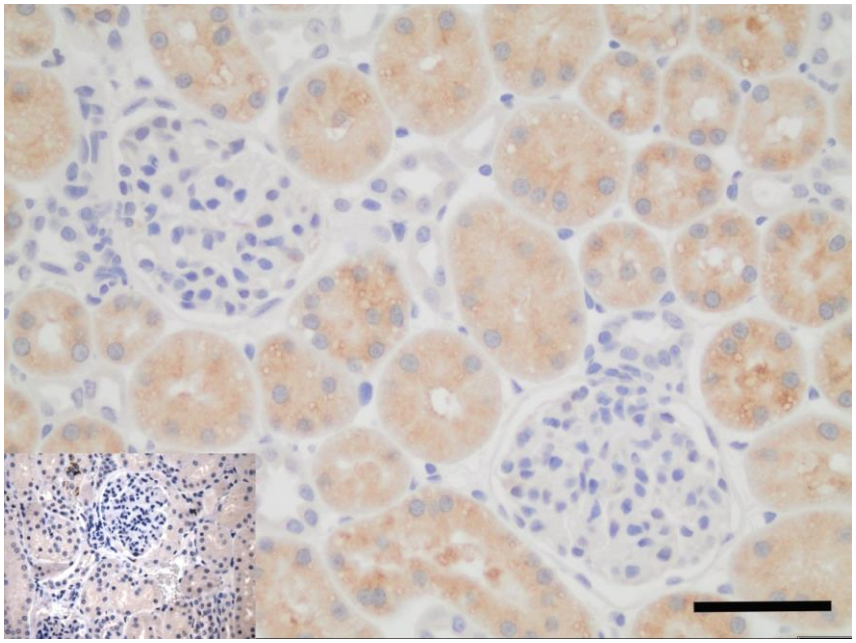

Figure 4.1 Alpha-enolase immunohistochemistry of the kidney from a healthy research cat prior to vaccination (Week 0). There is minimal staining in glomeruli and mild, monochromatic staining of tubules. Black bar = 50 μm. Inset: negative control.

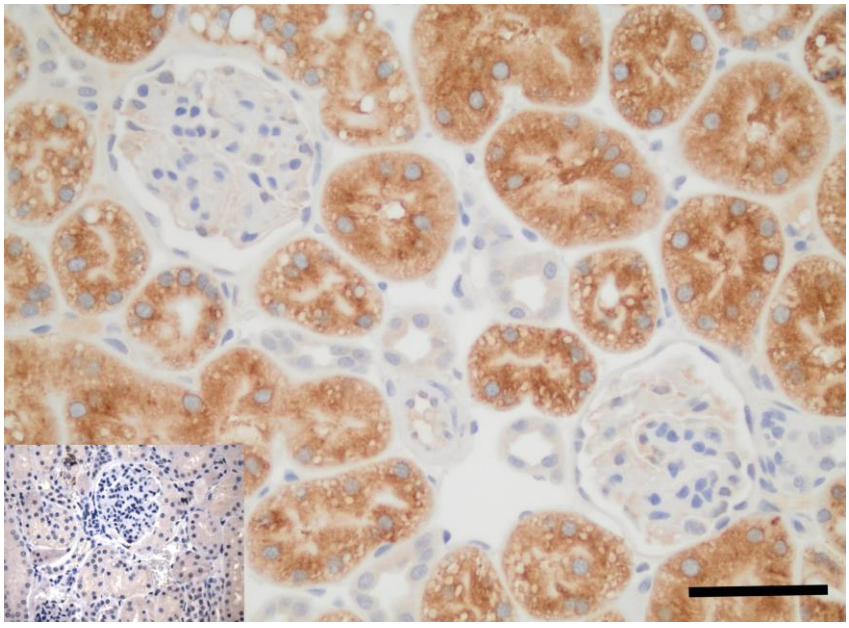

Figure 4.2 Alpha-enolase immunohistochemistry of the kidney from a healthy research cat 2 weeks after vaccine hyperinoculation (Week 16). There is minimal to mild staining in glomeruli with moderate, monochromic staining of tubules. Black bar = μm. Inset: negative control.

Table 4.1 Mean and standard deviation (SD) absorbance values for antibodies against α-enolase and CRFK cell antigen prior to vaccination (Week 0) and after vaccination (Weeks 4, 8, 12, 16) with a FVRCP vaccine in six cats.

Week	**0**	**4**	**8**	**12**	**16**
Anti-enolase	0.57 ± 0.16	2.00 ± 0.74	3.48 ± 0.72	3.82 ± 0.47	3.88 ± 0.43
Anti-CRFK	0.28 ± 0.03	0.66 ± 0.23	1.38 ± 0.39	1.63 ± 0.37	1.81 ± 0.4

4.4.4 Lymphocyte Proliferation Assay

Lymphocytes from hyperinoculated cats proliferated significantly (P = 0.028) more in the presence of α-enolase when compared to the unvaccinated control cats confirming the induction of a cell-mediated immune response toward α-enolase contained within the vaccine. No significant difference was found in lymphocyte proliferation between hyperinoculated study cats and unvaccinated control cats for the feline spleen, kidney, and CRFK cell antigen (Figure 4.3).

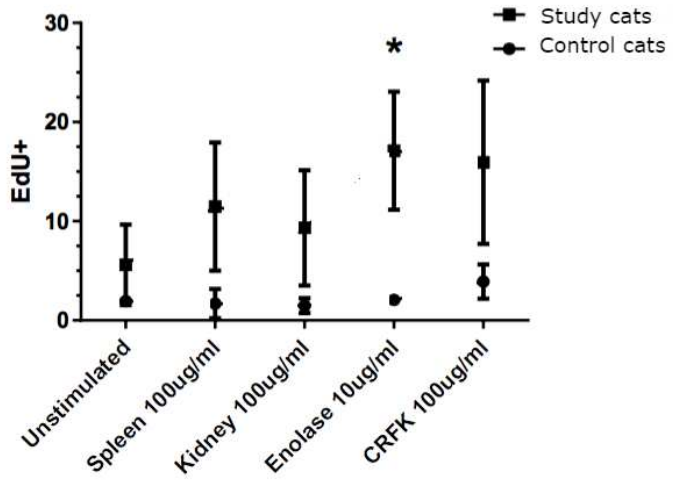

Figure 4.3 Lymphocyte proliferation assay against spleen, kidney, α-enolase, Crandell-Rees feline kidney (CRFK) cell antigens for 4 study cats and two unvaccinated control cats.

4.4.5 Serum Inflammatory Cytokines and Chemokines and Human Kidney Injury Biomarkers

Two of 19 analytes (Fas, IL-2) were excluded for analysis because they were detectable in an insufficient number of samples to make a meaningful comparison. Serum concentrations of 3 analytes were significantly different at Week 16 when compared to Week 0 (Flt-3L, GM-CSF, CCL5/RANTES) and 4 analytes approached significance (IFN-γ, IL-4, CXCL12, IL-8; Table 4.2).

Urinary calbindin, uromodulin, and RBP4 were detected in all 6 cats at least one time point (Week 0 and/or Week 16). Although the median urine concentrations of the 3 markers decreased over the 16-week period, no significant difference in the urine concentrations at Week 16 when compared to Week 0 was found for calbindin ($P = 0.312$), uromodulin ($P = 0.438$), and RBP4 ($P = 0.844$). The remainder of the analytes were not detected at any time point.

4.5 Discussion

In the United States, veterinary pharmaceutical companies use the CRFK cell line or other cell lines to propagate the feline viruses contained in FVRCP vaccines (both parenteral and intranasal). Vaccination contamination with the α-enolase antigen likely occurs in all vaccines that propagate the viruses with any cells line since this glycolytic pathway enzyme is in all mammalian cells. During virus purification for vaccine production, it is impossible to remove all CRFK proteins, thus CRFK proteins are contained in the vaccine. As a consequence, cats immunized with the vaccine become exposed and may mount an immune response against CRFK cell proteins as well as the vaccine viruses. Based on results of the indirect ELISA and lymphocyte proliferation assay, our study confirms that the parenteral FVRCP vaccine used in our study contains CRFK cell antigens (including α-enolase) and that hyperinoculation with the

Table 4.2 Summary of 19 serum cytokine, chemokine, and growth factor concentrations (pg/mL) in 6 hyperinoculated cats prior to vaccination (Week 0) and 2 weeks after a 14-week series of FVRCP vaccines (Week 16; 8 vaccines total).

	Week 0	**Week 16**	
Variable (pg/mL)	**Mean +/- SD**	**Mean +/- SD**	**P-value**
Flt-3L	134.4 +/- 30.7	58.2 +/- 27.3	0.004*
IL-12 p40	288 +/- 131.2	198 +/- 103	0.213
SCF	105 +/-12.7	97.7 +/- 87.7	0.854
	Median (range)	**Median (range)**	**P-value**
GM-CSF	0 (0)	10.1 (0-37.9)	0.018*
IFN-γ	0(0)	206 (0-477.3)	0.059
IL-1β	0 (0)	32.9 (0-124.1)	0.181
IL-4	0 (0-92.9)	508 (0-1690)	0.059
CXCL12	0 (0-1012)	1291 (289-1479)	0.063
IL-6	0 (0)	136 (0-380)	0.100
MCP-1	0 (0)	2713 (0-8042)	0.181
IL-13	0 (0)	54.5 (0-98.5)	0.100
IL-8	0 (0-16.8)	33 (0-77.9)	0.059
PDGF-BB	0 (0)	448 (0-1012)	0.174
CCL5	5.8 (0-38.7)	49.9 (19-168)	0.031*
IL-18	0 (0)	112 (0-1225)	0.181
TNF-α	0 (0)	33.4 (0-188)	0.181
CXCL1	0 (0-8.4)	6.5 (0-20.4)	0.281

Abbreviations: CCL2, C-C motif chemokine ligand 2; CCL5/RANTES, C-C motif chemokine ligand 5; CXCL1, C-X-C motif chemokine ligand 1; CXCL12, C-X-C motif chemokine ligand 12; CXCL8, C-X-C motif chemokine ligand 8; IL-8, interleukin 8; Flt3L, fms-related tyrosine kinase 3 ligand; GM- CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon gamma; IL-12 (p40), interleukin 12 subunit p40; IL-13, interleukin 13; IL-18, interleukin 18; IL-1β, interleukin 1 beta; IL- 2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; PDGF-BB, platelet-derived growth factor subunit B; SCF, stem cell factor; sFas, soluble Fas cell surface death receptor; TNF-α, tumor necrosis factor alpha.

vaccine leads to both a humoral and cell-mediated immune response toward these antigens in healthy cats. The detection of antibodies against CRFK cell antigen after parenteral FVRCP vaccination is consistent with previous findings.[7] To note, the previous study used the same vaccine as our study[d] in addition to 2 other parenteral FVRCP vaccines[r,s] and found that 5 of the 6 healthy cats administered a parenteral vaccine (4 times over 56 weeks) were positive for anti-CRFK antibodies at least once during the study.[7] Two cats in the previous study were vaccinated (4 times over 56 weeks) with a modified-live intranasal/intraocular FVRCP vaccine[t] and they did not develop anti-CRFK antibodies exceeding the positive cutoff value on ELISA.[7] Similar to our study, there was no histologic evidence of permanent renal damage on biopsies associated with administration of FVRCP vaccines, therefore this 16-week model cannot be used to study interstitial nephritis in cats.

To date, there has been no documented direct link between CRFK-contaminated FVRCP vaccines and feline interstitial nephritis. Although intranasal FVRCP vaccines may not induce a systemic immune response towards CRFK cell proteins, it is unknown whether it is a safer option compared to a parenteral FVRCP vaccine. In addition, an intranasal FVRCP vaccine is not an appropriate option for all cats based on their lifestyle and risk of exposure to feline viruses, thus intranasal FVRCP vaccines cannot be used in place of parenteral FVRCP vaccines in all cases. We encourage veterinarians to avoid over-vaccination and to use individualized vaccination protocols as recommended by the American Association of Feline Practitioners.[33]

Hyperinoculation with a parenteral FVRCP vaccine containing CRFK cell proteins (including α-enolase) over the 16-week study did not lead to clinical, hematologic, serum biochemical, urine, or histopathological abnormalities consistent with acute kidney injury or interstitial nephritis. In comparison, a previous study showed that interstitial nephritis was

induced in 50% of cats previously hypersensitized with purified CRFK cell lysate 13 times over a 2-year period.[8] The reason why our study did not lead to histologic evidence of interstitial nephritis is likely multifactorial. First, it is possible that the cats in our study received too little CRFK cell lysate or α-enolase antigen over the 16-week period. Second, formation of interstitial nephritis secondary to an immune-mediated reaction towards CRFK cell lysates may be idiosyncratic and a consequence of the strength of the immune response mounted by the individual cat. Lastly, 16 weeks may not be enough time to identify histopathologic evidence of interstitial nephritis and lesions may have been missed on the renal biopsy if lesions were localized in the kidney.

Alpha-enolase immunoreactivity in the kidneys has been evaluated by immunohistochemistry in healthy people and in people with autoimmune lupus nephritis. In normal human kidneys, α-enolase is highly expressed in tubules and almost undetectable in glomeruli.[14] This was consistent with a previous study in cats[17] and our study prior to vaccination. Renal biopsies from systemic lupus erythematous (SLE) patients had higher α-enolase expression in the tubules and had some expression in the glomeruli in variable sites.[34] The renal biopsies obtained at Week 16 in our study exhibited similar findings. In addition, the data in SLE patients shows that α-enolase is overexpressed in sites of renal inflammation[16] and that anti-enolase serum antibodies correlate with active renal disease.[35] These findings may indicate that in our study the higher renal tubular and glomerular α-enolase immunoreactivity scores and anti-enolase antibody absorbance values noted at the end of the 16-week study supports subclinical renal injury without dysfunction. The mechanism behind α-enolase overexpression and mechanisms of renal injury induced by anti-enolase antibodies in people is unclear but hypothesized to be due to direct cell damage or interference with normal α-enolase

function by autoantibodies.[29] The exact cause of the greater α-enolase immunoreactivity in renal tubules post-vaccination is unknown. An *in vitro* experiment using CRFK cells to determine whether the enolase up-regulation in the renal tubular cells is a direct effect of cytokines on the enzyme is warranted.

With the exception of Flt-3L, IL-12 p40, SCF, all detectable pro-inflammatory cytokine (IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-13, IL-18, and TNF-α), chemokine (CXCL1, CXCL12, CCL5, MCP-1) and growth factor (GM-CSF, PDGF-BB) analytes increased over the 16-week study. Significant changes in serum analyte concentrations over the 16-week study was only found for Flt-3L, GM-CSF, and CCL5; this is likely due to large intra-individual variation in the serum analyte concentrations and type II error. The increasing serum concentrations of a majority of the analytes is most easily attributable to a normal immune response to repeat vaccination. However, interestingly the same 3 of 6 cats did not have detectable serum concentrations at the end of the 16-week study for multiple analytes that would be expected to be increased with vaccination (IL-1β, IL-2, IL-18, TNF-α). In addition, the serum analyte concentrations between cats at the end of the study were highly variable (e.g. serum MCP-1 concentration was undetectable for 3 cats and markedly elevated for the other 3 cats at Week 16). Therefore, the increasing serum concentrations of these cytokines, chemokines, and growth factors over the 16-week study is unlikely to be completely attributable to normal immune response to vaccination and other sources of inflammation (such as renal) should be considered. In addition, it is presumed that the young cats in our study at enrollment were healthy, however it is possible the 3 cats that did not have detectable serum concentrations of the 4 aforementioned analytes had an impaired immune system which could explain this finding.

Although no statistical differences in urine concentrations of the human kidney injury markers calbindin, uromodulin, and RBP4 before and after hyperinoculation were found, the concentrations of the three molecules were decreased at Week 16 when compared to Week 0 in 5/6 cats for calbindin and 4/6 cats for uromodulin and RBP4. These 3 molecules are markers of tubular damage in people. Calbindin is involved in the regulation of calcium absorption in the distal nephron and decreased urinary calbindin concentrations are indicative of decreased renal expression due to distal nephron injuries.[36] Uromodulin is expressed solely in the thick ascending limb. Although absolute excretion of uromodulin declines with reduction in total nephron mass in patients with CKD, urinary concentrations are increased preceding onset of CKD.[37] Retinol binding protein-4 is a marker of proximal tubular damage and urinary concentrations increase in humans[38] and dogs[39] with tubular injury. With the exception of KIM-1,[40] none of the human renal injury markers measured in study have been measured in cats with kidney disease. However, uromodulin and RBP4 are part of the urine proteome in cats, and cats with CKD had an increased expression of RBP4 and decreased expression of uromodulin based on gel electrophoresis.[41] Based on our results, it is likely the human assay cross-reacts with the feline protein for the detected molecules calbindin, uromodulin, and RBP4; however, caution should be taken when interpreting the findings since the human kidney injury marker bead panel has not been validated in cats. Further evaluation of calbindin, uromodulin, and RBP4 using a validated feline-specific assay is warranted to determine the role these markers have in cats with documented tubular injury and CKD.

There were several limitations to our study. We did not quantify the amount of CRFK cell components or α-enolase contained in the lot of vaccines that the cats received. This makes it difficult to make direct comparisons between our study and previous studies utilizing CRFK

cell lysates, although authors believe that that the propensity to develop renal inflammation may be idiosyncratic.[8] Second, the study was designed to be intentionally shorter in duration compared to previous studies[7,8] in order to evaluate the protocol for development of a model to study interstitial nephritis in cats. Although not evaluated, it is possible that interstitial nephritis would be documented after a booster vaccination 1 year later as found in a previous study.[8] Lastly, small sample size and the use of purpose-bred research cats makes it difficult to ascertain the clinical significance of the study findings in client-owned cats.

In conclusion, hyperinoculation with a market leading FVRCP parenteral vaccine over a 16-week period was not associated with detectable renal dysfunction, renal inflammatory disease, or glomerular disease. However, antibody and cell-mediated immune response towards α-enolase, increased α-enolase immunoreactivity scores in renal tubules and glomeruli, and higher serum concentrations of select inflammatory cytokines and chemokines within this 16-week model was found and thus this model warrants further investigation with potentially larger study sizes and longer follow-up periods. This study in conjunction with previous studies supports a link between interstitial nephritis and sensitization to α-enolase.

ENDNOTE

[a]Milliplex MAP Feline Cytokine/Chemokine Magnetic Bead Panel; Millipore-Sigma, Burlington, MA

[b]Milliplex Human Kidney Injury Magnetic Bead Panel, Panels 1 and 3, Merck, Damstadt, Germany

[c]Novibac Feline 3-HCP, Merck Animal Health, Millsboro, DE

[d]Felocell 3, Zoetis, Florham Park, NJ

[e]Fel-O-Vax PCT + Calicivax, Boehringer-Ingelheim Vetmedica, St. Joseph, MO

[f]Ultra Fel-O-Vax FVRCP, Boehringer-Ingelheim Vetmedica, St. Joseph, MO

[g]Purevax Feline 3, Merial, Duluth, GA

[h]Enolase 1 Recombinant Protein, Novus Biologicals, Littleton, CO

[i]Immulon 2HB; Thermo Fisher Scientific, Waltham, MA

[j]Goat-anti-Cat IgG-HRP(heavy chain only), Seracare, Milford, MA

[k]KPL Sureblue™ 1 component TMB Microwell Peroxidase Substrate; Seracare, Milford, MA

[l]Multiskan Ascent; Thermo Fisher Scientific, Waltham MA

[m]LSM Lymphocyte Separation Medium, MP Biomedicals, Santa Ana, CA

[n]Invitrogen, Carlsbad, CA

[o]Sigma Aldrich, Saint Louis, MO

[p]Beckman Coulter, Fort Collins, CO

[q]Bio-Plex 200, Bio-Rad Laboratories, Hercules, CA

[r]Fel-O-Vax, Fort Dodge Animal Health, Overland Park, KS

[s]PureVax, Merial, Athens, GA

[t]Trivalent intranasal/intraocular vaccine, Heska Corp, Fort, Collins, CO

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CHAPTER 5: EVALUATION OF THE FECAL MICROBIOME AND SELECT MICROBIAL METABOLITES IN CATS WITH CHRONIC KIDNEY DISEASE[1]

5.1 Summary

Background: Chronic kidney disease (CKD) in people causes a functional intestinal dysbiosis with alteration of the microbial composition and production of microbial metabolites. Metabolites altered by dysbiosis include straight-chain short-chain fatty acids (SCFAs) and the major gut-derived uremic toxins indoxyl sulfate (IS) and p-cresol sulfate (pCS). Straight-chain and branched-chain (BCFA) short-chain fatty acids are metabolites produced by colonic microbiota via metabolism of complex polysaccharides and protein, respectively. The uremic toxins IS and pCS are also products of protein metabolism by colonic microbiota. Characteristics of the fecal microbiome and fecal fatty acid concentrations with correlation to serum IS and pCS concentrations are unknown in cats with CKD.

Objectives: The objectives of these studies were to a) characterize the fecal microbiome and fecal SCFA concentrations in cats with CKD and b) correlate findings with serum IS and pCS concentrations.

Animals: Client-owned cats with CKD (IRIS stages 2, 3, and 4) and senior (≥ 8 years) healthy control cats.

Methods: Prospective, cross-sectional study. Voided fecal samples were analyzed by sequencing of 16S rRNA genes and *E. coli* quantitative PCR (qPCR). Fecal samples were analyzed using

[1] Summers SC, JM Quimby, A Isaiah, et al. 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(2):662-669.

Summers SC, JM Quimby, RK Phillips, et al. Preliminary evaluation of fecal fatty acid concentrations in cats with chronic kidney disease and correlation with indoxyl sulfate and p-cresol sulfate. J Vet Intern Med 2019;34(1):206-215.

stable isotope dilution gas chromatography-mass spectrometry to determine fecal concentrations of SCFAs. Serum concentrations of IS and pCS were measured using liquid chromatography tandem mass spectrometry.

Results: Cats with CKD had significantly decreased fecal microbial diversity and richness. *E. coli* qPCR showed no significant difference in bacteria count between healthy senior cats and CKD cats. No significant differences in fecal straight-chain SCFAs were found between groups. Fecal concentrations of the BCFA isovaleric acid were significantly higher in cats with CKD compared to healthy control cats ($P = 0.017$). Cats with IRIS CKD stage 3 and 4 had significantly higher fecal isovaleric acid concentrations compared to healthy senior cats ($P = 0.026$). Cats with IRIS CKD stage 2 ($P = 0.01$) and stages 3 and 4 ($P = 0.0006$) had significantly higher serum IS concentrations compared to healthy senior cats. No significant difference was found in serum IS concentrations between stage 2 and stages 3 and 4 CKD cats. The pCS concentrations were not significantly different between CKD cats and healthy control cats. Total fecal concentrations of BCFAs and serum IS concentrations correlated positively with serum creatinine concentration, blood urea nitrogen, and pCS concentrations.

Conclusions: The study demonstrated that CKD is associated with a functional dysbiosis in cats, particularly in late stage disease. 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. Fecal BCFA concentrations and serum IS concentrations were higher in cats with clinical evidence of muscle wasting, providing evidence for protein malassimilation in cats with CKD.

5.2 Introduction

An alteration of the gut microbiome has been associated with many illnesses in humNA including chronic kidney disease (CKD). The uremia associated with CKD has been shown to negatively impact the gut microbiome in humans and rats.[1,2] Influx of urea, uric acid, and oxalate into the colon raises the luminal pH and causes a domination of microbiota that consume these substrates instead of indigestible carbohydrates effectively causing an intestinal dysbiosis.[3,4]

In humans and rats with CKD, intestinal dysbiosis is characterized by a shift in the microbiota to a less diverse community that is dominated by certain bacterial families.[1] These alterations in the intestinal microbiome leads to change in the function and production of microbial metabolites. Important metabolites of the colonic microbiota are short-chain fatty acids (SCFAs) which consist of the straight-chain SCFAs acetic acid, propionic acid, butyric acid, valeric acid, and the branched-chain fatty acids (BCFA) isovaleric acid and isobutyric acid. The straight-chain SCFAs are the most abundant SCFAs in the human intestinal tract, representing 90-95% of the SCFA present in the colon.[5] Straight-chain SCFAs are major end-products of saccharolytic fermentation of complex polysaccharides (including non-digestible dietary fiber) and epithelial-derived mucus, and are essential nutrients vital for both intestinal and host health.[6] They have several beneficial local and systemic effects including promotion of colonic motility, facilitation of lipid and glucose metabolism, blood pressure regulation, and anti-inflammatory properties.[7-12] In contrast, BCFAs represent only a small portion (5%) of total SCFA production, and are produced when protein passes through the small intestine unabsorbed and protein-derived branched chain amino acids are fermented by microbiota in the colon.[6,13,14] Branched-chain SCFAs and other products of protein fermentation in the colon are considered deleterious

to the gut, and may promote inflammation as well as have negative effects on motility in a rodent model.[13-15]

Additional end-products of microbial fermentation of protein in the colon are the protein-bound uremic toxins indoxyl sulfate (IS) and p-cresol sulfate (pCS). Indoxyl sulfate and pCS accumulate in systemic circulation because toxin-producing bacterial families are more prevalent in dysbiosis and reduce glomerular filtration rate.[16] Indoles are produced by the metabolism of dietary tryptophan by tryptophanase in intestinal bacteria including *Escherichia coli* (*E. coli*), *Proteus vulgaris*, and *Bacteroides* spp.[17-19] 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*.[18-20] Indole and p-cresol are absorbed into the blood, metabolized to IS and pCS in the liver, and excreted in the urine.[18,19] 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,[21,22] promoting renal fibrosis,[23,24] and by stimulating progression of glomerular sclerosis.[25] These uremic toxins also contribute to morbidity and mortality by impairing the nervous system,[26] lowering erythropoietin (EPO) production,[27] altering bone turnover,[28,29] and increasing the risk of cardiovascular disease.[30-32]

Various studies have evaluated the gastrointestinal microbiome of cats in health and in disease,[33-40] but the fecal microbiome in cats with CKD has not been characterized. In veterinary medicine, IS concentrations have been correlated to the severity of CKD in cats and dogs,[41] and were found to be an independent predictor for disease progression.[42] However, the role of pCS and SCFAs in patients with CKD has not been explored in veterinary medicine. The primary aim for this chapter was to characterize the fecal microbiome and evaluate fecal SCFAs in CKD cats

and compare to healthy senior control cats. A secondary aim was to correlate findings to serum IS and pCS concentrations. The hypothesis was that cats with CKD would have an altered fecal microbiome, altered fecal fatty acid profile, and high serum concentrations of IS and pCS when compared to healthy senior cats. In addition, concentration of microbial metabolites of protein catabolism (IS, pCS, BCFAs) will have a positive correlation to severity of azotemia, to muscle condition score, and to each other. This will be investigated by collecting serum and voided fecal samples from client-owned cats with CKD and healthy senior cats and performing fecal 16S ribosomal RNA (rRNA) gene sequencing, fecal stable isotope dilution gas chromatography-mass spectrometry to measure fecal fatty acid concentrations, and serum liquid chromatography with tandem mass spectrometry to measure serum IS and pCS concentrations.

5.3 Study Design and Selection of Cats

This was a prospective, cross-sectional study. To be eligible for inclusion, CKD and senior cats underwent a thorough evaluation that included a review of the past medical record, complete physical examination (including 9-point body condition score [BCS; Nestle Purina, St. Louis, MO] and muscle condition score [MCS]), minimum database consisting of CBC, serum biochemistry panel, and urinalysis, serum total thyroxine (T4) concentration, blood pressure, fecal flotation, and urine protein:creatinine ratio (if urine dipstick testing detected ≥ 1+ protein). Evidence of azotemic CKD was defined as a serum creatinine concentration > 1.6 mg/dL on at least 2 time points (over at least 3 months) together with urine specific gravity < 1.035 on at least 1 occasion or an increase in serum creatinine concentration on at least 2 time points together with an increase in symmetric dimethylarginine (SDMA) concentration (>14 μg/dL) interpreted in combination with clinical history and physical examination findings consistent with CKD. The

CKD cats were staged based on International Renal Interest Society (IRIS) guidelines.[43] Senior cats (≥ 8 years) were recruited from employees, students, and staff of the veterinary teaching hospital. Senior cats were considered healthy based on an unremarkable client history and past medical record review, physical examination, and normal laboratory test results including serum creatinine concentration < 1.6 mg/dL and USG > 1.035.

Exclusion criteria based on review of available history, examination and diagnostics tests included complications of CKD such as acute obstructive or neoplastic urinary disease, urinary tract infection, recent hospitalization (< 2 months), medications known to significantly alter the intestinal microbial composition received within 6 weeks before enrollment (e.g. antibiotics, antacids, probiotics) or diseases known to alter the intestinal microbial composition in cats including uncontrolled hyperthyroidism, diabetes mellitus, and known or suspected gastrointestinal disease including intestinal parasitism, food- or antibiotic-responsive chronic enteropathy, and chronic diarrhea.[44] A client questionnaire was provided to the owner and the following information was obtained: diet (brand name and amount offered of wet or dry food or both), current medications or supplements, medications or supplements administered in previous 3 months, appetite and fecal score at the time of enrollment, clinical signs of constipation, and frequency of vomiting. A table with descriptions of MCS, appetite, fecal, and vomiting scores is provided in Table 5.1. The project was approved by the Clinical Review Board (#2016-080) at Colorado State University and all owners gave written informed consent before participation.

Table 5.1. Clinical scoring system used to determine muscle condition, appetite, consistency of feces, and frequency of vomiting.

Score Parameter	**Description**
Muscle Score	0 = normal 1 = mild muscle loss 2 = moderate muscle loss 3 = severe muscle loss
Appetite Score	0 = none of the food consumed 1 = 25% of ration consumed 2 = 50% of ration consumed 3 = 75% of ration consumed 4 = all food consumed
Fecal Score	1 = very hard and dry 2 = firm but not hard 3 = normal, little or no segmentation, moist 4 = very moist, log shaped 5 = very moist, piles 6 = texture but no defined shape 7 = watery puddle
Vomiting Score	0 = Never 1 = Rare (1-2 times a year) 2 = Once monthly 3 = Once weekly

5.4 Description of Cats

Thirty cats with CKD (17 cats had IRIS stage 2 CKD, 11 cats had IRIS stage 3 CKD, 2 cats had IRIS stage 4 CKD) and 11 healthy senior cats were enrolled in the study. Descriptions of age, body condition score, clinical scores, hematologic and biochemical variables of cats in each group (healthy senior cats, stage 2 CKD cats, and stages 3 and 4 CKD cats) are presented in Table 5.2.

Table 5.2 Characteristics of study groups including healthy senior cats, IRIS stage 2 CKD cats and IRIS stage 3 and 4 CKD cats.

	Healthy senior cats (n = 11)	**CKD stage 2 (n = 17)**	**CKD stages 3 & 4 (n = 13)**
Variable (reference interval)	**Mean +/- SD**	**Mean +/- SD**	**Mean +/- SD**
Age (years)	10.5 +/- 1.3[a]	14.5 +/- 3.2[b]	13.4 +/- 3.9
Hematocrit (32 - 47 %)	41 +/- 4[a]	36.5 +/-4[b]	34.5 +/- 5[b]
	Median (range)	**Median (range)**	**Median (range)**
Body condition score (1-9)	5 (4-8)	5 (2-8)	5 (3-7)
Muscle condition score (0-3)	0 (0-1)[a]	1 (0-2)[b]	1 (0-3)[b]
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)

Rows bearing a different superscript letter are significantly different from one another ($P < 0.05$).

5.4.1 Ultrasound Findings and Clinical Scores

Twenty-two of 30 (73%) cats with CKD had a urinary ultrasound examination with 19/22 cats having changes consistent with chronic renal degenerative disease, 2/29 cats with apparently normal renal architecture, and 1/29 cats with chronic unilateral obstructive ureteroliths and concurrent renal degenerative disease. No sonographic evidence of neoplasia or pyelonephritis was identified in the CKD cats. Abdominal imaging was not performed on healthy senior cats.

Healthy senior cats were significantly younger compared to the IRIS CKD stage 2 cats (P = 0.004). No significant difference was found in age of IRIS CKD stage 3 and 4 cats compared to healthy senior cats and IRIS CKD stage 2 cats. When clinical scores (BCS 1-9, MCS 0-3, fecal score 1-7, vomiting score 0-3, appetite score 0-4) were compared between healthy senior cats and CKD cats, BCS was not significantly different between healthy senior cats and CKD cats. Despite a similar BCS, MCS and thus severity of muscle atrophy was significantly more prominent in CKD cats (median score, 1; range, 0-3) compared to healthy senior cats (median score, 0; range, 0-1; P = 0.008). When MCS was compared between stages of CKD, IRIS stage 2 (P = 0.035) and IRIS stage 3 and 4 (P = 0.0004) CKD cats had significantly more muscle atrophy when compared to healthy senior cats, but no difference was found between IRIS CKD stage 2 and stage 3 and 4 cats (P = 0.316; Figure 5.1). CKD cats had significantly lower appetite scores (P = 0.004) and higher vomiting scores (P = 0.009) compared with healthy senior cats. Stage 2 (P = 0.008) and stages 3 and 4 (P = 0.007) CKD cats had lower appetite scores compared to healthy senior cats. Stage 2 CKD cats had higher vomiting scores compared to healthy senior cats (P = 0.005). No difference was found in fecal scores among groups.

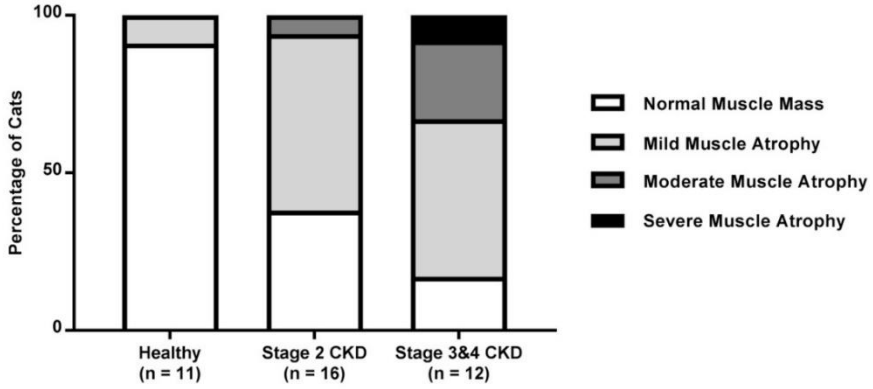

Figure 5.1 Percentage of cats with normal muscle mass and muscle atrophy (mild, moderate, severe) for healthy senior cats, IRIS CKD stage 2 cats, and IRIS CKD stage 3 and 4 cats.

5.4.2 Clinicopathologic Values

As expected, compared with the healthy senior group, CKD cats had a significant increase in serum concentrations of creatinine ($P < 0.001$) and BUN ($P < 0.001$). Additionally, the CKD cats had significantly lower hematocrit ($P = 0.003$) and higher serum total calcium concentration ($P = 0.04$) compared with healthy senior cats. No difference was found in serum phosphorus and potassium concentrations between groups. Serum total T4 concentration at time of enrollment excluded hyperthyroidism. Two CKD cats had serum total T4 concentration < 1.0 µg/dL which was suspected to be associated with non-thyroidal illness. All healthy senior cats and most CKD cats (24/30) were negative for proteinuria on urine dipstick testing. Of the 6/30 CKD cats that had ≥ 1+ protein on urine dipstick testing, only 3 had borderline proteinuria (0.22, 0.23, 0.24 UPC), and the remaining cats were non-proteinuric (UPC < 0.2).

5.4.3 Cat Diet and Current Medications

For IRIS CKD stage 2 cats, 3/17 (18%) of cats ate exclusively a commercial renal diet, 2/17 (12%) ate a combination of commercial renal diet and maintenance diet, and 12/17 (71%) ate variable commercial maintenance diets. For IRIS CKD stage 3 and 4 cats, 6/13 (46%) cats ate exclusively a commercial renal diet and 7/13 (54%) ate variable commercial maintenance diets. Healthy senior cats were being fed variable commercial maintenance diets.

Current medications at the time of enrollment for the CKD cats included transdermal mirtazapine gel (4/30 cats), amlodipine (4/30 cats), PO potassium supplementation (3/30 cats), and transdermal methimazole, alendronate, levothyroxine, maropitant, psyllium powder, aluminum hydroxide, glucosamine/chondroitin joint supplement, PO buprenorphine, and polyethylene glycol (1 cat each). None of the healthy senior cats were on medications at the time of enrollment.

5.5 Characterization of the Fecal Microbiome in Cats with Chronic Kidney Disease

5.5.1 Materials and Methods

5.5.1a Sequencing of 16S Ribosomal RNA Genes

Fecal microbiome analysis was performed on all enrolled cats (30 CKD cats and 11 healthy senior control cats). A voided 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 rRNA gene as previously described.[45] Briefly, DNA was extracted following the manufacturer's instructions (Mo Bio Power soil DNA isolation kit, Mo Bio Laboratories, Carlsbad, CA). The DNA then was amplified and sequenced (Illumina, Inc., San Diego, CA) using primers 515 F (5-GTGC CAGCMGCCGCGGTAA-3) to 806 R (5-GGACTACVSGGGTATCTAAT-3) at MR DNA Laboratory (Shallowater, TX). Sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.[46] Sequences were filtered for chimeras using 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 version 13.8 database.[47] 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, respectively, 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. 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. Chao1 and Shannon indices account for the number of observed OTUs and the evenness of bacterial species distribution within the fecal sample.

To compare the bacterial communities among samples, beta diversity was evaluated with the phylogeny based weighted and unweighted UniFrac[48] distance matrix and visualized using Principal Coordinate Analysis (PCoA) plots. Weighted UniFrac gives importance to the abundance of OTUs present in the fecal sample and unweighted does not.

5.5.1b Quantitative E. coli PCR

Quantitative PCR (qPCR) was used as described previously[39] to evaluate *E. coli* bacterial counts between healthy control cats and CKD cats.

5.5.1c PICRUSt

To compare differences in the functional potential of the fecal microbiome among groups, the software Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was performed based on the 16S rRNA genes that were found in the Greengenes phylogenetic tree of 16S rRNA gene sequences.[49]

5.5.1d Statistical Analysis

For statistical analysis between the stages of CKD, stage 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 $P < 0.05$ 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 senior 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 a commercial software (JMP Pro 11, SAS Software Inc., Cary NC). Because most datasets did not meet the assumptions of normal distribution, comparisons between healthy and CKD groups were determined using non-parametric Kruskal-Wallis tests (healthy senior cats and stage of CKD [stage 2, stages 3 and 4]) or a Mann-Whitney U test (healthy senior cats vs CKD stages 2-4) in Prism software (Prism 7, Prism Graphpad Inc., La Jolla, CA). 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[50] 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,[51] a web-based application to evaluate the correlations between bacterial taxa and IS and pCS serum concentrations. Linear discriminant analysis effect size (LEfSe) was used to elucidate the bacterial taxa and functional genes that were associated with healthy senior 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 (LDA) score = 3.0.

5.5.2 Results

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, respectively, to account for unequal sequencing depth. When alpha diversity, as described by Chao 1 and Shannon diversity indices, was compared between CKD cats and healthy senior cats, the Chao1 diversity index was significantly lower in CKD cats (median, 4286; range, 2729-6171) when compared to healthy senior cats (median, 5099; range, 2258-6633; P = 0.028). The Shannon diversity index was lower in CKD cats (median, 5.9; range,

4.1-7.3) compared to healthy senior cats (median, 6.4; range, 3.8-7.4) but this finding was not statistically significant (P = 0.062). 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 senior cats (median, 2575; range, 1274-3208; P = 0.026; Figure 5.2). When the CKD cats were analyzed by IRIS stage and compared to healthy senior cats, the Chao1 diversity index was significantly lower in stages 3 and 4 CKD cats compared to healthy control cats (P = 0.033). No significant differences were found in Shannon diversity index (P = 0.113) or species richness (number of unique OTUs; P = 0.062) among IRIS stage 2 CKD cats, stages 3 and 4 CKD cats, and healthy senior cats (Table 5.3).

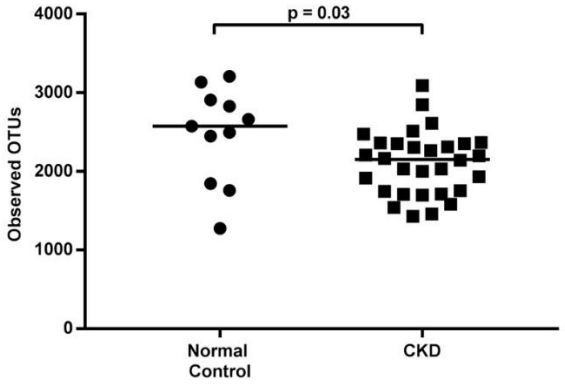

Figure 5.2 Scatter plot of the number of observed Operational Taxonomic Units (OTUs) in cats with chronic kidney disease (CKD) and healthy senior control cats.

Table 5.3 Summary of alpha diversity indices between healthy senior cats, IRIS CKD stage 2 cats, and IRIS CKD stage 3 and 4 cats.

	Healthy senior cats (n = 11)	**CKD stage 2 cats (n = 17)**	**CKD stages 3 & 4 cats (n = 13)**
Variable	**Median (range)**	**Median (range)**	**Median (range)**
Observed OTUs	2575 (1274-3208)	2198 (1541-3092)	2032 (1427-2511)
Chao1	5099 (2558-6633)[a]	4635 (2729-6171)	4151 (2960-4773)[b]
Shannon Index	6.4 (3.8-7.4)	6.0 (4.8-7.3)	5.9 (4.1-6.7)

Rows bearing a different superscript letter are significantly different from one another (P < 0.05).

For evaluation of beta diversity, no significant difference was found in microbial communities between CKD cats and healthy senior 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 stage 2 cats and stages 3 and 4 cats compared to healthy senior cats (weighted R = -0.04; unweighted R = 0.06). Figure 5.3 shows the PCoA plots representing the phylogeny based weighted and unweighted UniFrac distance matrix for (a) healthy senior cats and CKD cats and (b) healthy senior cats, IRIS CKD stage 2 cats, and IRIS CKD stage 3 and 4 cats.

a **b**

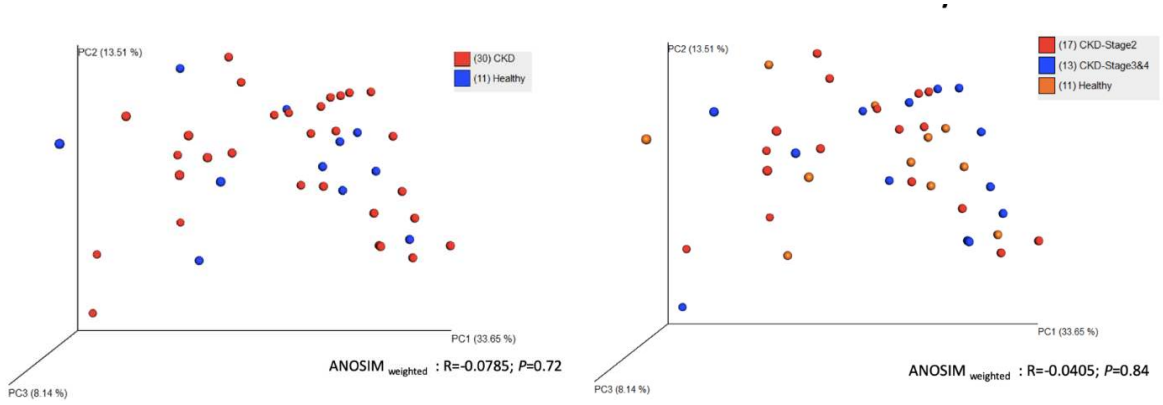

Figure 5.3 Principal Coordinate Analysis (PCoA) plots representing the phylogeny-based weighted and unweighted UniFrac distance matrix for (a) healthy senior cats and cats with CKD and (b) healthy senior cats, IRIS CKD stage 2 cats, and IRIS CKD stage 3 and 4 cats.

When individual bacterial groups were analyzed based on LEfSe, several bacterial taxa were identified as being significantly different among the groups based on univariate statistics. When comparing healthy senior cats to CKD cats, CKD cats had significantly lower relative abundances of the genera *Holdemania, Adlercreutzia, Eubacterium, Slackia*, and *Mogibacterium*. When comparing healthy senior cats to CKD stage 2 cats, the genera *Eubacterium* and

Adlercreutzia were lower and the genus *Prevotella* was enriched in CKD stage 2 cats. When healthy senior cats were compared to CKD stages 3 and 4 cats, the genera *Aldercreutzia* and *Eubacterium* were lowered and the genus *Prevotella* was enriched in CKD stage 3 and 4 cats. Genus *Prevotella* within class *Prevotellaceae* was lower in cats with CKD, whereas *Adlercreutzia* and *[Eubacterium]* were lower in the CKD stage 3 and 4 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 senior cats and CKD cats. The functional genes for ribosome biogenesis, translation factors, inorganic ion transport and metabolism were found to be different between healthy senior cats and CKD cats, however these features were not significant after correcting for multiple comparisons.

5.6 Characterization of Fecal Fatty Acids in Cats with Chronic Kidney Disease

5.6.1 Materials and Methods

5.6.1a Fecal Fatty Acid Assay

Fecal straight-chain SCFA and BCFA concentrations were measured in all enrolled cats (30 CKD cats and 11 healthy senior control cats). Fecal concentrations of straight-chain SCFAs (i.e. acetic acid, propionic acid, butyric acid, valeric acid) and BCFAs (i.e. isobutyric acid, isovaleric acid) were measured using a stable isotope dilution gas chromatography-mass spectrometry (GC-MS) assay as previously described[52] with some modifications. Briefly, the fecal samples were weighed, diluted 1:5 in extraction solution (2N hydrochloric acid), and frozen at -80°C until analysis. After thawing, fecal samples were homogenized using a multi-tube vortexer for 30 minutes at room temperature, then fecal suspensions were centrifuged for 20

minutes at 2,100 ∗ g and 4°C. Supernatants then were collected using serum filters (Fisherbrand serum filter system, Fisher Scientific Inc, Pittsburgh, Pa). From each sample, 500 µL of supernatant was mixed with 25 µL of internal standard (200 mM heptadeuterated butyric acid) and extracted using diethyl ether on a C18 solid phase extraction column (Sep-Pak C18 1 cc Vac Cartridge, Waters Corporation, Milford, MA). The organic phase of the samples was derivatized using *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) at room temperature for 60 minutes. A gas chromatograph (Agilent 7890A, Agilent Technologies Inc, Santa Clara, CA) coupled with an electron ionization mass spectrometer (Agilent 5975C, Agilent Technologies Inc, Santa Clara, CA) was used for chromatographic separation and quantification of the derivatized samples. Separation was achieved using a DB-1 ms capillary column (Agilent Technologies Inc, Santa Clara, CA). The GC temperature program was as follows: 40°C held for 0.1 min, increased to 70°C at 5°C/min, 70°C held for 3.5 min, increased to 160°C at 20°C/min, and finally increased to 280°C at 35°C/min, then held for 3 min. The total run time was 20.53 min. The mass spectrometer was operated in electron impact positive-ion mode with selective ion monitoring at mass-to-charge ratios (*M/Z*) of 117 (acetate), 131 (propionate), 145 (isobutyrate and butyrate), 152 (heptadeuterated butyrate; internal standard), and 159 (isovalerate and valerate). Quantification was based on the ratio of the area under the curve of the internal standard and each of the fatty acids. The lower detection limits of fecal concentrations of acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate were 1.33 µmol/g, 0.43 µmol/g, 0.12 µmol/g, 0.03 µmol/g, 0.02 µmol/g, and 0.05 µmol/g respectively. To take into account differences in water content among fecal samples, final concentrations of fecal SCFAs were adjusted by fecal dry matter (DM) and expressed as µmol/g of fecal DM.

5.6.1b Statistical Analysis

Cats with IRIS CKD stage 3 and 4 were combined for statistical analysis. When fecal fatty acid concentrations were compared to clinical scores and dietary consumption, the latter were grouped as follows for analysis: MCS (normal muscle mass [MCS 0] versus muscle atrophy [MCS 1-3]), vomiting score (normal vomiting score [0-1] versus abnormal vomiting score [2-3]), BCS categories (too thin [BCS 1-4] versus ideal body weight [BCS 5] versus overweight [BCS 6-9]), appetite (ate >75% of food offered by owner versus ≤75% of food offered by owner), and consumption of renal diet (exclusively consuming a renal diet versus consuming a maintenance diet). Data sets were assessed for normality using the Shapiro-Wilk test and were analyzed using Prism (Version 8.2.1, Graph Pad Software Inc, La Jolla, CA). In general, a Mann-Whitney U test was used for comparison between 2 groups and Kruskal-Wallis with Dunn's post hoc analysis used for comparison among ≥ 3 groups. Two-tailed Spearman correlation coefficient (rho) was computed to evaluate the association between straight-chain SCFAs and BCFAs and the following variables: hematocrit and serum creatinine, blood urea nitrogen (BUN), calcium, phosphorus, potassium. For all analyses, a value of $P < 0.05$ was considered significant, and data are represented as median and range.

5.6.2 Results

For healthy senior cats, the mean percentages of the total SCFA fecal concentration for acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid, and isobutyric acid were 60%, 22%, 12%, 2%, 3%, and 2%, respectively. For cats with CKD, the mean percentages of the total SCFA fecal concentration for acetic acid, propionic acid, butyric acid, valeric acid, isovaleric acid, and isobutyric acid were 58%, 21%, 11%, 4%, 5%, and 3%, respectively. Fecal fatty acid concentrations for healthy senior control cats, IRIS CKD stage 2 cats, and IRIS CKD

stage 3 and 4 cats are presented in Table 5.4. No significant differences among CKD cats (IRIS stages 2-4 combined), IRIS CKD stages (stage 2, stage 3 and 4), and healthy senior cats were found in total fecal straight-chain SCFA, total fecal BCFA concentrations, or individual fecal concentrations of acetic acid, propionic acid, butyric acid, valeric acid, and isobutyric acid. Fecal concentrations of isovaleric acid were significantly increased in cats with CKD (median, 7.7 μmol/g; range, 2.5-65.1) compared to healthy senior cats (median, 4.3 μmol/g; range, 2.9-7.4; P = 0.014). When compared between stages of CKD, IRIS CKD stage 3 and 4 cats had significantly higher (P = 0.025) fecal concentrations of isovaleric acid compared to healthy senior cats, but no significant difference was found between IRIS CKD stage 2 cats and IRIS CKD stage 3 and 4 cats, or between IRIS CKD stage 2 cats and healthy senior cats (Figure 5.4).

Table 5.4 Fecal fatty acid concentrations in healthy senior cats, IRIS CKD stage 2 cats, and IRIS CKD stage 3 and 4 cats.

	Healthy senior cats (n = 11)	CKD Stage 2 (n = 17)	CKD Stages 3 & 4 (n = 13)
Variable	Median (range)	Median (range)	Median (range)
Fecal Straight-Chain SCFA			
Acetic (μmol/g)	130.4 (28.0-371.4)	145.5 (31.2-346.7)	204.4 (42.9-320.9)
Propionic (μmol/g)	42.1 (10.7-153.8)	57.2 (8.1-270.3)	46.0 (10.7-143.2)
Butyric (μmol/g)	23.9 (7.0-237.6)	20.8 (4.9-122.2)	22.1 (2.3-88.4)
Valeric (μmol/g)	3.0 (0.4-23.1)	4.3 (0.2-47.4)	2.7 (0.4-47.6)
Total SCFA (μmol/g)	203.2 (47.5-635.0)	280 (50.0-533.2)	298.2 (59.1-487.8)
Fecal BCFA			
Isovaleric (μmol/g)	4.3 (2.9-7.4)[a]	6.2 (3.1-23.9)	9.5 (2.5-65.1)[b]
Isobutyric (μmol/g)	3.4 (2.4-6.6)	4.8 (1.9-16.5)	5.9 (1.2-25.9)
Total BCFA (μmol/g)	7.8 (5.3-14.0)	10.1 (4.9-40.4)	15 (3.7-90.9)

Rows with a different superscript letter are significantly different from one another (P < 0.05).

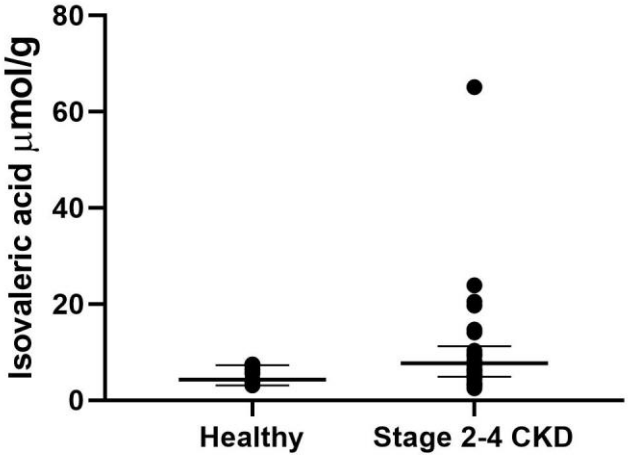

Figure 5.4 Fecal isovaleric acid concentrations (median and interquartile range) in healthy senior cats and cats with chronic kidney disease (CKD).

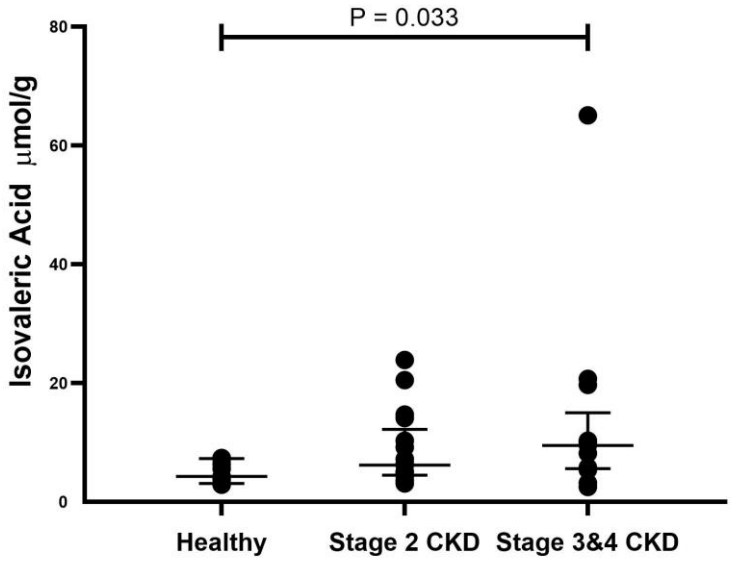

Figure 5.5 Fecal isovaleric acid concentrations (median and interquartile range) in healthy senior cats, IRIS stage 2 CKD cats, and IRIS stage 3 and 4 CKD cats.

When total fecal straight-chain SCFA, total fecal BCFA, and individual fecal fatty acid concentrations (i.e. acetic acid, propionic acid, butyric acid, isovaleric acid, isobutyric acid, valeric acid) were compared to clinical scores (BCS, MCS, vomiting score, and appetite score

categories) for all enrolled cats, no significant difference was found for BCS, vomiting score, or appetite score categories. For MCS, fecal total BCFA concentrations (P = 0.008), isovaleric acid concentrations (P = 0.008) and isobutyric acid concentrations (P = 0.022) were significantly higher in cats with muscle atrophy (MCS 1-3) compared to cats with a normal muscle mass (MCS 0; Table 5.5; Figure 5.6).

Table 5.5 Fecal branched-chain fatty acid concentrations in study cats with normal muscle mass and cats with muscle atrophy.

	Normal Muscle Mass (MCS 0) (n=16)	**Muscle Atrophy (MCS 1-3) (n=23)**	**P-value**
Fecal BCFA			
Isovaleric (μmol/g)	4.5 (2.9-10.3)	9.4 (2.5-65.1)	0.008
Isobutyric (μmol/g)	3.5 (2.2-7.2)	5.9 (1.2-25.9)	0.022
Total BCFA (μmol/g)	8.2 (5.3-16.8)	14.6 (3.7-90.9)	0.008

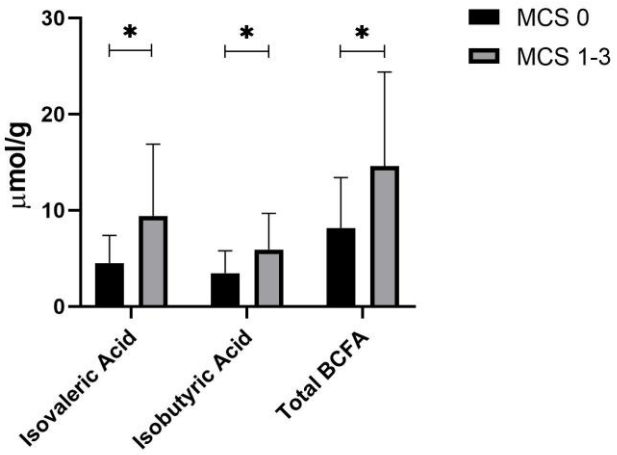

Figure 5.6 Fecal isovaleric acid, isobutyric acid, and total branched-chain short-chain fatty acid (BCFA) concentrations in healthy senior and CKD cats with normal mass (muscle condition score [MCS] 0) and with muscle atrophy (MCS 1-3).

No significant differences in total or individual fecal SCFA concentrations were found when all CKD cats consuming exclusively a prescription renal diet were compared to those not exclusively consuming a renal diet. No significant differences in fecal SCFA concentrations were found between cats of the same CKD stage exclusively consuming a renal diet in comparison to those not exclusively consuming a renal diet, however, the highest concentrations of isovaleric acid in IRIS CKD stage 3 and 4 cats were observed in those cats not consuming a renal diet (n = 6; median, 14.7 μmol/g; range, 3.2 – 65.1) versus IRIS CKD stage 3 and 4 cats that were consuming a renal diet (n = 6; median, 7.6 μmol/g; range, 2.5 – 10.1).

For correlation between fecal fatty acid concentrations and renal laboratory variables, neither total nor individual concentrations of fecal straight-chain SCFAs correlated with renal laboratory variables. Total fecal concentrations of BCFAs weakly correlated with serum creatinine (rho, 0.36; P = 0.023) and BUN (rho, 0.40; P = 0.009) concentrations. When individual concentrations of BCFA were assessed, fecal isovaleric acid concentrations were weakly correlated with serum creatinine (rho, 0.39; P = 0.013) and BUN (rho, 0.44; P = 0.004) concentrations.

When analysis was performed excluding an outlier stage 3 CKD cat that had extremely high fecal isovaleric acid concentration, CKD cats (all cats IRIS stage 2-4) still had significantly higher fecal concentrations of isovaleric acid compared to healthy senior cats (P = 0.023). When individual stages of CKD were compared to healthy senior cats, no significant difference in fecal isovaleric acid concentrations was found between healthy senior cats and IRIS stage 3 and 4 CKD cats (P = 0.052; Figure 5.7). Fecal total BCFA (P = 0.013), fecal isovaleric acid (P = 0.013) and fecal isobutyric acid (P = 0.034) concentrations continued to be significantly increased in cats with muscle atrophy. For the correlation analysis, total fecal concentrations of

BCFAs still weakly correlated with serum creatinine (rho, 0.32; P = 0.044) and BUN (rho, 0.37; P = 0.021) concentrations. Fecal isovaleric acid concentrations continued to weakly correlate with serum creatinine (rho, 0.35; P = 0.026) and BUN (rho, 0.41; P = 0.009) concentrations.

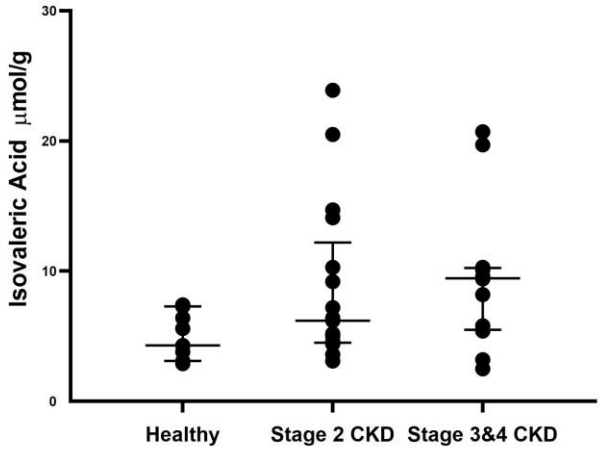

Figure 5.7 Fecal isovaleric acid concentrations (median and interquartile range) in healthy senior cats, IRIS stage 2 CKD cats, and IRIS stage 3 and 4 CKD cats (single outlier excluded).

5.7 Evaluation of Serum Indoxyl Sulfate and P-cresol Sulfate Concentrations and Correlation with Fecal Microbiota and Fatty Acid Concentrations

5.7.1 Materials and Methods

5.7.1a Assays for Serum Indoxyl Sulfate and p-Cresol Sulfate Concentrations

Serum IS and pCS concentrations were measured in healthy control cats (n = 10) and compared to CKD cats (16 IRIS stage 2 CKD cats and 12 IRIS stage 3 and 4 CKD cats; n = 28). An inadequate amount of serum was available to perform IS and pCS concentrations on 3 cats. Total IS and pCS serum concentrations were determined by liquid chromatography with tandem mass spectrometry modified from a published method[53] 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, CA).

The calibration curves were constructed by spiking standard IS (Alpha Asear, Haverhill, MA) 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, MO). 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 +/- 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 100–50000 ng/mL for both IS and pCS. The linearity of the curves was > $r^2 = 0.99$ using $1/x^2$ weighting. As measured in cat serum, the lower limit of quantitation (LLOQ) 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.

5.6.1b Statistical Analysis

Data was analyzed with Prism (Version 8.2.1, Graph Pad Software Inc, La Jolla, CA). Cats with IRIS CKD stage 3 and 4 were combined into one group for statistical analysis. Median IS and pCS concentrations between healthy control cats and CKD cats were compared using the Mann-Whitney U test. Healthy senior cats and the stage of CKD (stage 2, stages 3 and 4) were compared using Kruskal Wallis testing with Dunn's post hoc analysis. When serum IS and pCS

concentrations were compared to clinical scores and dietary consumption, the latter were grouped as follows for analysis: MCS (normal muscle mass [MCS 0] versus muscle atrophy [MCS 1-3]), vomiting score (normal vomiting score [0-1] versus abnormal vomiting score [2-3]), BCS categories (too thin [BCS 1-4] versus ideal body weight [BCS 5] versus overweight [BCS 6-9]), appetite (ate >75% of food offered by owner versus ≤75% of food offered by owner), and consumption of renal diet (exclusively consuming a renal diet versus consuming a maintenance diet). Spearman correlation coefficient (rho) was computed to evaluate the association between IS and pCS serum concentrations and the following variables: number of unique OTUs, fecal fatty acid concentrations, hematocrit, and serum creatinine concentration, and serum urea (BUN), 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.

5.7.2 Results

5.7.2a Serum Indoxyl Sulfate and p-Cresol Sulfate Analysis

Serum IS concentrations were found to be significantly higher in CKD cats (median, 1210 ng/mL; range, 165-2860) compared to healthy senior cats (median, 4700; range, 746-27,600; $P < 0.0001$). Serum concentrations of IS and pCS for healthy control cats, IRIS stage 2 CKD cats, and IRIS stage 3 and 4 cats are shown in Table 5.6. Significantly higher IS concentrations were seen in stage 2 CKD cats ($P = 0.024$) and stages 3 and 4 CKD cats ($P = 0.0008$) in comparison to healthy senior cats (Figure 5.8). No significant difference was found in serum IS concentrations between stage 2 CKD cats and stage 3 and 4 CKD cats. The serum pCS concentrations were not significantly different between CKD cats and healthy senior cats (Figure 5.9).

Table 5.6 Serum indoxyl sulfate and p-cresol sulfate concentrations (median and range) in healthy senior cats, IRIS stage 2 CKD cats, and IRIS stage 3 and 4 CKD cats.

Variable	Healthy senior cats (n = 10)	CKD stage 2 cats (n = 16)	CKD stages 3 & 4 cats (n = 12)
Indoxyl sulfate (ng/mL)	1210 (165-2860)[a]	3370 (746-10300)[b]	6385 (1020-27600)[b]
p-Cresol sulfate (ng/mL)	2905 (901-7220)	6940 (34-30600)	5300 (189-35300)

Rows with a different superscript letter are significantly different from one another ($P < 0.05$).

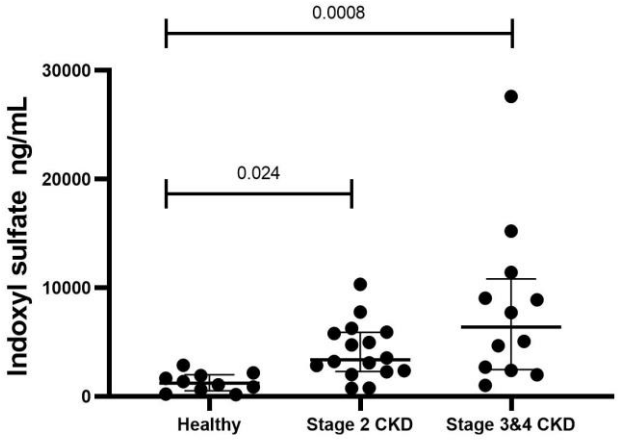

Figure 5.8 Serum indoxyl sulfate concentrations (median and interquartile range) in healthy senior cats, IRIS stage 2 CKD cats, and IRIS stage 3 and 4 CKD cats.

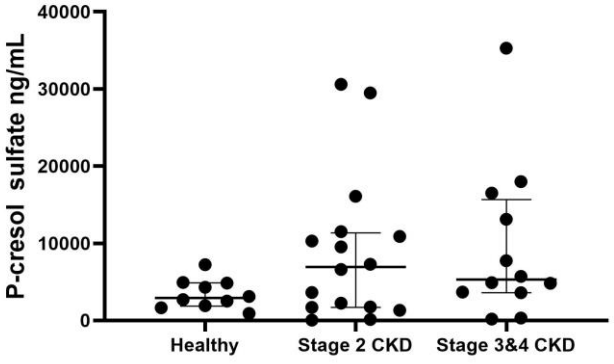

Figure 5.9 Serum p-cresol sulfate concentrations (median and interquartile range) in healthy senior cats, IRIS stage 2 CKD cats, and IRIS stage 3 and 4 CKD cats.

5.7.2b Correlation to Clinicopathologic Values and Comparison to Clinical Scores

Serum concentrations of IS were found to be correlated to hematocrit (rho, -0.68; $P < 0.001$), BUN (rho, 0.72; $P < 0.001$), creatinine (rho, 0.64; $P < 0.001$), and pCS (rho, 0.42; $P = 0.009$). Unlike IS, serum pCS concentrations did not significantly correlate to serum creatinine or BUN. No correlation of either IS or pCS concentrations to serum phosphorus, calcium, sodium, and potassium concentrations was found.

When serum IS and pCS concentrations were compared to clinical scores (BCS, MCS, vomiting score, and appetite score categories) for all enrolled cats, no significant difference was found for BCS, vomiting score, or appetite score categories. However, when serum 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 reported by the owner at enrollment, serum concentrations of IS tended to be higher in cats that had a worse appetite ($P = 0.070$), but no significant difference in pCS concentrations was seen between the groups. No significant difference was found in serum IS and pCS concentrations between CKD cats that exclusively consumed a renal diet compared to CKD cats that consumed a commercial maintenance diet. Serum IS concentrations were significantly higher in cats with muscle atrophy (MCS 1-3) compared to cats without muscle atrophy (MCS 0; $P = 0.005$). No significant difference was found in serum pCS concentrations between cats with and without muscle atrophy ($P = 0.286$).

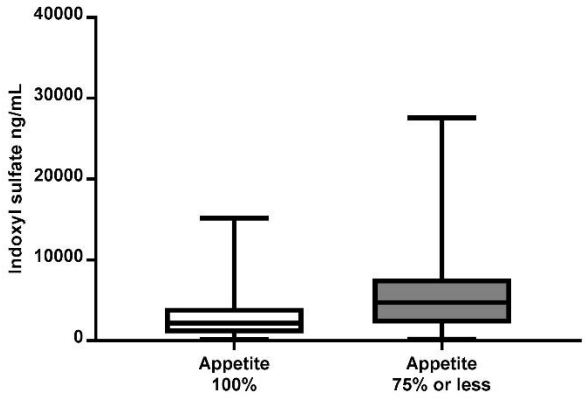

Figure 5.10 Serum indoxyl sulfate concentrations (median and range) in healthy and CKD cats that ate 100% of food offered and in those that ate 75% or less of food (based on owner report of appetite score).

5.7.2c Correlation with Fecal Microbiome

The genera *Bifidobacterium* (rho, -0.41; P = 0.012) and *Eubacterium* (rho, -0.40; P = 0.013) were significantly correlated with pCS serum concentrations, whereas the genus *Mogibacterium* (rho, -0.32; P = 0.047) was significantly correlated with serum IS concentrations.

5.6.2d Correlation with Fecal Fatty Acid Concentrations

Neither total nor individual concentrations of fecal straight-chain SCFAs correlated with serum IS or pCS concentrations, with the exception of fecal concentration of valeric acid which was positively correlated with pCS serum concentration (rho, 0.48; P = 0.005). Total fecal concentrations of BCFAs correlated with serum pCS (rho, 0.35; P = 0.030) concentrations. When individual concentrations of BCFA were assessed, fecal isovaleric acid concentrations correlated with serum pCS (rho, 0.34; P = 0.034) concentrations but not significantly with serum IS concentrations (P = 0.070).

When analysis was performed excluding an outlier stage 3 CKD cat that had extremely high fecal isovaleric acid concentration, total fecal concentrations of BCFAs still weakly

correlated with serum pCS (rho, 0.33; P = 0.046) concentrations. Fecal isovaleric acid concentrations continue to correlate with serum pCS concentrations (rho, 0.32; P = 0.046).

5.8 Discussion

Decreased fecal bacterial species diversity and richness in CKD cats is consistent with previous assessment of the intestinal microbiome in humans with CKD and rat CKD models.[1,54,57] 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.[1,54-57] In vitro studies in rats have shown that several bacterial families generate toxic compounds such as p-cresol and indole.[58,59] 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 bacterium in humans,[17,20] 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,[20,60] but in our study a negative correlation was found between the genus *Bifidobacterium* and serum pCS concentrations.

The reasons for differences in the fecal microbiome 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.[37] 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.[61] This variability makes it difficult to incriminate a single bacterial species as the culprit for increased serum IS and pCS serum concentrations in CKD patients. Also, using 16S rRNA gene sequencing to

characterize the bacterial composition compounds this issue because the technique lack sensitivity beyond the genus level and the technique only looks at relative abundances of taxa rather than a quantitative comparison. 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.[62]

Another goal of the study was to characterize fecal straight-chain and branched-chain SCFA concentrations in cats with CKD and compare these findings to a population of healthy senior cats. A prominent finding was significantly higher fecal concentrations of the BCFA isovaleric acid in CKD cats compared to control cats and its positive correlation with serum creatinine, BUN, and pCS concentrations. Both BCFAs and the uremic toxins IS and pCS are produced when amino acids escape absorption in the small intestine and are metabolized by the colonic microbiota.[13] Because cats with CKD have higher serum IS and pCS and fecal isovaleric acid concentrations,[41,44] these findings taken together suggest that cats with CKD may have protein malassimilation in the upper small intestinal tract. Similar findings are seen in both rat models and in humans with end-stage renal disease (ESRD) where increased abundance of proteolytic bacteria and increased amounts of undigested amino acids in the colon have been described.[63,64] Protein malassimilation also is documented in humans with CKD.[65] In 1 study in humans, the percentage of dietary protein-derived leucine that appeared in plasma after consumption was significantly decreased (41 +/- 5%) compared to healthy controls (61 +/- 4%).[66] This finding is of particular interest because leucine is an amino acid that results in formation of BCFAs in the colon.

Increased concentrations of fecal isovaleric acid and serum IS and pCS in cats with CKD may have implications for gastrointestinal health and motility. Although straight-chain SCFAs,

primarily butyrate and propionate, have been shown to have a prokinetic effect by promoting contraction of smooth muscle in the colon in *ex vivo* studies evaluated in colonic tissue from dogs,[67] cats,[68] and guinea pigs,[69] BCFAs and other products of protein fermentation in the colon are considered deleterious to the gut. For example, isovaleric acid has been shown to cause colonic smooth muscle relaxation in an *ex vivo* rodent model.[15] In addition, decreased gastrointestinal motility has been linked directly to uremia by causing dysbiosis and colonic inflammation secondary to increased uremic toxin exposure in rodent models.[70-72] In an *ex vivo* rodent model, incubation of colons with the gut-derived uremic toxins IS and pCS, but not urea, was shown to decrease force of contraction by 66% and 55%, respectively.[72] This finding supports the idea that uremia directly causes impaired colonic motility, although other factors such as dehydration and hypokalemia also may be factors. Constipation is a common problem in humans on hemodialysis and has been shown to negatively impact health-related quality of life.[73,74] Although the prevalence of constipation in cats with CKD is unknown, CKD in cats is associated with an increased risk of constipation.[75] Preliminary data from an owner survey study by Jones et al. showed that cats with CKD defecated less frequently when compared to healthy cats ($P = 0.02$). It is possible that higher fecal isovaleric acid and serum IS concentrations in CKD cats might contribute to constipation. In our study population, only 2/30 CKD cats had a fecal score of 1 (small hard feces) that may be associated with constipation, and no owners reported concern for overt signs of constipation in their CKD cats. However, subtle signs of constipation are difficult to detect for cat owners, and normal feces does not exclude the possibility of decreased frequency of defecation. Additional studies assessing the relationships among microbial metabolites, defecation frequency, and stool quality are warranted.

Cats with CKD did not have significantly different fecal straight-chain SCFA concentrations as compared to healthy senior cats. Factors that affect straight-chain SCFA concentrations include amount and type of fermentable carbohydrate consumption, composition and diversity of the intestinal microbiota, colonic transit time, and interactions between microbes and the host.[76-78] In humans with CKD and ESRD, it is commonly recommended to decrease dietary intake of fruits and vegetables to limit potassium intake and prevent hyperkalemia.[79] This decrease in dietary fiber leads to a decrease in bacterial groups known to produce straight-chain SCFAs in the colon, in particular butyric acid.[56,80] Although fecal SCFA concentrations has not been evaluated in humans, it is presumed that humans with CKD have reduced production of SCFA in the colon by bacteria because of reduced fiber intake. Dietary fiber restriction to prevent hyperkalemia is not recommended in cats with CKD because hypokalemia is a more common sequela of the disease. Therefore, microbial SCFA production may not be affected in cats as it is presumed in humans with CKD.

Although cats are obligate carnivores, and comparison to humans may not be appropriate, it has been documented that dietary protein and fiber intake and nutrient sources affect both the microbiome composition of cats and microbial metabolite production in healthy cats, similar to what is observed in humans.[81,82-84] Specifically, the fiber source has been shown to affect fecal SCFA concentrations in healthy cats.[81] A previous study evaluated the effect that 3 fiber sources with differences in fermentability, solubility, and prebiotic potential had on fecal microbial metabolites in healthy cats. Fecal acetate and propionate concentrations increased in cats that were fed a highly soluble, fermentable fiber (pectin) whereas fecal butyrate and BCFAs increased after supplementation with rapidly fermented, prebiotic fiber (fructooligosaccharide) and pectin.[81] In our study, the diets of the enrolled cats were variable and unfortunately the fiber

source and intake for the cats could not be accurately determined. In addition, the specific microbiota responsible for SCFA production has yet to be determined in veterinary medicine. In the present study, no specific bacterial taxa were associated with the CKD cats compared to healthy senior cats.[44] To further define the link between gut microbial composition and SCFA production in cats, shotgun metagenomic analysis on fecal samples in conjunction with targeted analysis of fecal SCFAs in cats on a well-defined diet is needed to determine the bacterial groups responsible for fecal acid production in the colon of cats.

A few observations are important to note when evaluating differences in fecal SCFAs in CKD cats exclusively consuming a prescription renal diet versus those CKD cats consuming a maintenance diet in our study. Although most commercial renal diets are highly digestible and therefore moderately restricted in fiber content, in our study CKD cats consuming a renal diet did not have statistically different fecal straight-chain SCFA concentrations compared to cats fed a commercial maintenance diet. Additionally, renal diets are often lower in protein and highly digestible, which may explain the observed lower fecal isovaleric acid concentrations in CKD cats consuming a renal diet as compared to those that were not consuming a renal diet, most notably in the advanced stage IRIS CKD 3 and 4 cats. This difference however was not statistically significant, which may be a consequence of small sample size. In addition, serum IS and pCS concentrations did not differ between cats fed exclusively a renal diet and cats fed a fed a commercial maintenance diet. Further evaluation of the link between dietary intake and fecal microbial metabolites could help advance our understanding of the potential benefits provided by nutritional modifications on cats with CKD.

In humans, serum pCS concentrations are higher in hemodialysis and CKD patients than in controls.[54,85] 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 13/28 (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 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,[21-25] contribute to multi-organ dysfunction,[26,27,30,86] and are associated with increased mortality in human CKD patients.[26,27,30] In addition, because removal by hemodialysis in human patients is markedly lower for protein-bound IS and pCS than for urea and creatinine,[87] human medicine has focused on strategies to decrease production of IS and pCS including modulation of bacterial growth in the colon by dietary management,[88] prebiotics, probiotics, and target adsorption of uremic toxins by the use of adsorbents.[2,18,58] The generation of IS and pCS can be modulated by selectively increasing saccharolytic and reducing 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.[54,89] In particular, increasing dietary fermentable fibers has been shown to restore the colonic gut barrier and microflora composition in mice with CKD,[90] and increasing fiber and decreasing protein intake have been shown to decrease IS and pCS concentrations.[60,88,91] Adsorbents such as sevelamer hydrochloride[92,93] (Renalgel, Genzyme, Cambridge, MA) and AST-120[92,93] (Kremezin, Kurecha Chemical Industry, Tokyo, Japan) also are used to limit intestinal absorption of IS and pCS. In veterinary medicine, there has been no investigation into

strategies to decrease gut-derived uremic toxins in CKD patients and, based on a growing body of literature,[41,42] further exploration as a potential therapeutic target seems warranted.

Lastly, cats with muscle atrophy (MCS 1-3) had significantly higher serum IS concentrations and fecal isovaleric acid, isobutyric acid, and total BCFA concentrations as compared to cats with normal muscle mass (MCS 0). This is an interesting finding considering that all of the listed metabolites are products of protein fermentation in the colon. Previous literature suggests that weight loss and muscle wasting in human ESRD patients is caused, at least in part, by impaired small intestinal protein digestion and absorption as well as by alterations in protein metabolism and inadequate protein intake.[94,95] This scenario may be further complicated by age-related changes in cats, with decreased protein digestibility described as an aspect of impaired digestive function in senior cats.[96] In our study, 1 IRIS CKD stage 3 cat was an outlier compared to the other enrolled cats and had an extremely high fecal isovaleric acid concentration (Figure 5.4). When investigated, it was discovered that this patient was being fed a partially raw, high protein diet (Instinct Dry, Nature's Variety, St. Louis, MO), and was the only cat in the study to be receiving a high protein diet. Taken overall, increased fecal BCFA concentrations may represent an indirect indicator of the efficiency of protein malassimilation in cats with CKD.[97] Interestingly, this IRIS stage 3 CKD cat had a serum IS concentration (7,700 ng/mL) near the group median and the concentrations was not notably elevated. This discrepancy noted in this stage 3 cat fed a high protein diet may be explained by difference in protein sources within the diets. Further evaluation of fecal amino acid concentrations and correlation to serum IS and pCS and fecal fatty acid concentration is 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 senior cat population and the study

exclusion criteria, an age-matched healthy control population (i.e. 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, 2/30 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.[98] 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 and fiber intake was not possible due to the common practice of feeding a mixture or rotation of diets. Thus, analysis of a possible relationship between percent dietary protein and fiber and serum uremic toxin and fecal fatty acid concentrations, respectively, was not possible.

In summary, the study demonstrated that CKD is associated with a functional dysbiosis in cats with CKD, particularly in cats with late stage disease. 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 suggesting early therapeutic interventions to potentially reduce accumulation of gut-derived uremic toxins would be beneficial in the management of feline CKD. Fecal BCFA concentrations and serum IS concentrations were higher in cats with clinical evidence of muscle wasting, providing evidence for malassimilation of protein in cats with CKD.

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CHAPTER 6: MANIPULATION OF THE FECAL MICROBIOME, GUT-DERIVED UREMIC TOXINS, AND CLINICAL SCORES IN CATS WITH CHRONIC KIDNEY DISEASE USING *ENTEROCOCCUS FAECIUM* STRAIN SF68

6.1 Summary

Background: Chronic kidney disease (CKD) is common in senior cats and is associated with weight loss, decreased appetite, and vomiting. *Enterococcus faecium* strain SF68 (SF68) is a commercially available probiotic that includes hydrolyzed chicken and liver as a palatability enhancer. This probiotic has shown to have immune modulating effects in dogs and cats. Probiotics can alter the gut microbiome, and in people with CKD probiotics decrease serum concentrations of gut-derived uremic toxins.

Objective: To compare clinical and renal clinicopathologic values in client-owned cats with CKD that are fed SF68, the palatability enhancer without the SF68 bacterium (palatability enhancer group), or a commercially available renal diet alone (control group).

Animals: 24 client-owned cats with chronic kidney disease (IRIS Stages 1-3).

Methods: Prospective, randomized, placebo-controlled pilot study. CKD cats were fed SF68 (n=8), the palatability enhancer without the SF68 bacterium (n=8), or no supplement (n=8) for 8 weeks. Body weight, blood pressure, renal laboratory values, electrolytes, and urine protein-to-creatinine ratio at enrollment (Week 0) to the end of the 8-week study period (Week 8) were compared among groups. The proportion of vomiting episodes documented by the owners over the 8-week study period were compared in the SF68 and palatability enhancer groups. Characterization of the fecal microbiome using 16S ribosomal RNA gene sequencing and measurement of serum indoxyl sulfate (IS), p-cresol sulfate (pCS), and trimethylamine-n-oxide

(TMAO) concentrations using liquid chromatography with tandem mass spectrometry were performed.

Results: Short-term supplementation with SF68 or the palatability enhancer in cats with CKD was accepted well. While some owners thought their cats had increased food consumption with the study supplement, changes in body weights among groups were not statistically different at Week 8 when compared to Week 0 (P = 0.82). Cats fed SF68 had similar incidence rates of vomiting over the course of the study when compared to the palatability enhancer group. Renal clinicopathologic values were stable in all of the groups during the 8-week study and did not vary significantly among groups. Fecal microbial richness, diversity, and community structure did not change over the 8-week study in cats fed SF68. Serum concentrations of IS, pCS, and TMAO were not statistically different at Week 8 when compared to Week 0 in the SF68 and palatability enhancer groups.

Conclusions: Cats with IRIS CKD stages 1, 2 or 3 had stable disease over the 8-week study period regardless of group. The probiotic SF68 had no appreciable effect on the fecal microbial communities or serum concentrations of uremic toxins. The palatability enhancer in the commercial product may help to augment appetite in some cats.

6.2 Introduction

Chronic kidney disease (CKD) is one of the most common syndromes affecting senior cats and is associated with high morbidity and mortality.[1,2] Cats with CKD often exhibit weight loss, decreased appetite, and intermittent vomiting, which may result in dehydration, worsened azotemia and electrolyte imbalance, and subsequently the need for hospitalization and fluid diuresis.[3,4] Morbidity associated with CKD may influence owners to decide to euthanize as they perceive their cat as having a poor quality of life. Because CKD is an irreversible and

progressive disease, current management options in cats focus on diet and other supportive care measures to control nausea, maintain body weight and hydration, and the treatment of common disease sequelae such as hypertension and anemia.[5]

In veterinary medicine, evidence exists that some probiotics can be beneficial for the management of acute and chronic enteropathies,[6-8] chronic kidney disease[9] as well as induce immune modulation[7,10,11] and lessen antibiotic associated diarrhea.[12] In humans, probiotics reduce blood concentrations of gut-derived uremic toxins, in particular p-cresol sulfate (pCS), by modulating the gut environment in patients with CKD, and in a mouse model the multi-strain probiotic VSL#3 was shown to alleviate renal dysfunction caused by ischemia and reperfusion.[13-16] *Enterococcus faecium* strain SF68 (SF68) is an orally administered commercially available probiotic frequently used as a dietary supplement in both dogs and cats (Purina® ProPlan® Veterinary Diets; Fortiflora™ Probiotic Supplement). In addition to the potential benefits of SF68 on the gut microbiome, the product also contains hydrolyzed chicken and liver that serves as a palatability enhancer which can augment appetite in some cats and dogs. This probiotic is sprinkled on food which is preferable to being medicated with a pill for a subset of cats. In addition, the immune modulating properties of SF68 could potentially influence the classic lymphoplasmacytic interstitial nephritis that occurs in most cats with CKD.[17]

The primary objective of this pilot study was to compare the fecal microbial community profile, serum concentrations of gut-derived uremic toxins (pCS, indoxyl sulfate [IS], trimethylamine-n-oxide [TMAO]), and clinical and renal clinicopathologic parameters among CKD cats fed either SF68, palatability enhancer without SF68 bacterium, or no dietary supplement for 8 weeks. The hypotheses were that the palatability enhancer contained within the probiotic would improve appetite in the cats, and that the probiotic SF68 would result in

changes to the fecal microbiome, serum concentrations of gut-derived uremic toxins, and renal clinicopathologic values.

6.3 Materials and Methods

6.3.1 Selection of Cats

All client-owned cats with CKD that presented from January 2017 to December 2018 were eligible for enrollment into the study population. The cats underwent a thorough evaluation that included a complete physical examination, minimum database consisting of complete blood cell count (CBC), serum biochemistry panel, and urinalysis, urine protein:creatinine (UPC) ratio, total thyroxine concentration, blood pressure, and an ultrasound of the urinary system. Physical examination and Doppler sphygmomanometry blood pressure were performed by one trained individual for consistency. Cats were considered to have CKD based on a creatinine concentration >1.6 mg/dL and urine specific gravity (USG) <1.035 in addition to ultrasound changes consistent with chronic degenerative renal disease. The CKD cats were staged based on the International Renal Interest Society (IRIS) guidelines.[18]

Exclusion criteria included recent use of any commercially available probiotic within 6-weeks of enrollment, a creatinine concentration > 5.0 mg/dL, intravenous diuresis within the previous 2 months prior to enrollment, current urinary tract infection, uncontrolled hypertension, and concurrent disease that could negatively impact the potential for survival in the short-term future (e.g. neoplasia, uncontrolled hyperthyroidism, obstructive ureterolithiasis). Cats were allowed to continue on any previously prescribed medications during the study and owners were instructed to continue the current diet throughout the study. If changes in the medication or diet regimens were required during the study, then the cat was removed from the study. For cats in

the SF68 and palatability enhancer groups, a questionnaire was provided to the owner and the following information was obtained: diet, current medications or supplements, and fecal and vomiting score at the time of enrollment (Table 6.1). The project was approved by the Institutional Animal Care and Use Committee at Colorado State University, and all owners gave written informed consent prior to participation.

Data from a group of CKD cats that were recruited between July 2018 to February 2019 for a separate 8-week diet study (Purina NF Early Care and Advanced Care, Nestle Purina® PetCare) were used as the control group. These cats had the same exclusion criteria, inclusion criteria, and initial diagnostic testing as described above and did not receive probiotics (including SF68), antibiotics, or antacids during the study period.

6.3.2 Study Design

This prospective, blinded, placebo-controlled pilot study was performed at the Colorado State University Veterinary Teaching Hospital. The probiotic SF68 and the palatability enhancer without the SF68 bacterium were supplied by Nestle Purina® PetCare. Cats were randomized by household to be fed 1 gram of either SF68 (1×10^8 CFU/g) with the palatability enhancer (SF68 group) or the palatability enhancer without SF68 (palatability enhancer group; similar quantity as compared to the commercial product) mixed with food daily for 8 weeks. All members of the staff involved in the trial and the cat owners were blinded to the whether the cats were fed SF68 or the palatability enhancer. Both supplements were cultured at the end of the study to confirm the treatment groups and to confirm viability of the probiotic. The owners of cats fed either SF68 or the palatability enhancer were provided a daily monitoring sheet to record daily vomiting episodes, fecal score, and the approximate amount of supplement consumed each day (Table 6.1). To record the amount of the study supplement consumed each day, owners were

instructed to mix the supplement with a portion of canned food or dry food at mealtime and to estimate the amount consumed.

Clinicopathologic data from a group of CKD cats that were enrolled in a separate renal diet study (Purina NF Early Care and Advanced Care, Nestle Purina® PetCare) but were not fed SF68 or the palatability enhancer were used as a control group. All cats in the control group were fed the renal diet for at least 4 weeks prior to enrollment in the present study. All cats in the 3 groups were evaluated for physical examination, Doppler blood pressure, CBC, serum biochemistry panel, urinalysis, and UPC ratio 4 and 8 weeks after enrollment.

Table 6.1 Clinical scoring system used to determine appetite, consistency of feces, and frequency of vomiting.

Score Parameter	Description
Study Supplement	0 = none of the food consumed 1 = 25% of ration consumed 2 = 50% of ration consumed 3 = 75% of ration consumed 4 = all food consumed
Fecal	1 = very hard and dry 2 = firm but not hard 3 = normal, little or no segmentation, moist 4 = very moist, log shaped 5 = very moist, piles 6 = texture but no defined shape 7 = watery puddle
Vomiting	0 = Never 1 = Rare (1-2 times a year) 2 = Once monthly 3 = Once weekly

6.3.3 16S Ribosomal RNA Gene Sequencing of Feces

A fresh voided 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 (rRNA) gene. Extraction of DNA of the fecal samples and a blank control was performed following the manufacturer's instructions (QIAamp PowerFecal DNA Kit, Qiagen, Germantown, MD). The DNA was then amplified and sequenced (Illumina, Inc, San Diego, CA) using primers 515F to 806R at Novogene (Sacramento, CA). Sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0.[19] Sequences were filtered for chimers using UCHIME algorithm.[20,21] Sequences analysis were performed by Uparse software[22] using all the effective tags. Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, Mothur software was performed against the SSUrRNA database of SILVA Database[23] for species annotation at each taxonomic rank (kingdom, phylum, class, order, family, genus, species).[24] To obtain the phylogenetic relationship of all OTUs representative sequences, the MUSCLE (Version 3.8.31)[25] can compare multiple sequences rapidly. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences. Subsequent analysis of alpha diversity and beta diversity were all performed based on this output normalized data.

To evaluate microbial community diversity within each fecal sample, alpha diversity was measured with rarefied richness and the Chao1 and Shannon diversity indices. Rarefied richness provides a number of observed OTUs and is used as a proxy for bacterial species. The Chao1 diversity index is a measure of the expected total richness. Shannon diversity indices evaluate the richness and evenness of the OTUs contained with each fecal sample. To visualize the differences in the microbial communities among samples based on composition, beta diversity the phylogeny-based weighted UniFrac distance was calculated and a Principal Coordinate

Analysis (PCoA) ordination plot was made to visualize this distance matrix.[26] UniFrac is a measure used to test if the phylogenetic lineages between samples are significantly different.

6.3.4 Serum Uremic Toxin Assay

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 Colorado State University Proteomics and Metabolomics Facility. Serum total indoxyl sulfate, pCS, and TMAO concentrations were determined by liquid chromatography with tandem mass spectrometry on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. The extraction and acquisition methods were developed based on previously published guidelines.[27] The authentic standards pCS (A8895) was purchased from APExBio Technology, IS (potassium salt, #16926) from Cayman Chemical, TMAO from Sigma-Aldrich, trimethylamine-d9 N-oxide (TMAO-D9, sc-475042) from Santa Cruz Biotechnology, p-cresol sulfate-d7 (pCS-D7 potassium salt, DLM9786-0.01) from Cambridge Isotope. For sample preparation, internal standard mix contained 0.8 µg/mL of TMAO-D9 and 6.7 µg/mL of pCS-D7 dissolved in 10% methanol in water. Frozen serum samples were thawed on ice. To 50 µL of sample, 50 µL of internal standard mix, and 400 µL of cold methanol were added to each aliquot. The mixture was vortexed for 5 s and then incubated at -20°C overnight. Then the samples were centrifuged at 17000 g and 4°C for 15 min. The supernatant was recovered and 50 µL of supernatant was directly transferred to autosampler vials to be used for TMAO and IS analysis. Another aliquot of 50 µL of supernatant was further diluted by mixing with 1000 µL of 50% methanol in water, to be used for pCS analysis. A quality control (QC) pool was generated by mixing 30 µL of each experimental sample extract.

Standard curves in spiked cat serum were linear over the range of 0 to 2 µg/mL for IS and TMAO and 0 to 100 µg/mL for pCS. The linearity of the curves was > $r^2 = 0.99$. The percent coefficient of variation of QC samples were 3.5% for IS, 2.8% for pCS, and 2.4% for TMAO. Limits of detection (LOD) and limits of quantification (LOQ) were calculated as 3 times or 10 times the standard deviation of the blank divided by the slope of the calibration curve, respectively. The limit of quantitation (LOD) and limits of quantification (LOQ) for IS was 0.0004 µg/mL and 0.0015 µg/mL, for pCS was 0.0026 µg/mL and 0.0086 µg/mL, and for TMAO was 0.0013 µg/mL and 0.0042 µg/mL, respectively.

6.3.5 Statistical Analysis

Prior to the analyses, the data was assessed for normality by the Shapiro-Wilk test. Descriptive statistics for the following continuous clinicopathologic values were performed: body weight, blood pressure, UPC ratio, and serum concentrations of creatinine, blood urea nitrogen (BUN), phosphorus, total calcium, potassium, and sodium. To compare treatment effects among the three groups (SF68 group, palatability enhancer group, control group), the percent change in each clinicopathologic variable from enrollment (Week 0) to the end of the study period (Week 8) was calculated and the difference between groups was evaluated by the ANOVA test for parametric data and Kruskal-Wallis test for non-parametric data. In addition, the absolute values of each clinicopathologic variable for each group at Week 8 were compared to Week 0 either by paired t test for parametric data or Wilcoxon matched-pairs signed rank test for non-parametric data. To evaluate the overall potential for an effect on appetite induced by the palatability enhancer, cats in the palatability enhancer group and SF68 group were combined, the percent change in each variable from Week 0 to Week 8 was calculated, and the percent change was compared to the control group by the two-sample t test for parametric data and Mann-

Whitney test for non-parametric data. The proportion of vomiting episodes documented by the owners over the 8-week study period and the proportion of cats that vomited at least once in the SF68 group and palatability enhancer group were evaluated by Fisher's exact test. Analysis of similarity (ANOSIM) using Bray-Curtis distance matrix was used to determine whether community structure significantly differed between groups at Week 8 and between Week 0 and Week 8 in the SF68 group and palatability enhancer group. The R value signified the strength of the factors on the samples and ranges between 0 and 1. An R value closer to 1 indicates high separation between groups and R value close to 0 indicates no separation between groups. Linear discriminant analysis (LDA) effect size (LEfSe) was used to capture the bacterial taxa with significant differences in relative abundance among groups. An LDA effect size cutoff was set to 4. The larger the LDA effect size the more the bacterial taxa explain the differentiating phenotypes within each group. To determine the effect the probiotic has on serum IS, pCS, and TMAO concentrations, the concentrations at Week 8 were compared to Week 0 within each group by Wilcoxon matched-pairs signed rank test. For all analyses, a value of $P < 0.05$ was considered significant.

6.4 Results

6.4.1 Cat Descriptions

Twenty-seven cats with CKD were enrolled and 24 cats completed the 8-week study. One cat that was fed SF68 was removed from the study within the first week after clinical signs of hematochezia and tenesmus developed. These clinical signs resolved within 24 hours of discontinuing the study supplement. Two cats (both fed SF68) were removed from the study on Week 4 due to illness unrelated to CKD and the study. One cat developed a corneal ulceration

from a cat scratch that required antibiotic therapy and one cat had an incidental large hiatal hernia that required surgical repair. Eight CKD cats were enrolled in the SF68 group (IRIS stage 1, n=1; stage 2, n=4; stage 3, n=3). Eight CKD cats were enrolled in the palatability enhancer group (IRIS stage 1, n=2; stage 2, n=6). Eight CKD cats were enrolled in the control group (IRIS stage 1, n=2; stage 2, n=4; stage 3, n=2). One cat in the palatability enhancer group was excluded after completion of the 8-week study for suspect dietary intolerance to the hydrolysate contained within the study supplement (see Potential Adverse Events section 6.4.5). For statistical analysis, there were 8 cats in SF68 group, 7 cats in palatability enhancer group, and 8 cats in the control group. An inadequate amount of serum was available to perform IS and pCS concentrations in 1 cat in the SF68 group.

All cats were domestic short or long-haired cats except for one Siamese cat in the SF68 group. The mean age for all cats was 13 +/- SD 4.2 years. Thirteen cats were spayed female and eleven cats were castrated male. Twenty-two cats were fed exclusively a renal diet (including the 8 control cats fed Purina NF Early Care or Advanced Care depending on CKD stage) and two cats in the palatability enhancer group were fed a prescription high fiber diet (Royal Canin Fiber Response). As these were client-owned CKD cats, many cats were on a medical regimen at enrollment that was maintained throughout the study period. Medications administered to study cats by their owners during the study period included cyanocobalamin subcutaneous injections (4/24 cats), glucosamine chondroitin supplement (3/24 cats), subcutaneous fluids (3/24 cats), amlodipine (2/24 cats), gabapentin (2/24 cats), oral potassium supplement (3/24 cats), transdermal mirtazapine ointment (3/24 cats), and azithromycin, aspirin, fluticasone inhaler, topical imidacloprid, omeprazole, fish oil, buprenorphine, budesonide, and prednisolone (one cat each).

6.4.2 Clinical Parameters and Clinicopathological Data

Table 6.2 shows clinical and clinicopathologic variables for CKD cats in each group at Week 0 and Week 8. All cats in SF68 group and 7/8 cats in the palatability enhancer group accepted and ate > 75% of the supplements daily throughout the 8-week study. One cat in the palatability enhancer group ingested < 50% of the supplement for the first 23 days of the study but was included in the final data analysis. At enrollment, the median reported fecal score was 2 (range, 1-3) and no diarrhea (fecal score > 5) was noted during the study period for all cats that completed the study.

Body condition score and muscle score was static throughout the study period in all three groups. Body weights for cats in the SF68 group (P=0.75), palatability enhancer group (P=0.46), and control group (P=0.75) were not significantly different at Week 8 when compared to Week 0. No significant difference in the percent change in body weight over the 8-week study among the study groups was found (P = 0.82). No significant difference in the percent change in body weight over the 8-week study was found when the results from the SF68 and palatability enhancer groups were combined and compared to the control group (P = 0.56).

The median appetite score at enrollment for the SF68 group and the palatability enhancer group was 1 (range, 0-2) and 1 (range, 0-1), respectively. The median vomiting score for the SF68 group and palatability enhancer group was 2 (range, 0-3). The proportion of vomiting episodes reported by the owner in the SF68 group (4.8%) and palatability enhancer group (6.1%) during the study period was not statistically different between the two groups (P = 0.45). The proportion of cats in each of the study supplement groups (SF68 group, 71%; palatability enhancer group, 75%) that vomited at least once during the study period was not statistically

Table 6.2 Clinicopathologic variables for CKD cats that were fed SF68 probiotic, palatability enhancer without SF68 bacterium (palatability enhancer group), or no supplement (control group) for 8 weeks.

	Palatability Enhancer Group (n=7)		SF68 Group (n=8)		Control Group (n=8)	
Variable (reference interval)	Mean +/- SD		Mean +/- SD		Mean +/- SD	
	Week 0	Week 8	Week 0	Week 8	Week 0	Week 8
Creatinine (0.8-2.4 mg/dL)	2.1 +/- 0.6	1.9 +/- 0.5	2.6 +/- 0.9	2.7 +/- 0.9	2.2 +/- 1.0	2.3 +/- 1.1
Phosphorus (3.0 – 6.0 mg/dL)	3.7 +/- 0.6	4.3 +/- 0.8	3.8 +/- 0.6	4.0 +/- 0.8	3.6 +/- 0.5	3.9 +/- 0.7
Potassium (3.7-5.4 mEq/L)	4.9 +/- 0.5	4.7 +/- 0.5	4.4 +/- 0.5	4.4 +/- 0.5	4.7 +/- 0.5	4.7 +/- 0.5)
Blood pressure	134 +/- 16	135 +/- 14	143 +/- 11	138 +/- 16	143 +/- 13	133 +/- 20
	Median (range)		Median (range)		Median (range)	
	Week 0	Week 8	Week 0	Week 8	Week 0	Week 8
Body weight (kg)	4.7 (3.2-8.0)	4.7 (3.3-8.3)	4.6 (2.4-7.6)	4.6 (2.4-7.7)	4.2 (2.7-5.4)	4.1 (2.6-5.7)
Body condition score (BCS)	5 (5-9)	6 (5-9)	6 (2-8)	6 (2-8)	5 (4-6)	5 (4-6)
Muscle condition score (MCS)	1 (0-2)	1 (0-2)	1 (0-3)	1 (0-3)	2 (1-3)	2 (1-3)
BUN (18-35 mg/dL)	36 (27-55)	34 (29-70)	43 (33-67)	44 (32-66)	39 (24-51)	41 (20-55)
Total calcium (9.2-11.1 mg/dl)	10.0 (9.7-14.2)	10.1 (9.7-11.9)	10.5 (9.3-11.3)	10.4 (9.4-11.5)	10.3 (9.6-14.8)	10.2 (9.3-13.1)
Sodium (149-157 mEq/L)	152 (151-155)	152 (150-155)	152 (148-155)	152 (150-156)	154 (151-158)	153 (148-155)
UPC ratio (<0.2)	0.23 (0.09-0.63)	0.18 (0.11-2.14)	0.50 (0.1-1.91)	0.51 (0.08-2.34)	0.12 (0.08-0.22)	0.18 (0.1-0.36)

different between the groups (P = 0.36). A vomiting score at enrollment and record of the number of vomiting episodes throughout the study period were not obtained for the control group.

No significant difference in the percent change in blood pressure from Week 0 to Week 8 was found among the three groups (P = 0.39). No significant difference in the percent change from Week 0 to Week 8 was found for UPC ratio and serum creatinine, BUN, phosphorus, calcium, potassium, and sodium concentrations among the three study groups. No significant difference in the absolute laboratory values from Week 0 to Week 8 was found in each group (Table 6.2).

Except for UPC, no significant difference in serum biochemical parameters was found during the 8-week study period when the SF68 and palatability enhancer groups were combined and compared to the control group. The percent change in the UPC ratio was significantly higher in the control group (median, +38%) compared to the combined SF68/palatability enhancer groups (median, -14%; P=0.01).

6.4.3 Fecal Microbiome Analysis

Fecal microbiome analysis was performed on a fecal sample collected at Week 0 and Week 8 for 6 cats in the palatability enhancer group and for 7 cats in the SF68 group. Analysis was performed on a fecal sample collected at Week 8 for 6 cats in the control group. A fecal sample at Week 0 was not available from the cats in the control group. The median number of quality sequences for the 32 fecal samples was 517,730 (range, 78,250 to 694,923). The relative abundances of taxa at the genus level between groups (control group, Week 8; palatability enhancer group, Week 0; palatability enhancer group, Week 8; SF68 group, Week 0; SF68 group, Week 8) are presented in Figure 6.1. No significant differences were found when alpha

diversity, as described by Chao 1 and Shannon diversity indices, was compared between Week 0 and Week 8 for the SF68 group and the palatability enhancer group. Alpha diversity indices also did not differ between the three groups at Week 8. Species richness, as described by the number of unique OTUs, was not statistically different between Week 0 and Week 8 for the palatability enhancer group and the SF68 group nor between the three groups at Week 8.

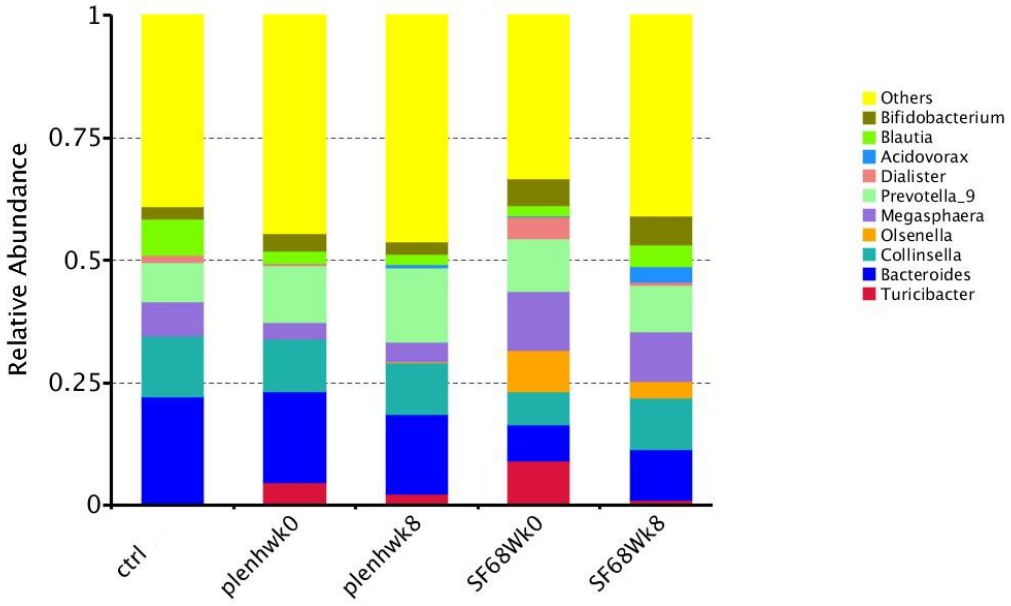

Figure 6.1 Relative abundances of taxa (genus level) in the control cats (Week 8), in cats that were fed palatability enhancer (Week 0 and Week 8), and in cats that were fed SF68 probiotic (Week 0 and Week 8).

Based on ANOSIM, community microbial structure did not significantly differ between Week 0 and Week 8 for the palatability enhancer group ($P = 0.98$; $R = -0.17$) or the SF68 group ($P = 0.97$; $R = -0.12$). The fecal community structure in the palatability enhancer group was different compared to the control group at Week 8 ($P = 0.012$; $R = 0.26$). No difference in the microbial communities was found between the SF68 group and the control group at Week 8 ($P = 0.19$; $R = 0.07$). The beta diversity data using weighted UniFrac distance matrix is displayed

using a principal coordinate analysis (PCoA) plot (Figure 6.2). Each dot represents the microbial community within one fecal sample. The closer the dots, the more similar the bacterial taxa are between the two fecal samples.

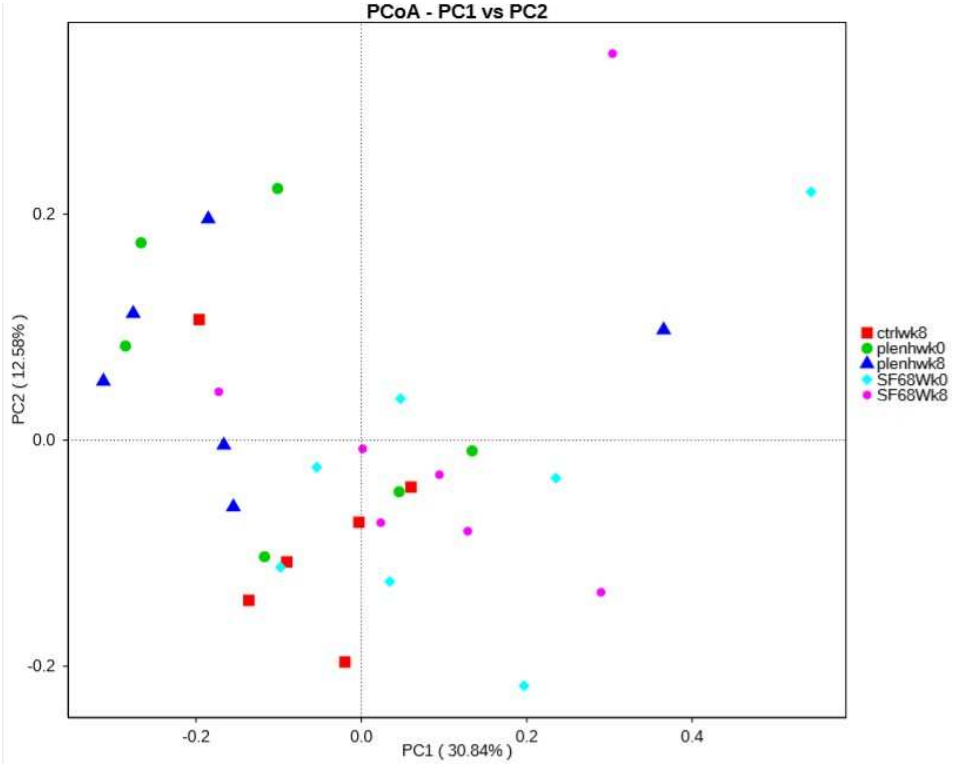

Figure 6.2 Principal coordinate analysis plot representing each fecal sample in the three study groups (Control [ctrl], Palatability Enhancer [plenh], SF68) at the two study time points (Week 0 or Week 8).

When individual bacterial groups were analyzed based on LDA effect size, bacterial taxa were identified as being significantly different among groups. When comparing groups at Week 8, the relative abundance of the genus *Olsenella* was higher and the relative abundances of the genus *Lachnospiraceae*, genus *Blautia*, and species *Bacteroides plebeius* were lower in the SF68 group compared to the control group. No differences in the individual bacterial taxa were found between Week 0 and Week 8 in the SF68 group or the palatability enhancer group.

The control sample from the DNA extraction procedure generated 439 raw reads. A total of 278 reads remained after pre-processing and chimera filtering. The main bacterial taxa belonged to the genera *Bacteroides, Collinsella,* and *Megasphaera* and unclassified taxa. Considering the low number of reads compared to the fecal samples, the bacterial taxa isolated from the control sample likely reflect the kitome, rather than contamination.

6.4.4 Uremic Toxin Serum Concentrations

The serum concentrations of IS, pCS, and TMAO were performed in 8 control cats, 7 cats supplemented with the palatability enhancer, and 5 cats supplemented with commercial SF68 product (total 20 CKD cats). No significant difference was found in serum IS, pCS, and TMAO concentrations at Week 8 when compared to Week 0 for the SF68 group and the palatability enhancer group. Indoxyl sulfate was greater at Week 8 (median, 2435; range, 1155-3986 ng/mL) compared to Week 0 (median, 1838; range 997-2575 ng/mL) in the control group ($P = 0.008$); no difference in pCS and TMAO between Week 0 and Week 8 was found in the control group.

6.4.5 Potential Adverse Events

No adverse events were noted in the control group fed a prescription renal diet for 8 weeks. One cat fed the palatability enhancer was removed from the study on day 7 after reported persistent tenesmus and hematochezia. The cat continued to eat well and reportedly enjoyed the supplement. The clinical signs resolved within 24-48 hours after discontinuation of the supplement. This cat had a reported history of food intolerance and was suspected to be intolerant of study powder. One cat in the SF68 group was found to have incidental mild tachypnea on examination at Week 8. Thoracic radiographs showed severe pleural effusion and alveolar pattern in the left cranial lung lobe. This cat had a history of grade III/VI parasternal heart murmur of unknown etiology and it was presumed that she was in congestive heart failure,

although neoplasia or infection could not be excluded. Owner declined further evaluation, and it was recommended that subcutaneous fluids be discontinued. Cat 6 was reportedly doing well at home 2 weeks later after finishing the study and discontinuation of subcutaneous fluids. The blood pressure and sodium of this remained static and within the normal reference range throughout the study. One cat excluded from statistical analysis in the palatability enhancer group developed mildly elevated liver enzyme activities (alkaline phosphatase, alanine transferase) and, unlike other cats, had lost weight (0.74 kg) during the study period despite a good appetite. This cat was rechecked 3 months after discontinuation of the supplement and the liver enzyme activities had normalized and his body weight had increased to baseline. It was presumed that this cat had occult food intolerance and reactive hepatopathy. For this reason, this cat was excluded from statistical analyses.

6.5 Discussion

While probiotics have shown to be beneficial in humans with CKD,[15] this is the first study evaluating the effect of probiotics on clinical parameters, clinicopathologic variables, serum uremic toxin concentrations, and fecal microbial composition in cats with CKD. With the exception of 2 cats that may have had intolerance to the palatability enhancer contained within the study powders, the results suggest that most cats with CKD will consume the *E. faecium* SF68 probiotic for at least 8 weeks and in many cases is well tolerated. This is also the first study that reports the effect of feeding the commercial renal diet Purina NF Early Care and Advanced Care to client-owned cats with CKD. The diet was accepted by all cats and eaten for the duration of the 8-week study and the cats were stable throughout the trial based on clinical and laboratory parameters. A potential limitation was that the diet trial generating the control data was not performed in parallel to the probiotic study but was rather used as a 3rd

randomization group. However, with the exception of not recording vomiting events or daily fecal score and no collection of a fecal sample at enrollment to evaluate fecal microbial composition, data collected for the control group was the same as for the cats randomized into the SF68 or palatability enhancer groups.

Cats with CKD are well documented to commonly suffer from poor appetite and weight loss.[3,28,29] In a recent survey,[28] many owners of CKD cats (43%; 466/1079) reported that their cats had an abnormal appetite and 52% of these owners reported that their cats had a poor appetite or required coaxing to eat 5-7 days a week. Accurate determination of total daily food intake was not possible in the current study due to the common practice of free feeding dry food or to the difficulty of monitoring in a multi-cat household. Therefore, body weight was used as a surrogate measurement of caloric intake during the study. Unlike many other commercially available probiotics for cats, SF68 includes a chicken and liver-based hydrolysate to add flavor to the product that is appetizing for cats. We hypothesized that one potential beneficial effect of the SF68 product when fed to cats with CKD would merely relate to the fact that many cats are stimulated to eat more due to the palatability enhancer. To assess that hypothesis, the results from the palatability enhancer and SF68 groups were combined and the percent body weight change over the 8-week study were compared to the control group. Significant differences were not detected leading to rejection of this hypothesis. However, several owners reported that they perceived that their cats ate better and enjoyed their food more with the addition of the study supplements. In fact, owners of the 5/16 cats opted to continue their cats on the commercially available SF68 probiotic product after the study. The owner of one cat with IRIS CKD stage 3 in the SF68 group that was removed from the study at Week 4 (incidental hiatal hernia that required surgery) reported that her cat ate better with the study supplement and within 4 weeks the cat

gained 6% body weight. Since dysrexia is a common problem in CKD cats, the use of SF68 added to food to augment appetite in cats with CKD should be considered by practitioners and may be effective at alleviating signs of dysrexia and weight loss for some cats. A study with larger numbers of cats and a longer duration of supplementation should be considered.

When the laboratory results from the palatability enhancer and SF68 groups were combined and compared to the control group, no significant findings were noted except for a significant increase in UPC ratio for the control group over the 8-week study. However, this finding was not clinically significant for several reasons. First, the majority of cats (19/23) including in the final analysis had only a mild UPC ratio < 0.5. Second, previous studies have shown that the variation of UPC ratios observed in cats with values within the reference range suggest that serial UPC ratios need to differ by as much as 90% to conclude a high level of confidence that a cat's magnitude of proteinuria has increased.[30] This only occurred in 1 cat in the palatability enhancer group (UPC, Week 0: 0.33; Week 8: 2.14) whom also had a history of chronic pancreatitis.

Probiotics, in addition to synbiotics and prebiotics, have been used in people in hope to improve gut dysbiosis and improve clinical outcomes by reducing microbial production of protein-bound uremic toxins, in particular pCS.[15,31] According to a meta-analysis of randomized controlled trials in people with renal disease, probiotics have been shown to reduce serum concentrations of pCS.[15] In our study, the SF68 probiotic did not significantly alter the microbial community diversity or community structure over the 8-week study period. The significant differences in microbial community structure at Week 8 between cats fed the palatability enhancer and control cats likely reflects individual cat differences in the fecal microbiome. In addition, we found that the SF68 probiotic did not significantly change serum gut-derived uremic

toxin concentrations. However, serum was available at both time periods (Week 0 and Week 8) for only 5 cats fed the commercial SF68 product. Due to the small sample size, it is difficult to truly ascertain the affect the SF68 probiotic had on the production of the uremic toxins. Although SF68 did not result in a significant change in serum uremic toxin concentrations in this 8-week pilot study, further evaluation of probiotics, particularly high-dose multi-strain synbiotics, in cats with CKD warrants further investigation.

There have been several studies that support probiotic SF68 having immune modulating effects in cats. In the first study, cats supplemented with SF68 for 22 weeks had statistically significant increases in CD4+ lymphocyte counts compared to cats fed the palatability enhancer as a placebo.[11] Additionally, cats infected with feline herpesvirus-1 and fed SF68 had less clinical signs of upper respiratory disease over time when subjected to housing changes to induce mild stress when compared to cats in the control group which suggested immune modulation.[32] In addition to fibrosis and tubular atrophy on renal histopathology, cats with idiopathic CKD also commonly have lymphoplasmacytic nephritis which may suggest an underlying autoimmune etiology.[17] Inflammatory cytokines such as interleukin-18 and transforming growth factor beta-1 have been linked to ischemic injury and renal fibrosis in people.[33,34] Therefore, modulation of the immune system could either have detrimental or positive effects on cats with CKD. The failure to detect worsening of renal parameters in the SF68 group of cats when compared to the palatability enhancer group or control group described here suggests that any immune modulation induced by the bacterium within 8 weeks was not detrimental. However, positive effects on renal laboratory values were also not noted. Whether any immune modulating effects induced by the bacterium had peaked by 8 weeks of supplementation is not known and full evaluation of the immune-enhancing effects of SF68 probiotic in this population of cats was not

explored. Therefore, whether the immune modulating effects of SF68 were of benefit or detriment cannot be definitively concluded in cats with CKD. Further studies should be performed feeding the probiotic to larger numbers of cats with CKD for longer time periods and concurrently evaluating immune function.

The commercial SF68 probiotic includes calcium iodate. This is important to consider as chronic kidney disease is a risk factor for incidental total hypercalcemia in cats.[35] Three of the sixteen cats that completed the 8-week study and were fed the study powder had a total hypercalcemia at enrollment. One of these three cats was excluded from the final statistical analysis after a suspected occult food intolerance was highly suspected after study conclusion. However, even with the addition of this cat in the statistical analysis, serum calcium concentrations were not different at the end of the study compared to at enrollment in the 2 study supplement groups. However, considering the small sample size, the authors encourage monitoring serum calcium in CKD cats receiving the commercial product.

In cats, the commercially available SF68 probiotic product has been shown to reduce episodes of acute diarrhea in a shelter,[7] improve stool character with chronic intractable diarrhea,[36] and lessen antibiotic-associated diarrhea.[12] Multi-strain probiotics and synbiotics have been reported to alleviate clinical signs of constipation in cats with chronic constipation and improve stool character in cats with chronic diarrhea.[6,8] At the time of enrollment in the present study, the majority of cats with CKD that were fed the study supplement had a normal fecal score (2 or 3) and one cat that were fed SF68 had a fecal score of 1 consistent with constipation. None of the owners reported that their cats exhibited signs of constipation at any time point during the study. Fecal scores were unchanged over the 8-week period in both study powder groups, including the one cat with constipation. Although several owners reported improvement

in frequency in vomiting episodes with both study supplements, there was no significant difference in the proportion of vomiting episodes between the two supplement groups. A prospective, blinded, cross-over study would be necessary to evaluate the potential positive effect SF68 has on vomiting and fecal constituency in cats with CKD.

There were several limitations to this study. First, the sample sizes were low in this pilot study which is compounded by 1 cat in the palatability enhancer group being removed from statistical analysis due to the potential for an intolerance to the product. Second, the groups were not matched by IRIS CKD stage, but rather by household. Three households included a total of 7 cats enrolled in the study, and cats in the same household were fed the same study supplement to prevent cross-contamination. Therefore, the IRIS CKD stages enrolled in each group varied. Since weight loss and poor appetite are more pronounced in later stage disease,[3] this may have affected full interpretation of the effect of the probiotic on appetite and body weight. Lastly, a history of suspect or known gastrointestinal disease was not an exclusion criteria in this pilot study. One cat in the SF68 group had suspect inflammatory bowel disease and received oral budesonide and one cat in the palatability enhancer group received intermittent prednisone for treatment of chronic pancreatitis. Neither of these cats showed evidence of an adverse reaction towards the study supplement.

In conclusion, cats with IRIS CKD stages 1, 2 or 3 had stable disease over the 8-week study period regardless of group. The study showed that feeding the probiotic SF68 to a small group of CKD cats does not affect the fecal microbial community structure or serum concentrations of 3 major gut-derived uremic toxins. The palatability enhancer contained in the commercial SF68 product may be used to augment appetite in some CKD cats.

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CHAPTER 7: BIOLOGICAL VARIABILITY OF MAJOR GUT-DERIVED UREMIC TOXINS IN THE SERUM OF HEALTHY ADULT CATS

7.1 Summary

Background: Indoxyl sulfate (IS), p-cresol sulfate (pCS), and trimethylamine-n-oxide (TMAO) are gut-derived uremic toxins that have the potential to be therapeutic biomarkers in cats with chronic kidney disease. To understand the clinical application, reliable biological variation estimates of within-individual variation (CV_I) and group variation (CV_G) are needed to determine the individuality of these analytes. The individuality determines whether a population-based reference interval (index of individuality [IOI] ≤ 0.7: low individuality) or a subject-based reference interval (IOI ≥ 1.67: high individuality) is most appropriate when monitoring serial measurements.

Objectives: Primary objective was to determine short- and medium-term biological variation estimates to calculate the IOI and reference change value (RCV) for serum total IS, pCS, and TMAO concentrations in healthy, fasted adult cats. Secondary objective was to determine the effect recent feeding has on serum concentrations.

Animals: 12 healthy adult research cats

Methods: Prospective, cohort study. The study was performed in 3 parts: short-term biological variation (Part 1), effect of recent feeding on serum concentrations (Part 2), medium-term biological variation (Part 3). For Part 1 and 2, a jugular catheter was placed. For Part 1, after a 12-hour fast blood was collected every 2 hours over a 12-hour period (0600, 0800, 1000, 1200, 1400, 1600, and 1800). For Part 2, blood was collected after a 12-hour fast (0600). Cats were then fed and blood was collected every 2 hours over a 12-hour period (0800, 1000, 1200, 1400,

1600, and 1800). For Part 3, blood was collected by jugular venipuncture after a 12-hour fast 5 times over a 19-day period (interval, 3-6 days). Analytical variation was based on duplicate extraction and analysis of each serum sample. Serum total IS, pCS, and TMAO concentrations were determined by liquid chromatography-tandem mass spectrometry.

Results: Inter- and intra-individual variation was large for serum IS, pCS, and TMAO. For serum IS concentrations, the short-term biological variation estimates corresponded to a high IOI (1.96) and the medium-term biological variation estimates corresponded to a low IOI (0.65). The IOI was intermediate using both short-term and medium-term biological variation estimates for serum pCS concentrations (0.98 and 1.17, respectively) and TMAO (1.47 and 0.83, respectively) concentrations. The RCV for IS, pCS, and TMAO based on the medium-term biological variation estimates suggest that serum concentrations would have to decrease by 21.9%, 28.9%, and 52.2%, respectively, between serial measurements to suggest a significant change. Serum IS, pCS, and TMAO concentrations were significantly lower in the non-fasted state when compared to a fasted state at multiple time points over a 12-hour period. Serum concentrations differed between multiple collection time points for IS in the non-fasted ($P = 0.001$) and pCS in the fasted state ($P < 0.001$).

Conclusion: Our study provides biological variation estimates for the 3 major gut-derived uremic toxins IS, pCS, and TMAO. Feeding may reduce serum concentrations of pCS, IS, and TMAO over a 12-hour period in cats. To compare serial measurements, it would be prudent to collect samples at the same time of day and consistently in either a fasted or non-fasted state.

7.2 Introduction

In patients with chronic kidney disease, the accumulation of the major gut-derived uremic toxins indoxyl sulfate (IS), p-cresol sulfate (pCS), and trimethylamine n-oxide (TMAO) in

systemic circulation is, in part, a consequence of functional dysbiosis.[1,2] Liquid chromatography alone or in combination with mass spectrometry are common methods to measure IS, pCS, and TMAO in plasma or serum.[3,4] Assessment of these molecules, in particular IS and pCS, in people, dogs, and cats with CKD provides information on risk of disease progression[5-7] and in people are markers of dietary interventions.[8,9]

Understanding biological variation and knowing how to interpret data is of paramount importance for both research purposes and to assess clinical utility of measuring IS, pCS, and TMAO in cats. Analytical measurements all have random variability from multiple sources. In addition to expected changes in the physiological status of the patient and analytical imprecision, biological variation is a source of variability and describes the physiological random fluctuation around a homeostatic set point. Assessment of biological variation for an analyte is necessary in order to interpret clinical test results, including whether a population-based reference interval is appropriate for the analyte.

Estimates of biological variation include within-individual variation (CV_I) and between-individual (or group) variation (CV_G) which take into account analyzer variation (CV_A). Biological variation studies are most commonly performed on healthy individuals because often estimates are constant across age, geography and methodology. In people it was found that values obtained in serum established for healthy individuals are applicable to most chronic diseases.[10] Biological variation allows practitioners to determine if a change in a measured analyte is clinically significant (i.e. reference change value [RCV]), even if the value is within the population-based reference interval. To determine the applicability of population-based reference intervals, the index of individuality (IOI) must be determined for the analyte. The IOI describes the relationship between CV_I and CV_G and takes into account CV_A.

Although an established reference interval and biological variation estimates have been established in healthy humans for serum total IS and pCS,[11] evaluation of biological variation for IS, pCS, and TMAO has not been explored in veterinary medicine. In addition, it is unknown whether recent feeding affects serum concentrations of IS, pCS, and TMAO. Therefore, the primary aim of this chapter was to determine short and medium-term biological variation estimates, IOI, and RCV for serum total IS, pCS, and TMAO concentrations in healthy adult cats. The secondary aim was to measure the difference in serum concentrations in fasted versus unfasted samples to determine optimal testing conditions to use in the field. The hypothesis is that subject-based reference intervals should be used for monitoring of changes in serial results and that serum concentrations will gradually increase after feeding.

7.3 Materials & Methods

7.3.1 *Study Design and Animals*

The prospective, cohort study was performed in 3 parts. In **Part 1**, short-term biological variation estimates of serum concentrations of IS, pCS, and TMAO were determined in a fasted state. Serum samples were collected every 2 hours over a 12-hour period after a 12-hour fasting period. In **Part 2**, serum samples were collected every 2 hours over a 12-hour period after a meal. In **Part 3**, medium-term biological variation estimates of the 3 analytes were determined by collecting a fasted serum sample 5 times over a 19-day period.

Twelve research-bred adult cats were enrolled. The study population included 12 apparently healthy domestic short-haired cats (2 years of age). The cats were of mixed sex (6 male castrated, 4 female spayed, 2 female intact). Prior to enrollment, cats were maintained at a research facility and group-housed. They were fed a commercially available adult maintenance

dry diet (Meow Mix Original Choice, Big Heart Pet Inc, San Francisco, CA) for at least 8-weeks prior to enrollment. The nutrient content of the commercial diet is found in Table 7.1. The body weight, body condition score (BCS; Purina 1-9 point scale; Nestle Purina, St. Louis, MO), muscle condition score (MCS; WASAVA Global Veterinary Community, Ontario, Canada) and liver and kidney laboratory values from the cohort of cats included in this study are summarized in Table 7.2. All cats had a normal physical examination with the exception of one female intact female with a grade II/VI left parasternal heart murmur of unknown etiology and mild paraspinal muscle atrophy. A CBC, serum biochemistry, urinalysis, and total serum thyroxine were normal for all cats including a creatinine < 1.8 mg/dl and USG > 1.045. The cats were housed and cared for in accordance with a protocol that was approved by the Institutional Animal Care and Use Committee at the contract research facility that was used for the study (HQR protocol 170.045).

Table 7.1 The nutrient content of the commercial diet fed to the enrolled study cats.

Nutrient	**g/100 kcal ME**
Protein	8.74
Fat	3.61
Carbohydrates	11.07
Insoluble dietary fiber	2.44
Soluble dietary fiber	0.37

Table 7.2 Physical examination parameters and liver and kidney clinicopathologic variables for 12 study cats.

Variable (reference interval)	Median (range)
Body weight (kg)	4.5 (3.0-6.8)
BCS (score 1-9)	5 (5-6)
MCS (score 0-3)	0 (0-1)
Creatinine (0.8-2.4 mg/dL)	1.4 (0.9-1.7)
BUN (18-35 mg/dL)	23 (17-27)
ALP (10-80 IU/L)	43 (34-64)
ALT (30-140 IU/L)	27 (14-50)
GGT (0-0.5 IU/L)	0 (0)
Total Bilirubin (0.0-0.1 IU/L)	0 (0-0.1)
Total T4 (μg/dL)	2.2 (1.7-4.2)
USG (> 1.035)	1.057 (1.049-1.064)

7.3.2 Sample Collection

For **Parts 1 and 2**, cats were fasted for 12 hours and then sedated with intramuscular ketamine (20 mg per cat) and midazolam (10 mg per cat) for placement of jugular catheters. Aseptic placement of either 4 French 15-cm wire guided single lumen jugular catheter (MILACATH, MILA International, Inc, Florence, Kentucky, USA; 10/12 cats) or single lumen catheter-through-cannula jugular catheter (Cavafix Certo, B Braun Medical AG, Melsungen, Germany; 2/12 cats) were placed in either the left or right jugular vein approximately 16 hours prior to the start of Part 1 and 36 hours prior to the start of Part 2. After catheter placement, all cats were housed in a separate kennel and provided a litterbox and free choice water. All cats recovered uneventfully from sedation. The catheters were secured and heparin locked (100 U/ml heparin sulfate).

For **Part 1**, after an overnight fast (12 hours) a total of 7 blood samples were collected between 0600 and 1800 using a 3-syringe technique. For the 3-syringe technique, 1 ml of heparinized saline was flushed through the catheter and 3-ml of blood was aspirated into the syringe. The syringe with heparinized blood was detached, a second 2-3 mL of blood was collected using a 3-mL syringe, and un-heparinized blood was placed into a sterile red top tube. The heparinized blood was then returned to the cat and the catheter was flushed with heparinized saline. Blood samples (2-3 mL depending on body weight) were collected at 0600 (Hour 0), 0800 (Hour 2), 1000 (Hour 4), 1200 (Hour 6), 1400 (Hour 8), 1600 (Hour 10), and 1800 (Hour 12). After sample collection, cats were offered 50 grams of their regular adult maintenance diet for one hour in the PM. For **Part 2**, after a 12-hour fast a blood sample (2-3 mL) was collected (0600; Hour 0) and cats were offered 50 grams of food for one hour. All cats ate 75-100% of the food offered. Non-fasted blood samples (2-3 ml) were collected at 0800 (Hour 2), 1000 (Hour 4), 1200 (Hour 6), 1400 (Hour 8), 1600 (Hour 10), 1800 (Hour 12) using the same 3-syringe technique (Figure 7.1).

Sample collection and processing were standardized to minimize pre-analytical variation. At each blood draw time, cats were collected in the same sequential order. Blood samples were centrifuged at 3,000 g for 10 minutes and the serum was aliquoted and then stored at 4 °C for less than 12 hours before transferred to -80 °C freezer. Jugular catheters were then removed without sedation and cats were moved to group-housing for the remainder of the study.

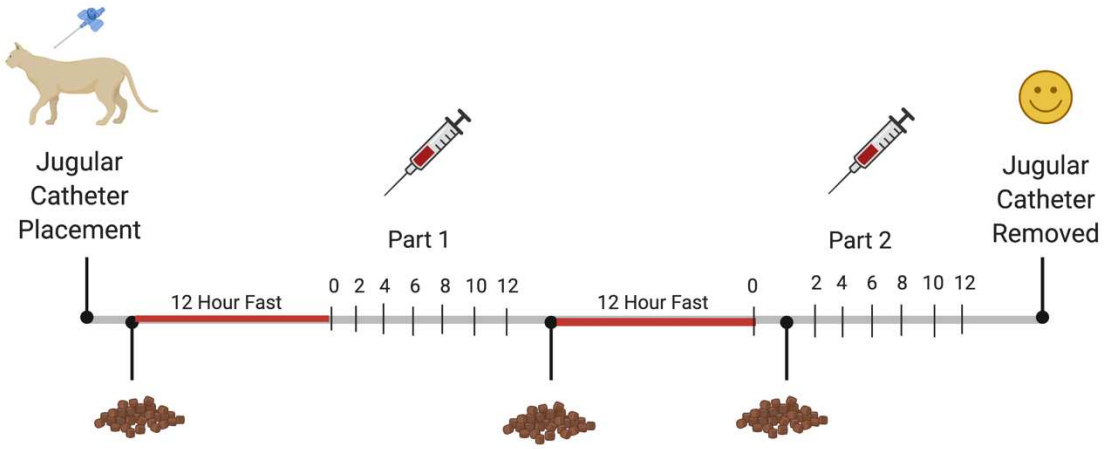

Figure 7.1 Schematic representing the timing of feeding and blood sampling for Part 1 and Part 2 of the study.

To determine medium-term biological variation, in **Part 3** blood samples were obtained 5 times over a 19-day period on days 0, 5, 8, 14, and 19 (range, 3-6 day interval). After an overnight 12-hour fast, each cat was sedated with an intravenous injection of ketamine (10-20 mg) and midazolam (5 -10 mg). A blood sample (3 mL) was collected between 0800 and 0900 by venipuncture of either the left or right jugular vein from each cat using a 22-gauge needle and 3-mL syringe by a single operator. After removal of the needle, blood was transferred into a 5-mL sterile collection tube without anticoagulant. Samples were centrifuged at 3,000 g for 10 minutes, the serum was aliquoted, and samples were placed in -80 °C within 3 hours of collection. Cats were collected in the same sequential order on each collection date so that sampling occurred at approximately the same time of day for each cat.

Serum samples from Parts 1-3 were stored at -80°C for 9 months before thawing and analysis at the Colorado State University Proteomics and Metabolomics Facility.

7.3.3 Uremic Toxin Assay

Total IS, pCS, and TMAO serum concentrations were determined by liquid chromatography-tandem mass spectrometry. Serum samples were prepared in three batches according to the three study parts (Part 1, Part 2, Part 3). Within each batch, samples were extracted in a randomized order and in duplicates. Each section batch, including its two replicates, were acquired within one acquisition batch in a randomized order different from its sample prep order.

The extraction and acquisition methods were developed based on previously published guidelines.[12] The authentic standards pCS (A8895) was purchased from APExBio Technology, IS (potassium salt, #16926) from Cayman Chemical, TMAO from Sigma-Aldrich, trimethylamine-d9 N-oxide (TMAO-D9, sc-475042) from Santa Cruz Biotechnology, p-cresol sulfate-d7 (pCS-D7 potassium salt, DLM9786-0.01) from Cambridge Isotope. For sample preparation, internal standard mix contained 0.8 µg/mL of TMAO-D9 and 6.7 µg/mL of pCS-D7 dissolved in 10% methanol in water. Frozen serum samples were thawed on ice. Each serum sample was extracted in duplicate and the difference of the 2 replicates demonstrates the variations generated from sample preparation and instrument acquisition. To 50 µL of sample, 50 µL of internal standard mix, and 400 µL of cold methanol were added to each aliquot. The mixture was vortexed for 5 s and then incubated at -20°C overnight. Then the samples were centrifuged at 17000 g and 4°C for 15 min. The supernatant was recovered and 50 µL of supernatant was directly transferred to autosampler vials to be used for TMAO and IS analysis. Another aliquot of 50 µL of supernatant was further diluted by mixing with 1000 µL of 50% methanol in water, to be used for pCS analysis. A quality control (QC) pool was generated by mixing 30 µL of each experimental sample extract.

The LC-MS/MS analysis was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a Waters UPLC T3 stationary phase (2.1 x 50 mm, 1.8 µM) column. Mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The analytical gradient was as follows: time = 0 min, 1% B; time = 0.65 min, 1% B; time = 2.85 min, 99% B; time = 3.5 min, 99% B; time= 3.55 min, 1% B; time =5 min, 1% B. Flow rate was 500 µL/min. Samples were held at 6°C in the autosampler, and the column was operated at 45°C. Injection volume was 1 µL. The capillary voltage of MS detector was set to 0.7 kV MS in negative ionization mode, and 0.6 kV in positive mode. Inter-channel delay was set to 3 msec. Source temperature was 150°C and desolvation temperature 550°C. Desolvation gas flow was 1000 L/hr, cone gas flow (nitrogen) was 150 L/hr, and collision gas flow (argon) was 0.15 mL/min. Nebulizers pressure (nitrogen) was set to 7 Bar. Autodwell feature was set for the collection of 12 points-across-peak. Indoxyl sulfate and TMAO were acquired in one injection using undiluted sample extract. P-Cresol sulfate after dilution was acquired separately.

All raw data files were imported into the Skyline open source software package.[13] Each target analyte was visually inspected for retention time and peak area integration. Peak areas were extracted for target compounds detected in biological samples and normalized to the peak area of the appropriate internal standard or surrogate in each sample. Normalized peak areas were exported to spreadsheet and absolute quantitation was obtained by using the linear regression equation generated for each compound from the calibration curve. Calibration curves were run in duplicates with each acquisition batch. Standard curves in spiked cat serum were linear over the range of 0 to 2 µg/mL for IS and TMAO and 0 to 100 µg/mL for pCS. The linearity of the curves was > $r^2 = 0.99$.

The quality control samples, injected after every 8 experimental samples, was from one homogenous quality control pool extract and the variations of quality controls as measured by the coefficient of variance (CV) was contributed from instrument acquisition. The percent CV of quality control samples were 3.5% for IS, 2.8% for pCS, and 2.4% for TMAO. Limits of detection (LOD) and limits of quantification (LOQ) were calculated as 3 times or 10 times the standard deviation of the blank divided by the slope of the calibration curve, respectively. The limit of quantitation (LOD) and limits of quantification (LOQ) for IS was 0.0004 µg/mL and 0.0015 µg/mL, for pCS was 0.0026 µg/mL and 0.0086 µg/mL, and for TMAO was 0.0013 µg/mL and 0.0042 µg/mL, respectively.

7.3.4 Statistical Analysis

Box and whisker plots were constructed (GraphPad Prism 8.3.0; GraphPad Software, La Jolla, CA) for each toxin in Part 1 (short-term) and Part 3 (medium-term) to qualitatively describe variance between individuals (CV_G) and variance within individuals (CV_I). Statistical analysis was performed according to previously reported guidelines using a specialized statistical software (SAS, version 9.4; SAS Institute, Cary, NC). [14]

Residual diagnostic plots were used to evaluate model assumptions of normality and equal variance. Based on these plots, a (natural) log transformation was used for each toxin in each study part. Log transformed data were assessed for outliers using a three step process. The data were evaluated for values falling outside three times the interquartile range (1) across all cats, (2) within each cat and (3) using cat level averages. For Part 1, no outliers were identified. For pCS in Part 2, a single time point for a single cat (both duplicates) was identified as an outlier. For pCS and TMAO in Part 3, a single time point for a single cat (both duplicates) was identified as an outlier. All outliers were identified when looking "within cat" but not at the

other levels. All observations were retained in the analysis because the outlying values were similar between duplicates and thus are unlikely to be due to analytical error.

A random effects model was fit using restricted maximum likelihood (REML) with SAS Proc Mixed. Cat and time point (nested within cat) were included in the model as random effects. Hence, the variance was partitioned into three components for each toxin: (1) CV_G, (2) CV_I, and (3) variation between duplicates (corresponding to CV_A).

Because analysis was done on the log transformed scale, the (back transformed) coefficients of variation were calculated using the following equation:

$$\text{CV} = \sqrt{(\exp(\sigma^2) - 1)}$$

The IOI was calculated from the CVs using the following reciprocal formula:

$$IOI = \frac{CV_G}{\sqrt{CV_I^2 + CV_A^2}}$$

For an analyte with a low degree of individuality (<0.7), a population-based reference interval may be used to detect clinical disease. If an analyte has a high degree of individuality (≥1.7) then the reference change value is more sensitive to determine if a change in an analyte concentration for an individual cat is clinically significant. For analytes with intermediate individuality (IOI 0.7-0.1.69), a population-based can be used, however a subject-based reference interval may detect mild changes in the health status of an individual.[14,15]

Because analysis was done on the log transformed scale, RCVs were calculated using the lognormal approach. Specifically:

$$\text{RCV}(\%) = \exp(\pm Z\sqrt{2 * (\sigma_I^2 + \sigma_A^2)})$$

Where Z = 1.96 for 2-sided interpretation and σ2 represents a log scale variance component.

To determine if the fasting state should be considered a variable when assessing serum concentrations collected at the same time of day, serum IS, pCS, and TMAO concentrations (from only cats that completed both Part 1 and Part 2 of the study; n=9) were compared between the fasted state and non-fasted state at hours 2, 4, 6, 8, 10, and 12 using a paired t test or Wilcoxon matched-paired signed rank test. To determine whether the time of day should be considered a variable when assessing serum concentrations, a one-way ANOVA with the Geisser-Greenhouse correction followed by a Tukey's post hoc analysis or a Friedman test with Dunn's post hoc analysis was used to compare serum IS, pCS, and TMAO concentrations between different time points over a 12-hour period in the fasted state (Hours 0, 2, 4, 6, 8, 10, 12) and a 10-hour period in the non-fasted state (Hours 2, 4, 6, 8, 10, 12), respectively. The average of the duplicates taken at each time point for each cat were used in the analysis. Data was log-transformed to meet the assumption of normality. If normality was not met then a non-parametric test was performed on the raw values.

7.4 Results

Of the 12 enrolled cats, 10 cats finished Part 1 and 9 cats finished Part 2 due to catheter malfunction or displacement. All 12 cats completed Part 3 of the study. In the 3 study parts, analytical variation was less than 25% of within individual variation ($CV_A < 0.25 \times CV_I$) for IS, pCS, and TMAO analytes, therefore CV_A contributed to no more than 6.25% of total variation indicating adequate precision. In general, the coefficients of variations between cats (CV_G) and within individual cats (CV_I) were large for the 3 toxins. Values of CV_G, CV_I, CV_A, and indices of individuality for serum IS, pCS, and TMAO concentrations in healthy adult cats from study

Part 1 and Part 3 are reported in Table 7.3. The reference change values for serum IS, pCS, and TMAO are reported in Table 7.4.

Table 7.3 Biological variation for each analyte expressed as coefficients of variation for group (or between cat variation; CV_G), individual variation (CV_I), and analytical variation (CV_A) and the index of individuality (IOI).

Analyte	**Median (range)**	**CV_G (%)**	**CV_I (%)**	**CV_A (%)**	**IOI**
	Short-term Biological Variation (n=10)				
Indoxyl sulfate	363 (90-1,541 ng/mL)	58.2	29.7	2.2	1.96
P-cresol sulfate	10,958 (3,013-46,666 ng/mL)	40.7	41.4	3.0	0.98
TMAO	828 (373-1,457 ng/mL)	30.4	20.6	2.3	1.47
	Medium-term Variation (n=12)				
Indoxyl sulfate	494 (101-1,917 ng/mL)	38.7	59.0	2.5	0.65
P-cresol sulfate	19,328 (3,266-77,609 ng/mL)	55.0	47.0	2.9	1.17
TMAO	888 (375-1,507 ng/mL)	19.7	23.7	2.7	0.83

Table 7.4 Reference change values for serum indoxyl sulfate, p-cresol sulfate, and trimethylamine-n-oxide (TMAO) in healthy adult cats.

Analyte	**Reference Change Value (%)**	
	Increase	**Decrease**
	Short-term Biological Variation (n=10)	
Indoxyl sulfate	44.6	224.4
p-Cresol sulfate	33.1	302.1
TMAO	56.6	176.6
	Medium-term Biological Variation (n=12)	
Indoxyl sulfate	21.9	455.7
p-Cresol sulfate	28.9	345.8
TMAO	52.2	191.7

For serum IS concentrations over a 12-hour period (Figure 7.2a), the variation between cats (CV_G) was greater than the within-cat variation (CV_I) corresponding to a high II and supporting the use of a subject-based reference interval. On the contrary, the CV_I for serum IS concentrations over a 19-day period (Figure 7.3a) was greater than CV_G corresponding to a low II. Therefore, a population-based reference interval is appropriate in this clinical scenario. For serum pCS concentrations, the CV_G and CV_I were similar over a 12-hour period (Figure 7.2b).

Over a 19-day period, serum pCS concentrations had greater CV_G when compared to CV_I (Figure 7.3b). For serum TMAO concentrations, the CV_G was greater than CV_I over a 12-hour period (Figure 7.2c) and the contrary was found over a 19-day period (Figure 7.3c). For serum pCS and TMAO concentrations, the II was intermediate using both short-term and medium-term biological variation estimates supporting the use of either a population-based or subject-based reference interval depending on the clinical picture.

Serum IS concentrations were significantly lower in the non-fasted state when compared to the fasted state at hours 1000 ($P = 0.006$), 1200 ($P = 0.005$), 1400 ($P = 0.050$), and 1800 ($P = 0.026$; Figure 7.4). Serum pCS concentrations were significantly lower in the non-fasted state when compared to the fasted state at hours 1000 ($P = 0.004$) and 1200 ($P = 0.008$; Figure 7.5). Serum TMAO concentrations were significantly lower in the non-fasted state when compared to the fasted state at hours 0800 ($P = 0.005$), 1000 ($P < 0.001$), 1200 ($P < 0.001$), 1400 ($P < 0.001$), 1600 ($P < 0.001$), and 1800 ($P < 0.001$; Figure 7.6).

In the fasted state, serum pCS concentrations were significantly different at Hour 2 compared to Hour 12 ($P=0.028$), at Hour 4 compared to Hour 10 ($P=0.012$) and Hour 12 ($P=0.0004$), Hour 6 compared to Hour 12 ($P=0.019$), and Hour 8 compared to Hour 12 ($P=0.042$). Serum IS and TMAO did not significantly differ between time points throughout a 12-hour period in the fasted state. Serum IS concentrations in the non-fasted state were significantly different at Hour 2 compared to Hour 8 ($P=0.043$), Hour 10 ($P=0.017$), and Hour 12 ($P=0.014$) and at Hour 6 compared to Hour 8 ($P=0.014$). In the non-fasted state, serum concentrations of pCS and TMAO did not significantly differ between time points.

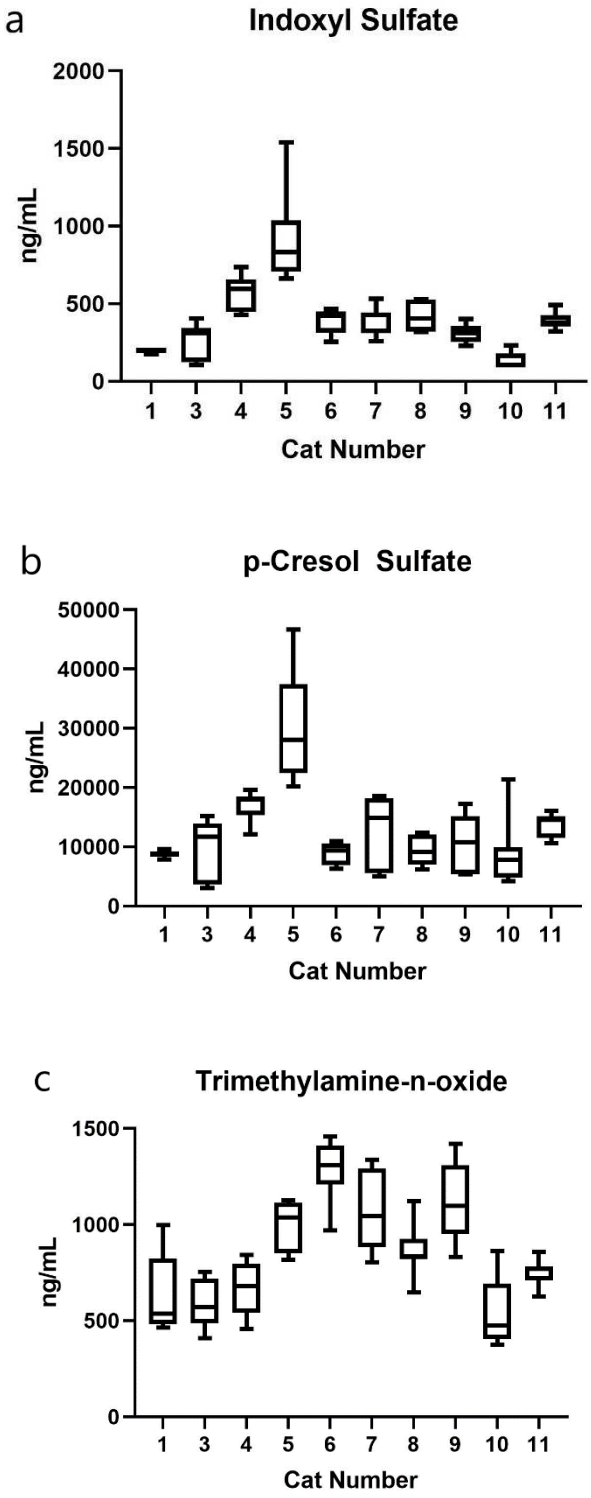

Figure 7.2 Box and whisker plots for serum indoxyl sulfate (a), p-cresol sulfate (b), and trimethylamine-n-oxide (c) concentrations in 10 healthy adult cats sampled every 2 hours over a 12-hour period in a fasted state.

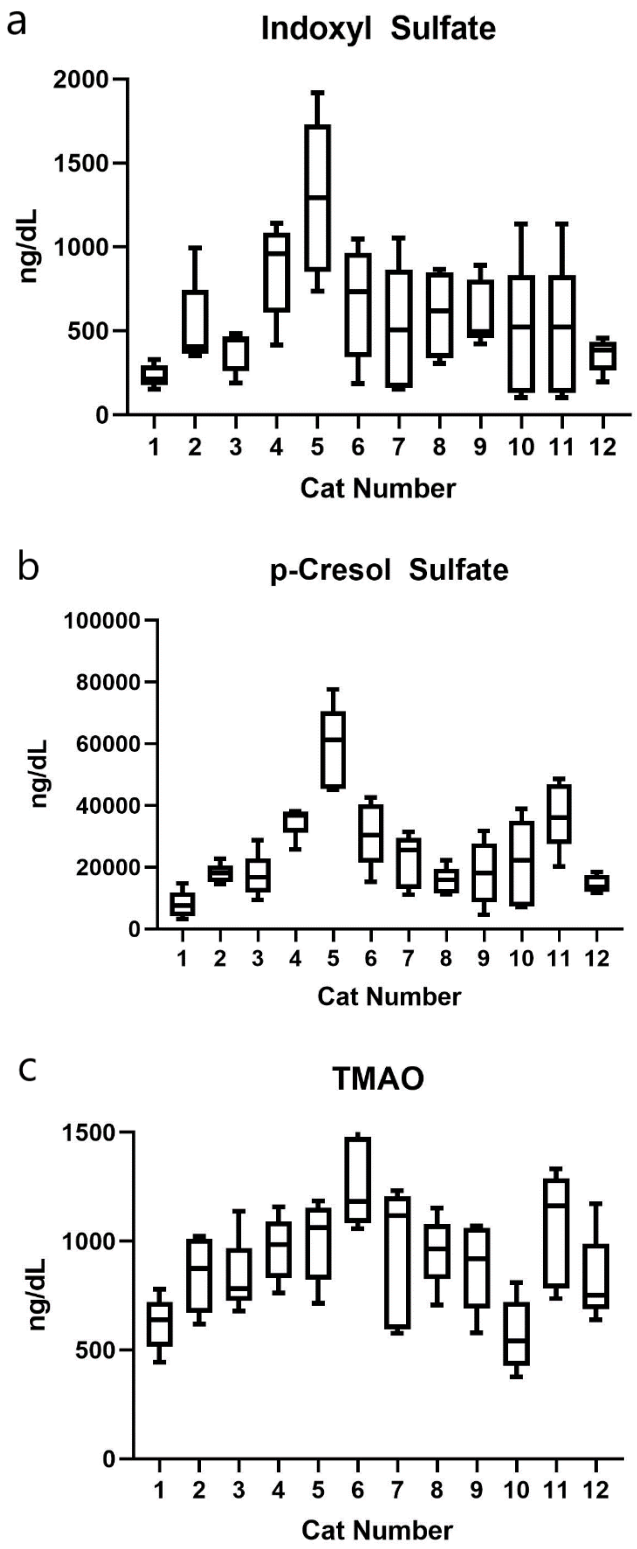

Figure 7.3 Box and whisker plots for serum indoxyl sulfate (a), p-cresol sulfate (b), and trimethylamine-n-oxide (c) concentrations in 10 healthy adult cats sampled 5-times over a 19-day period in a fasted state.

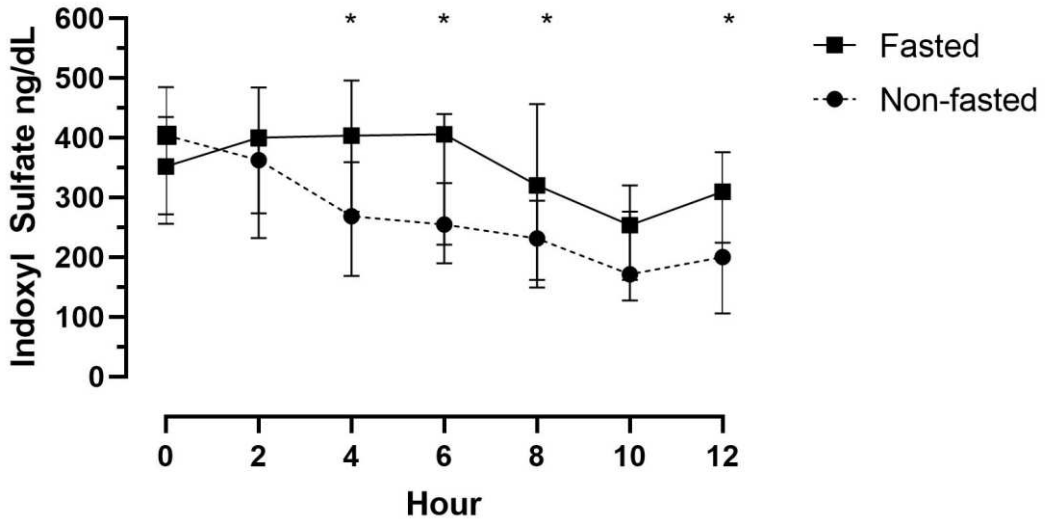

Figure 7.4 Serum indoxyl sulfate concentrations (median and interquartile range) in 9 healthy adult cats over a 12-hour period. Serum was collected after a 12-hour fast (Hour 0) and then every 2 hours in a fasted state and after a meal in non-fasted state (Hours 2, 4, 6, 8, 10, 12) in the same group of cats.

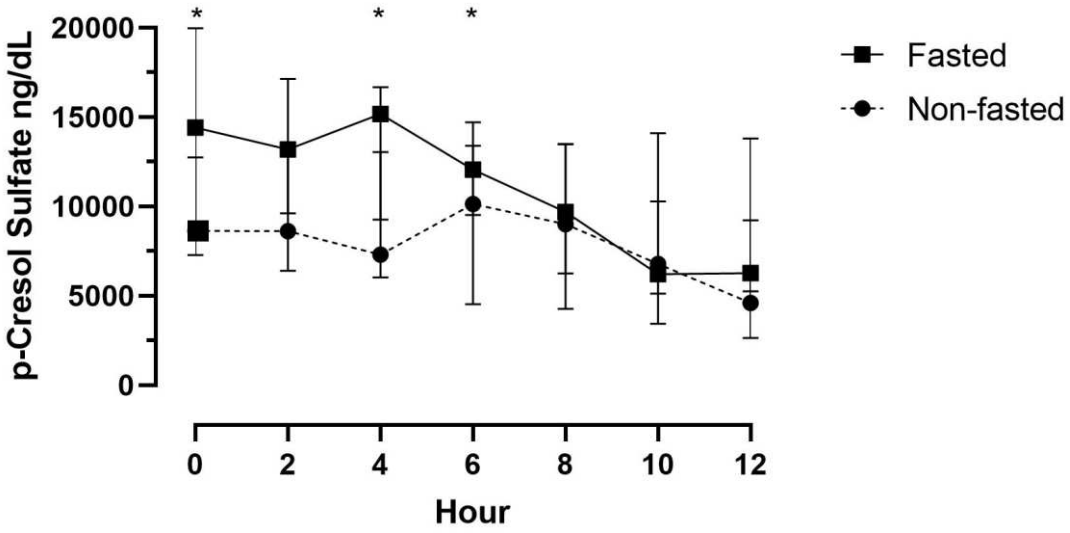

Figure 7.5 Serum p-cresol sulfate concentrations (median and interquartile range) in 9 healthy adult cats over a 12-hour period. Serum was collected after a 12-hour fast (Hour 0) and then every 2 hours in a fasted state and after a meal in non-fasted state (Hours 2, 4, 6, 8, 10, 12) in the same group of cats.

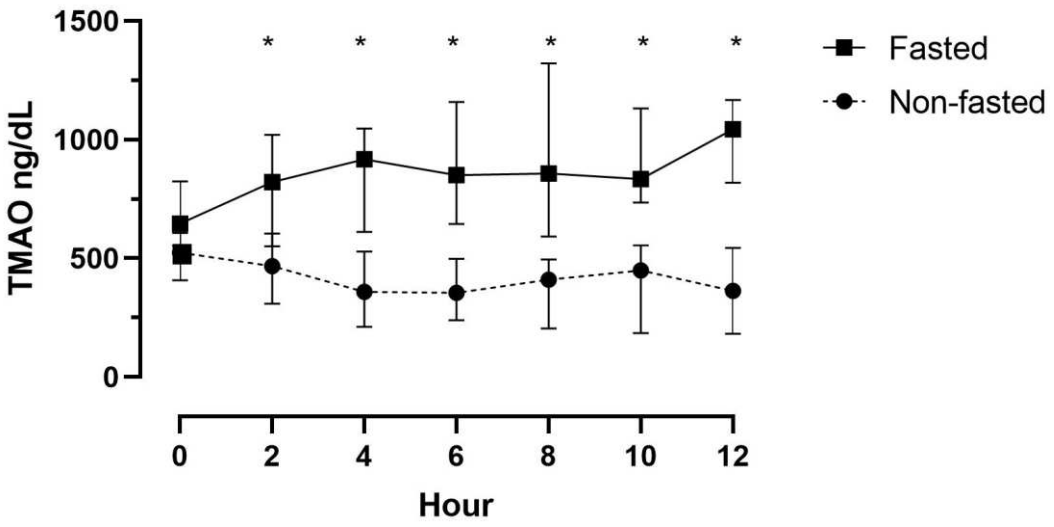

Figure 7.6 Serum trimethylamine-n-oxide (TMAO) concentrations (median and interquartile range) in 9 healthy adult cats over a 12-hour period. Serum was collected after a 12-hour fast (Hour 0) and then every 2 hours in a fasted state and after a meal in non-fasted state (Hours 2, 4, 6, 8, 10, 12) in the same group of cats.

7.5 Discussion

Our study determined the short-term and medium-term biological variation estimates for serum IS, pCS, and TMAO concentrations. In general, serum IS, pCS and TMAO concentrations have large intra- and inter-individual variability in healthy cats. Our findings are similar to a study in healthy people that evaluated the medium-term biological variation of total IS and pCS based on 8 samples collected daily on consecutive days in 10 healthy individuals. In this study by Pretorius et al.,[11] the CV_I and CV_G for total IS and pCS was 36% and 25% and 51% and 64%, respectively. The inter- and intra-variability is also substantially high in patients on hemodialysis.[16] This large variation has been attributed to the fact that these uremic toxins are end-products of protein catabolism in the colon. Urea, another product of protein catabolism, also has large intra- and inter-individual variation in cats.[17] In the studies in people, this notable variability was assumed to be due to changes in nutritional intake, however this is likely not the

case since in our study the cats were on the same diet and samples were collected after fasting. Other physiologic factors that may affect serum concentrations of IS, pCS, and TMAO are not well understood. Renal function, age, and gender are independent determinants of serum IS and pCS concentrations in people and host heritability also affect concentrations.[18] This is likely due to, at least in part, to the role these factors play in shaping the composition of the gut microbiome and therefore impacting amino acid processing in the intestines.[1,19,20]

Because IS, pCS, and TMAO are derived from dietary protein, we hypothesized that serum concentrations would increase after a meal. Indoxyl sulfate, pCS, and TMAO are end-products of amino acid fermentation in the colon and exclusively originate from the intestinal tract. Dietary protein is digested into peptides in the stomach and small intestine. A portion of undigested dietary proteins and peptides enter the colon and are depolymerized by bacterial proteases and peptidases into small oligopeptides and amino acids. In the distal colon, the amino acid tryptophan is fermented by bacteria to indoles and phenylalanine and the amino acid tyrosine are fermented to p-cresol. Indole and p-cresol are sulfated in the liver to indoxyl sulfate and p-cresol sulfate, respectively, and reversibly bind to plasma albumin.[21] For TMAO, bacteria generate trimethylamine from the amino acids choline and L-carnitine. In circulation, trimethylamine is oxidized to TMAO in the liver and is not protein bound.[22] We found that serum concentrations of IS, pCS, and TMAO were often lower in the non-fasted state when compared to the fasted state at multiple time points over a 12-hour period, thus rejecting our hypothesis. The reason for lower serum concentrations in the non-fasted state compared to the fasted state is unknown. Many environmental factors as well as age and the diet were controlled in our study. The only factors that differed between Part 1 and Part 2 were the day the samples were collected and the fasted state of the cats. Considering these factors, the lower serum

concentrations in the non-fasted state may be due day-to-day variation in intestinal transit time, stimulation of colonic motility by the gastric-colic reflex after a meal, differences in intestinal absorption of microbial metabolic byproducts, or physiologic variation in tubular secretion function.

For IS and pCS, serum concentrations gradually declined over a 12-hour period in both the fasted and non-fasted state. This is a similar finding in healthy people.[11] When measuring variance of metabolites, it is assumed that concentrations are relatively stable over time. Because a trend over time was appreciated in our study, the short-term biological variation coefficients of variance should be interpreted with some caution.

We found that serum concentrations vary depending on the time of day. In general, serum concentrations were different in the morning (0600-1000; Hours 0-4) when compared to the late afternoon (1400-1800; Hours 8-12) for IS and pCS. This suggests that to compare serum concentrations of IS and pCS between two time points in an individual, the serum sample should be collected at relatively the same time of day.

In most cases, veterinarians and researchers would be monitoring serial measurements with > 24 hour periods between sample collections, therefore the RCVs using medium-term biological variation estimates would be most appropriate in this scenario. The RCV is the percent change (either decrease or increase) between sequential measurements that suggest a significant change beyond random variation. Importantly, the RCV is calculated based on CV_I for the analyte and CV_A for the instrument upon which the specimens are analyzed, therefore an RCV value is not universal. An RCV is used to calculate a subject-based reference interval by applying the percent RCV to the mean analyte concentrations taken from an individual, ideally during health. An RCV can also be used to decide if a change between 2 measurements is

significant. In our study the RCV for IS, pCS, and TMAO based on the medium-term biological variation estimates suggest that serum concentrations would have to decrease by 21.9%, 28.9%, and 52.2%, respectively, between serial measurements to suggest a significant change. For example, a cat had a previous TMAO concentration of 300 ng/dL. A decrease to 120 ng/dL (a decrease of 60%) in a subsequent sample 2 weeks later represents an decrease greater than the RCV of 52.2%, therefore the change is likely a true relevant biological change. The RCV for an increase in serum concentration was large for IS (455.7%), pCS (345.8%), and TMAO (191.7%). To date, a population-based reference interval for serum IS, pCS, and TMAO has not been determined. In the meantime, the CV_I from our study and the CV_A for the instrument upon which the specimens are analyzed can be used to calculate an RCV to be applied to future measurements in either a research or clinical setting for cats to determine a significant change in the analyte concentration.

In human medicine, it has become common practice to apply biological variation values, including RCV, from healthy individuals to patients with chronic diseases. In many chronic diseases, this is an appropriate strategy.[10] One exception to this assumption is the biological variation of serum creatinine over 24 hours in patients with kidney disease in which the CV_I was higher in patients without CKD than in those with CKD (6.4% vs 2.5%) to which the authors attributed this difference to the effect of meat consumption.[10,23] Therefore, the RCV for serum creatinine based on the CV_I from individuals with CKD would be most appropriate rather than using the RCV determined from healthy individuals. The uremic toxins IS, pCS, and TMAO are metabolites of protein metabolism by colonic bacteria, and CKD patients have protein malassimilation allowing higher amounts of protein to enter the colon.[24,25] Therefore, it is plausible that serum concentrations of IS, pCS, and TMAO would be affected by dietary protein

consumption and this may be exaggerated in patients with CKD on variable diets. Determination of biological variation values for serum concentrations of IS, pCS, and TMAO in cats with CKD is warranted to assess the clinical significance of changes between serial laboratory values in this specific disease population.

This study has several limitations. Although the health status of the cats were thoroughly evaluated before enrollment, it is difficult to unequivocally prove health status and especially the absence of early kidney dysfunction. Imaging of the urinary tract and glomerular filtration rate determined by iohexol clearance or nuclear scintigraphy were not performed in these cats which may be more tools used to detect kidney dysfunction in cats. Second, varying sampling intervals ranging from 3-6 days were used to determine medium-term biological variation in our study. According to Freeman et al.,[14] initial studies of biological variation should use a standardized interval of 7 days between collections. Variable sampling intervals within a study will give variable coefficients of variation. In particular, greater duration between sample collection results in higher variation (most notably, CV_I).[26-28] Therefore, the shorter collection intervals in our study may have resulted in lower CV_I. Third, research cats were chosen for the study rather than healthy, young client-owned cats to minimize inter-individual variability due to homogeneity of living conditions and diet. Fourth, it is unknown whether the results of the study apply to cats with pathology. Therefore, caution should be applied when applying results to client-owned cats or cats with disease, notably CKD.

Our study provides biological variation estimates for the 3 major gut-derived uremic toxins IS, pCS, and TMAO. In conclusion, the results of this study demonstrate intermediate individuality for pCS and TMAO serum analytes. Serum IS concentrations have high individuality for short-term biological variation estimates and low individuality based on

medium-term biological variation estimates. Feeding may reduce serum concentrations of pCS, IS, and TMAO over a 12-hour period in cats. To compare serial measurements, it would be prudent to collect samples at the same time of day and consistently in either a fasted or non-fasted state.

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CHAPTER 8: CONCLUDING REMARKS AND FUTURE DIRECTION

8.1 Significance of Work

Chronic kidney disease in cats, especially senior cats, is a common diagnosis made by veterinarians, yet the etiology is often unknown at the time of diagnosis. Therapies known to slow progression of disease are limited to dietary manipulation and objective tools to monitor efficacy are needed. In addition, the role of the gut-renal axis in veterinary patients has not been elucidated. Based on the human literature, mechanisms to how renal diets work to slow progression of disease likely include changes in the production of microbial metabolites, both beneficial and deleterious. The veterinary community and cat owners are in need of answers. The work presented in this dissertation is intended to further our knowledge of possible etiologies of CKD, explore the potential role the gut microbiome and its metabolites play in the disease, and determine the clinical value of monitoring gut-derived uremic toxins in the serum of cats.

In Chapter 3 and 4, we evaluated two potential etiologies of CKD in cats, *Bartonella henselae* infection and frequent vaccination. In addition, Chapter 4, evaluated a potential model to study interstitial nephritis in a research setting. In Chapters 5 and 6 we characterized the gut microbiome, quantified the important microbial metabolites altered by uremia, and evaluated the effect of a commercial probiotic in cats with CKD. Lastly, in Chapter 7 we evaluated the clinical utility of measuring three major gut-derived uremic toxins in the serum of cats. This was important to better understand the biological variation of these molecules and determine their use as potential therapeutic biomarkers.

This body of work will impact practicing veterinarians and future research in feline CKD. Much of the information from these studies, in particular the studies on the gut microbiome

(Chapter 5-7), are translational to human medicine. Like cats, renal disease is common in people and diet is also a major therapy for the disease. Therefore, information gathered in this body of work in cats could benefit people as well as cats with kidney disease.

8.2 Specific Aim 1 (Chapter 3: *Bartonella henselae* in Cats with Kidney Disease)

Previously published retrospective studies in client-owned cats and prospective studies in experimentally-infected cats have associated *Bartonella henselae* with interstitial nephritis and urinary disease (in particular, hematuria) in cats.[1-4] However, no study to date has specifically look at the potential link between urinary disease, including kidney dysfunction, in cats and *B. henselae* infection. The primary aim of Chapter 3 was to determine the estimated prevalence rates of *B. henselae* IgG in serum and *Bartonella* spp. DNA in blood and urine of client-owned cats with evidence of kidney dysfunction and compare to an age-group matched cats (≥ 5 years) with normal kidney function within the same geographic location. In addition, we evaluated for an association between *B. henselae* seroprevalence and abnormalities on urinalysis in all cats. We found no differences in estimated *B. henselae* IgG seroprevalence rates between cats with and without kidney dysfunction, proteinuria, and hematuria. *Bartonella* spp. DNA was amplified from the blood of one of 106 cats with kidney dysfunction but none of the urine samples. Thus, the study did not support B. henselae as a cause of kidney disease in older cats.

The main limitations of this study design were that we were unable to differentiate acute versus chronic kidney disease as clinical history was unavailable, and we included only cats over the age of 5 years. Therefore, it would be interesting to evaluate for evidence of *B. henselae* infection, including PCR on renal tissue, obtained from client-owned cats with known CKD in a prospective design. In addition, since glomerulonephritis and *B. henselae* infection are more

common in younger cats the evaluation of the association between *B. henselae* infection and urinary disease (in particular proteinuria) in cats less than 3 years of age is still needed.

In conclusion, the work presented in this chapter support that veterinarians should not routinely perform *B. henselae* titers or PCR in older cats with kidney dysfunction, however further evaluation in younger cats, especially those with proteinuria, and cats with known CKD is needed.

8.3 Specific Aim 2 (Chapter 4: Evaluation of a Model of Interstitial Nephritis in Cats)

Annual or frequent vaccination was found to be a risk factor for the development of azotemic CKD in cats in a retrospective study.[5] A previous study showed that hyperinoculation with CRFK cell lysates containing the immunodominant antigen α-enolase over a 2-year period lead to the development of interstitial lymphoplasmacytic nephritis in 50% of the cats.[6] Interstitial nephritis is a common histopathology finding in cats with early idiopathic CKD.[7] Additionally, despite the high prevalence of CKD in cats and need to evaluate the disease in a controlled setting, a research model to study the disease in cats has not been established. The finding that hyperinoculation with CRFK cell lysates lead to histological findings that are similar to cats with early CKD makes this method a possible non-lethal model to study the disease in cats.

The primary aim of Chapter 4 was to evaluate whether hyperinoculation with a market leading FVRCP vaccine that contained α-enolase (8 vaccinations over a 16-week period) would induce renal histological changes consistent with feline interstitial nephritis and changes to the distribution of α-enolase as determined by immunohistochemical staining. This study demonstrated that hyperinoculation altered the immunoreactivity of α-enolase in renal tubules,

however did not change renal histopathology on H&E stain. In addition, hyperinoculated cats mounted both a humoral and cell-mediated immune response towards α-enolase contained within the vaccine. Curiously, some cats but not all had altered serum inflammatory cytokine production and changes to urine markers of tubular injury.

Although a direct link between vaccination and interstitial nephritis in cats has yet to be demonstrated, this study in conjunction with previous studies supports a link between interstitial nephritis and sensitization to α-enolase. This model warrants further investigation with potentially larger study sizes and longer follow-up periods. A potentially exciting area of kidney disease research that stems from the current work is the validation of new biomarkers of acute tubular injury. It is unknown whether increased tubular α-enolase immunoreactivity noted in the study corresponds to tubular injury, but this is of some concern. This study provides pilot data for the potential use of calmodulin and uromodulin as markers of acute tubular injury in cats. Validating a urine calmodulin and uromodulin assay for cats and using the assay in this model along with known markers of tubular injury (i.e. KIM-1) is a reasonable next step. To understand if greater α-enolase immunoreactivity in the renal tubules correlates to greater amount of α-enolase in the urine, validation of urinary α-enolase PCR could be validated. To date, evaluation of the role of α-enolase auto-antibodies in client-owned cats with CKD has not yet been performed. Future studies in client-owned cats with CKD and a known vaccination history evaluating serum α-enolase antibodies, urine α-enolase, and quantitative tissue levels of α-enolase antibodies in conjunction with renal immunohistochemical staining is warranted.

In conclusion, the work described in this chapter has increased our understanding of the potential link between vaccination and CKD in cats and has paved the foundation for addition research. While this model holds promise for kidney disease research in cats, additional work is

needed to determine the significance of altering α-enolase immunoreactivity within renal tubules with vaccination.

8.4 Specific Aim 3 (Chapter 5: Fecal Microbiome in Cats with Chronic Kidney Disease)

In human medicine, the impact of a disordered gut microbiome has been extensively researched in patients with end-stage renal disease, and several microbial metabolites are now therapeutic targets in the dietary management of the disease.[8-10] However, there is no information on the gut-kidney axis in veterinary patients. The aim of Chapter 5 was to characterize the fecal microbiome, evaluate fecal short-chain fatty acids (SCFAs) in cats with CKD and correlate findings to serum indoxyl sulfate (IS) and p-cresol sulfate pCS concentrations. This body of work demonstrated that cats with CKD, especially in late stage disease, have a functional dysbiosis associated with the increased serum concentrations of metabolites formed via protein metabolism in the colon, in particular isovaleric acid and indoxyl sulfate. Unlike in people, cats with CKD do not have altered straight-chain SCFAs. Interestingly, fecal BCFA concentrations and serum IS concentrations were higher in cats with clinical evidence of muscle wasting, providing evidence for malassimilation of protein in cats with CKD.

The findings of this work laid the framework for several future studies in cats with CKD. Although the work presented in this chapter supports that cats with CKD have protein malassimilation, to confirm serum and fecal amino acids should be performed in CKD cats and compared to both young and senior healthy cats. Additionally, this body of work showed us that the gut microbiome has strong potential as a therapeutic target in hope to reduce the production of harmful microbial metabolites, like IS, and limit protein malassimilation in hope to reduce kidney cachexia. Potential therapies that may alter microbial production of these toxins include

highly digestible protein-restricted diets, prebiotics (i.e. fiber), laxatives to treat constipation, probiotics/synbiotics, and fecal transplantation. Understanding the limitations of 16S rRNA gene sequencing, shotgun metagenomics would be a better tool to further explore the functional potential of the gut microbiome and quantify the altered abundances of bacteria in the gut.

In conclusion, the work presented in this chapter provides novel information as to the role the gut microbiome plays in the pathogenesis of CKD in cats. The gut microbiome is complex, but with the right tools can be better understood and the information gained used to find new therapies and potentially novel biomarkers for cats with CKD.

8.5 Specific Aim 4 (Chapter 6: Manipulation of the Gut Microbiome with SF68)

Studies have shown benefits of various probiotics in patients with kidney disease including reduction in blood pCS concentrations in people, alleviation of renal dysfunction caused by ischemia and reperfusion in a mouse model, and improvement in GFR in dogs with CKD. A commercial probiotic for cats (Purina® ProPlan® Veterinary Diets; Fortiflora™ Probiotic Supplement) combines the probiotic *Enterococcus faecium* strain SF68 (SF68) with a palatability enhancer. The aim of Chapter 6 was to evaluate the effect SF68 has on the fecal microbiome, serum concentrations of gut-derived uremic toxins (IS, pCS, TMAO), and clinical parameters when fed to cats with CKD. We found that supplementation of SF68 did not significantly change the fecal bacterial community structure, uremic toxin serum concentrations, or functional renal values, however all cats accepted the product when mixed with their food and clients reported improvement in clinical parameters (particularly, vomiting and appetite) in a subset of cats.

The work presented in this chapter is one of the first in the veterinary literature that evaluates the potential benefit of manipulating the gut microbiome in cats with CKD using probiotics. In general, this task is difficult since changes in composition and function of the microbiome in CKD patients is mediated by a change in the biochemical milieu and intraluminal pH of the gut, a factor that is difficult to change especially with end-stage disease.[8] Therefore, adding a potentially beneficial bacteria to the gut in the form of an oral supplement may not be enough. It is often with the use of a either a multi-strain probiotic or a synbiotic when an effect has been noted in serum concentration of IS or pCS in people with CKD.[11,12] Therefore, future studies may include evaluating the use of other probiotic/synbiotic products commercially available for cats or the use of prebiotics in cats with CKD. The potential appetite enhancer properties of the commercial product (SF68 bacterium with the palatability enhancer) is also an area that could be further explored in sick cats, however a longer study period may be needed to statistically appreciate a benefit.

In conclusion, the SF68 probiotic does not appear to have a beneficial effect in on the gut microbiome and gut-derived uremic toxin serum concentrations cats with CKD within an 8-week period. Future studies are needed to understand the role probiotics/synbiotics play in the treatment of cats with CKD.

8.6 Specific Aim 5 (Chapter 7: Biological Variation of Major Gut-Derived Uremic Toxins)

The biological variation estimates of serum IS, pCS, and TMAO concentrations is unknown in cats. The primary aim of Chapter 7 was to determine biological variation estimates and the Index of Individuality (IOI) for serum total IS, pCS, and TMAO concentrations in healthy adult cats. The secondary aim was to measure the difference in serum concentrations in

fasted versus unfasted samples to determine the effect of recent feeding on serum concentrations. We found that short-term and medium-term biological variation estimates for pCS and TMAO had intermediate individuality indicating that population-based reference intervals should be assessed in relation to subject-based reference intervals. For serum IS concentrations, short-term biological variation estimates corresponded to a high IOI (1.96) and the medium-term biological variation estimates corresponded to a low IOI (0.65). Recent feeding does appear to lower serum concentrations of IS, pCS, and TMAO. For serum pCS and IS concentrations, serum concentrations tended to decrease over time in a 12-hour period. When measuring and comparing serum concentrations, serum samples should be collected at a similar time of day, and ideally consistently in either a fasted or non-fasted state.

Often biological variation estimates from healthy individuals can be applied to individuals with disease, however this is not the case for serum creatinine in people. This raises concern that the biological variation estimates from healthy adult cats in our study may not be applicable to cats with CKD. Understanding this limitation, the RCV based on medium-term biological variation estimates was applied to the same uremic toxin assay used in cats with CKD from Chapter 6. Although some cats fed the SF68 probiotics had a significant decrease in serum IS, pCS, and TMAO (IS, 1 cat; pCS, 2 cats; TMAO, 1 cat) over the 8-week study, none of the cats had a significant increases in serum concentrations. A future study to determine the biological variation estimates of IS, pCS, and TMAO in cats with CKD is warranted to better understand the findings of Chapter 6.

In conclusion, the study presented in this PhD is the first to determine the effect recent feeding has on serum concentrations of the 3 major gut-derived uremic toxins. The study also provides guidelines on the optimal testing conditions and biologic variation estimates that can be

applied to IS, pCS, and TMAO measurements in cats. This is of particular importance for future studies evaluating whether or not a particular therapy can successfully reduce serum concentrations of these uremic toxins.

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