CHARACTERIZATION OF THE FECAL MICROBIOME IN DOGS RECEIVING MEDICAL MANAGEMENT FOR CONGENITAL PORTOSYSTEMIC SHUNTS

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ABSTRACT

Background: The GI microbiome has not been characterized in dogs being medically managed for congenital portosystemic shunts [CPSS].

Objectives: To characterize the fecal microbiome in a population of dogs being medically managed for CPSS.

Animals: 27 client-owned dogs.

Methods: Prospective cohort study enrollment of fecal samples was performed with follow-up data collected retrospectively. The overall fecal dysbiosis index [DI] and individual bacterial abundances were determined using real-time qPCR. Medical management, clinical findings, clinicopathologic, and outcome variables were collected, and logistic regression analyses were performed to evaluate associations between these variables and overall DI and bacterial abundances. Numerical variables were evaluated with general linear models.

Results: All dogs were administered a therapeutic hepatic diet and lactulose, while antibiotics were used in 22/27 (81.5%) dogs and acid suppressants in 7/27 (25.9%) dogs. Seventeen dogs (63.0%) had a DI > 2. The median DI in this population was 3.02 (range, 4.23-8.42), and the median DI in dogs receiving and not receiving antibiotics was 4.3 (range, -4.23 – 8.42) and 1.52 (range, -1.62 – 5.43), respectively.

No significant association between any of the analyzed variables and the DI was identified. The abundance of *E. coli* was positively significantly affected by the use of metronidazole ($p = 0.024$).

Conclusions and clinical importance: Dysbiosis appears to be common in dogs that are being medically managed for CPSS, though the clinical significance remains unclear.

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CHAPTER ONE

Introduction

The microbiota of the mammalian gastrointestinal (GI) tract consists of trillions of organisms performing a variety of crucial roles both locally and systemically.[1-3] Characterization of the GI microbiome has evolved from culture-based models to high-throughput DNA sequencing techniques or targeted quantitative polymerase chain reaction (qPCR) assays, which have allowed for the characterization of the microbiome at the genetic and species level in various species.[3-5]

Several studies have characterized bacterial populations for the GI flora in healthy humans and animals.[1-3] Subsequently, extensive research has been performed evaluating the GI microbiome characteristics in human and canine populations with specific pathologies such as chronic inflammatory enteropathies, diabetes, obesity, and hepatopathy-induced hepatic encephalopathy (HE).[6-10] By using the previously identified healthy control profiles of intestinal microbiota in comparison to the profiles from the diseased populations, the concept of a "dysbiosis index" (DI) was established, which focuses the analysis of shifts in the microbiome to certain important bacterial groups.[4] Certain alterations in the GI microbiome have been postulated to be a useful adjunct for identifying disease states or a potential monitoring parameter for treatment efficacy.[4-8,11]

The role of the GI microbiome in chronic hepatopathies and HE has been frequently investigated in humans, with ammonia being identified as a key player.[10,12,13] Significant differences have been reported in humans between the colonic microbiota of cirrhosis or HE patients and healthy control individuals, and some suggest the microbiome alterations in affected patients may contribute to more significant clinical signs of HE via alteration of the intestinal barrier function, an increased proportion of urease-producing bacteria, or in other ways.[11-13,14] Therefore, it has been postulated that manipulation of the microbiome may contribute to improved outcomes in these patients.

In dogs, the most common cause of HE is related to congenital portosystemic shunts (CPSS).[15] Single or multiple aberrant blood vessels allow portal venous blood to bypass the liver parenchyma with CPSS.[15,16] Medical management for dogs with PSS can include a restricted protein diet and administration of lactulose, antibiotics, anticonvulsants, or probiotics. Surgical attenuation of the CPSS is often recommended to restore more normal portal blood flow and improve liver function. Some individual components of medical management for CPSS have been investigated for effects on the microbiome in dogs.[4,15,17-21] Based on the literature, it is apparent that lactulose, diet, and certain antibiotics have the potential to alter the microbiome; however, these studies investigate effects in healthy dogs, people, or non-hepatic disease.[11,15,16,17-20] Because dogs being medically managed for CPSS are often prescribed these medications for long periods, this may lead to long term dysbiosis and potential subsequent health consequences.

There is a deficit in the literature as to the effects of these medications on the fecal microbiome and DI in dogs with CPSS and a complete lack of information as to whether these effects have any consequences on the clinical signs or outcomes of dogs with CPSS.

The aims of the current study were to characterize dysbiosis in dogs presenting with CPSS and to evaluate for associations between dysbiosis and clinical variables such as medical management, clinical signs, clinicopathologic findings, and postoperative outcomes. We hypothesized that medical management for CPSS would have a significant impact on the dysbiosis index, but that clinical outcome of the patient would not be affected regardless of the level of dysbiosis.

Materials and Methods

Case selection:
Dogs presenting to the University of Tennessee College of Veterinary Medicine (UTCVM) with a previous diagnosis of CPSS (confirmed either with transsplenic portal scintigraphy or computed tomography) were prospectively recruited from June, 2018 to August, 2019.

Inclusion criteria:
Dogs were included in the study following definitive diagnosis of CPSS using either CT/angiography or transsplenic nuclear portal scintigraphy and if fecal samples were able to be collected at the initial visit prior to surgical intervention for CPSS. All animals were enrolled following informed owner consent in accordance with the protocol approved by the University of Tennessee Institutional Animal Care and Use Committee (AUP #2641-0918). Animals were excluded if the fecal sample was not sufficient to be analyzed, if a fecal sample was not obtained, or if a fecal sample was not obtained prior to administration of perioperative antibiotics for the CPSS attenuation procedure.

Medical record review:
Information regarding patient signalment (breed size, sex, age, and reproductive status), weight, clinical signs prior to medical management (GI signs, neurologic signs, urinary signs), medications at the time of sample collection, clinical signs following initiation of medical management, preoperative clinicopathologic findings (complete blood count (CBC), serum biochemistry panel, pre- and post-prandial serum bile acids, and resting plasma ammonia), type of surgical intervention, postoperative clinical outcome, and postoperative albumin, BUN, cholesterol, and glucose values. Postoperative clinicopathologic and outcome data was obtained via a combination of medical record review and communication with referring veterinarians. Clinical management of the dogs was not altered for the study parameters, and each dog was managed at the discretion of the attending clinician, including adjustments to medical management perioperatively or postoperatively and surgical attenuation. In general, each dog was recommended to have

a follow-up serum biochemistry panel, CBC, and pre- and post-prandial serum bile acids performed between 3-6 months postoperatively. Neurologic signs included seizures, ataxia, head pressing, or mental obtundation. Gastrointestinal signs included vomiting, diarrhea, or regurgitation, and were considered distinct from anorexia or hyporexia. Lower urinary tract signs included hematuria, stranguria, or dysuria.

Closure of the CPSS following surgical intervention was suspected based on the following findings in follow-up blood analyses: normalization or improvement of serum bile acids[22] with improvement or normalization of hepatic synthetic factors such as albumin, glucose, cholesterol, or BUN.

Sample collection:
Fecal samples were either collected via free-catch fecal samples, digital rectal exam, or a fecal loop, and stored in a cryogenic storage container. Following fecal collection, the samples were immediately stored in a -80-degree Celsius freezer until all samples for study inclusion were collected and shipped for analysis. Samples were then shipped on dry ice and maintained in a -80 degree Celsius freezer at the Texas A&M Veterinary Medical Diagnostic Laboratory until the molecular diagnostics were performed.

Fecal quantitative PCR analysis:
DNA was extracted from each fecal sample (100 mg) using the MoBio Power soil DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's instructions. The qPCR assays were performed as previously reported by M.K. AlShawaqfeh, et al. In summary, qPCR reactions were performed using SYBR green-based reaction mixtures. The final total reaction volume was 10 µL. The final mix was composed of 5 µL SsoFast EvaGreen supermix (Bio-Rad Laboratories, CA, USA), 0.4 µL each of a forward and reverse primer, 2.6 µL of PCR water and 2 µL of normalized DNA. The PCR conditions were as follows: initial denaturation at 98°C for 3s and annealing for 3s. Melt curve analysis was performed post-amplification using these conditions: 95°C for 1 min, 55°C for 1 min and increasing incremental steps of 0.5°C for 80 cycles for 5s each. All samples were run in duplicate fashion. The qPCR data were expressed as the log amount of DNA (fg) for each particular bacterial group/10 ng of isolated total DNA. Each fecal sample was evaluated for the overall bacterial number, as well as the individual bacterial species including: Universal Bacteria, *Faecalibacterium*, *Turicibacter*, *Streptococcus*, *E. coli*, *Blautia*, *Fusobacterium*, and *C. hiranonis*. The overall dysbiosis index was then calculated for each dog as previously described by AlShawaqfeh, et al.[4]

The reference ranges for the bacteria tested to determine if the abundance was normal or abnormal were expressed as log DNA/gram of feces, and included Universal Bacteria (10.6-11.4), *Faecalibacterium* (3.4-8.0), Turicibacter (4.6-8.1), *Streptococcus* (1.9-8.0), *E. coli* (0.9-8.0), *Blautia* (9.5-11.0), *Fusobacterium* (7.0-10.3), and *C. hiranonis* (5.1-7.1).[4] The DI was classified as normal DI (< 0), a moderate shift (0-2), or a significant shift (> 2).

Statistical analysis:
Normality tests were conducted on numeric variables using Shapiro-Wilke tests. Descriptive statistics were calculated. Normally distributed data are presented as a mean ± SD, and non-normally distributed data are expressed as median and range. Logistic regression analysis was used to evaluate the effects of clinical variables (clinical signs, medical management, clinicopathologic values, and outcome data) on the categorical outcomes of bacterial abundance (normal or abnormal) and DI (normal, equivocal, or dysbiosis). The effects of clinical variables on numeric outcomes (such as clinicopathologic data) were evaluated using general linear models. Diagnostic analysis was conducted to examine model assumptions for normality and equal variance using Shapiro-Wilk test and Levene's test respectively. Ranked transformation was applied if diagnostic analysis exhibited violation of normality and equal variance assumptions. Post hoc multiple comparisons were performed with Tukey's adjustment. Statistical significance was identified at the level of 0.05. Analyses were conducted in SAS 9.4 TS1M7 for Windows 64x (SAS institute Inc., Cary, NC).

Results

A total of 27 dogs were included in the study in accordance with the inclusion criteria. The study population included 17 (63.0%) male dogs and 10 (37.0%) female dogs. Ten (58.8%) male dogs were neutered, and 6 (60%) female dogs were spayed. Median age at the time of fecal collection was 10 months (range, 3-48). Median body weight at the time of collection was 4.9 kg (range, 1.5-32.8).
Prior to initiation of medical management, documented clinical signs in the dogs of the study included neurologic signs in 16/27 (59.3%), gastrointestinal signs in 13/27 (48.1%), episodic or persistent anorexia or hyporexia in 11/27 (40.7%), and lower urinary tract signs in 5/27 (18.5%).

At the time of fecal collection, medical management had been instituted previously for all dogs. Medical management consisted of varying combinations of a therapeutic hepatic diet (all dogs), lactulose (all dogs), antibiotics in 22/27 (81.5%), acid suppressants in 7/27 (25.9%), antiepileptics in 3/27 (11.1%), and probiotics in 2/27 (7.4%). The antibiotics most commonly prescribed were metronidazole in 17 dogs receiving antibiotics (77.3%), amoxicillin in six (27.3%), and one each of amoxicillin/clavulanic acid (Clavamox, Zoetis, Parsippany-Troy Hills, NJ) and neomycin (4.5%). The median duration of lactulose administration at the time of fecal collection was 50.5 days (range, 1-842). The median duration of antibiotic administration was 45 days (range, 22-842).

Following initiation of medical management, the following clinical signs remained present: four (14.8%) with hyporexia or anorexia, three (11.1%) with neurologic signs (none with seizures), three (11.1%) with distinct GI signs, and three (11.1%) with lower urinary tract signs. Only two dogs developed new clinical signs between the onset of medical management and fecal collection - one with hyporexia/anorexia and one with lower urinary tract signs.

In total, 22/27 (81.5%) dogs underwent surgical intervention for attenuation of CPSS including 14 (63.6%) with ameroid constrictor placement and eight (36.4%) with percutaneous transvenous coil embolization. Follow-up data was available in 20 (90.9%) dogs undergoing surgical intervention for the CPSS, and 13 (65.0%) had bloodwork findings suggestive of closure of the PSS based on interpretation of serum bile acids and hepatic synthetic factors.

Information pertaining to blood analyses performed during the same hospitalization for fecal collection can be found in Table 1. The bacterial abundance as measured in these dogs is available in Table 2. In total, 17/27 (63.0%) dogs in the study had a DI of > 2, which has previously been reported as the cutoff for dysbiosis in dogs.[4] The overall median DI in this population of dogs was 3.02 (range 4.23-8.42). The median DI in dogs receiving antibiotics was 4.3 (range -4.23 – 8.42), and the median DI in dogs not receiving antibiotics was 1.52 (range -1.62 – 5.43) ($p = 0.58$).

No significant effect on the DI from any of the components of medical management was noted. However, the abundance of *E. coli* was significantly positively affected by the use of metronidazole ($p = 0.024$). No significant effects on the DI or any individual bacterial species abundances were identified with amoxicillin, amoxicillin/clavulanic acid, or neomycin. Preoperative serum albumin had a significant negative impact on the DI ($p = 0.009$), whereas no other clinicopathologic variables had a significant relationship. *C. hiranonis* abundances were significantly positively affected by preoperative serum albumin ($p = 0.035$), serum GGT ($p = 0.04$), and serum cholesterol ($p = 0.023$). *E. coli* abundances were significantly negatively affected by increasing preoperative platelet count ($p = 0.04$), lymphocytes ($p = 0.049$), and BUN ($p = 0.016$).

Discussion

The current study is the first in the veterinary literature to describe evaluation of the dysbiosis index in a cohort of dogs diagnosed with CPSS. Given the fact that many dogs with CPSS are administered medications to mitigate the clinical signs secondary to CPSS and that these medications are known to affect overall dysbiosis, evaluating these interactions is important to further understand treatment effects on these patients.[4,15,18-20] The overall rate of dysbiosis (DI >2) in the study population was relatively high at 63.0%, but no significant associations were identified between the DI and any individual component of medical management or clinical signs. Additionally, there was no significant association between the DI and clinical outcome or any of the clinicopathologic findings apart from a negative association with preoperative albumin. Additionally, significant associations between certain parameters and the abundance of individual bacterial species were also identified.

The role of the human GI microbiome in chronic hepatopathies and HE is well established.[10,21] Significant differences have been shown in the colonic microbiota of human patients with cirrhosis or HE and healthy control individuals, and some authors

Table 1. Blood analytes. Summarized data for clinicopathologic variables for the study population. Post-operatively, only synthetic hepatic factors and serum bile acids were analyzed. Numerical values for post-operative serum bile acids were not recorded, but rather if there was an improvement or not as compared to the preoperative values. All reference ranges are those used for the University of Tennessee College of Veterinary Medicine, aside from resting ammonia.[42]

Abbreviations: BUN, blood urea nitrogen; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; PCV, packed cell volume

Blood Analyte	Number of cases tested at presentation	Median (range)	Number of cases tested postoperatively	Median (range)	Reference Range
Serum Biochemistry					
Albumin (g/dL)	26	2.5 (1.8-3.6)	19	3.0 (2.4-4.1)	3.0-4.3
Globulins (g/dL)	26	3.45 (1.5-4.8)	N/A	N/A	2.6-4.7
Cholesterol (mg/dL)	25	178 (64-420)	14	229.5 (174-290)	74-255
BUN (mg/dL)	27	5 (2-13)	19	8.5 (4-26)	18-40
Glucose (mg/dL)	27	107 (51-121)	19	88 (80-195)	87-179
ALT (IU/L)	26	82.5 (31-516)	N/A	N/A	29-109
ALP (IU/L)	26	111 (41-441)	N/A	N/A	12-79
GGT (IU/L)	26	3 (2-13)	N/A	N/A	0-5
Total bilirubin (mg/dL)	26	0.4 (0.1-0.7)	N/A	N/A	0.1-0.7
Complete Blood Count					
PCV (%)	27	36.1 (28.0-50.5)	N/A	N/A	34-48
Total Protein (g/dL)	26	5.95 (3.3-7.3)	N/A	N/A	6.6-8.4
White blood cells (x10E3/uL)	26	13.6 (9.5-20.0)	N/A	N/A	4.7-15.3
Neutrophils (x10E3/uL)	26	9.2 (3.8-17.8)	N/A	N/A	2.00-9.20
Lymphocytes (x10E3/uL)	26	9.5 (1.19-15.8)	N/A	N/A	1.05-8.00

Table 1 continued

Platelets (x10E3/uL)	26	196.5 (103-395)	N/A	N/A	169-480
Liver Function Tests					
Pre-prandial Bile Acids (µg/dL)	22	182.2 (0-318.8)	18	N/A	0-30
Post-Prandial Bile Acids (µg/dL)	23	209.1 (69-382.3)	18	N/A	0-10
Resting Ammonia (µmol/L)	13	140 (66-864.4)	N/A	N/A	<70 µmol/L[42]

Table 2. Bacterial abundance. Summary of the various bacterial abundances present in the study population. Reference intervals were established in 120 healthy dogs.[4]

Bacteria	Abundance Reference Interval (log DNA/gram feces)	Median (range)	Number of Dogs Outside Reference Interval (%)	Number of Dogs Within Reference Interval (%)
Universal Bacteria	10.6-11.4	10.9 (10.43-13.22)	6 (22.2%)	21 (77.8%)
Faecalibacterium	3.4-8.0	7.28 (3.45-7.32)	2 (7.4%)	25 (92.6%)
Turicibacter	4.6-8.1	7.46 (3.25-9.13)	7 (25.9%)	20 (74.1%)
Streptococcus	1.9-8.0	6.05 (2.14-8.2)	1 (3.7%)	26 (96.3%)
E. coli	0.9-8.0	8.15 (0.88-8.77)	6 (22.2%)	21 (77.8%)
Blautia	9.5-11.0	11.23 (6.04-12.25)	14 (51.9%)	13 (48.1%)
Fusobacterium	7.0-10.3	9.69 (6.51-11.63)	8 (29.6%)	19 (70.4%)
C. hiranonis	5.1-7.1	3.07 (0.01-6.84)	19 (70.4%)	8 (29.6%)

suggest that the alterations in the microbiome in these patients may contribute to more significant clinical signs of HE or complications leading to mortality.[12,21] Investigation of the GI microbiome in dogs with hepatic pathology is more limited. A pilot study in 2015 conducted with 10 dogs revealed that one dog with chronic active hepatitis had Fusobacteria as the dominant bacterial phylum in the distal gut, as opposed to healthy dogs or dogs with distinct systemic pathology not involving the liver, in which the dominant organisms were Firmicutes or Proteobacteria, though clinical significance of this finding could not be determined.[23] Given the sparsity of literature, the role of dysbiosis in relation to hepatic dysfunction or pathology, including CPSS, in dogs has yet to be elucidated.

All dogs in the present study had been medically managed prior to fecal sample collection with medications to reduce ammonia production and absorption in the gut and the manifestation of correlated clinical signs. All dogs were fed a therapeutic hepatic diet at the time of fecal collection. Moderately protein-restricted diets that provide high-quality protein (bioavailable and with a balanced amino acid composition) are recommended for dogs with CPSS, as these diets have been shown to reduce the severity of HE scores in dogs with CPSS. [15,16,24] Though several studies on the effects of diet on intestinal microbiota in dogs have been published, none have been performed specifically with a reduced protein diet fed to dogs with hepatic pathology.[24-27] These studies show that diet has the potential to cause statistically significant shifts in the fecal microbiota with variable size effect, corroborating the results of several human studies investigating the effects of diet on the microbiome.[28-30] Given the other treatments administered in the dogs of this study, it is difficult to determine how significant the effects of the administered therapeutic hepatic diets were on the fecal microbiota of these dogs.

Lactulose was also administered to all dogs in the present study prior to fecal collection. Lactulose has been shown to cause a significant, reversible alteration of the microbiome in healthy dogs, but its effects on the microbiome have not been studied in dogs with any disease state.[15] Additionally, the effects of lactulose administered concurrently with antibiotics has not been studied in dogs. Contrary to the aforementioned study in dogs, one study in humans showed that lactulose did not have a significant impact on the microbiome in patients with cirrhosis.[31] Because all dogs in the present study were being treated with lactulose at the time of fecal sample collection, we cannot draw conclusions about the specific effect of lactulose in this population, though it may have contributed to the relatively high prevalence of dysbiosis (63.0%) in light of the previous canine study. This is an area that would benefit from potential future study.

No significant differences in the DI were noted between the 22 dogs (81.5%) receiving antibiotics and the five (18.5%) that were not. Previous studies performed in humans and dogs have shown both transient and long-lasting dysbiosis associated with antibiotic use, including the highly prevalent problem of *Clostridium difficile* in people.[18,32-34] Despite the lack of association with the DI, the present study identified a significant association between metronidazole and a greater abundance of *E. coli* ($p = 0.024$), in agreement with

the results of a previous study investigating the use of metronidazole in healthy dogs.[18] No significant correlation between the use of other antibiotics was identified in this study, though sample sizes were limited. The relationship between amoxicillin/clavulanic acid and the DI has previously been investigated in dogs, and no significant affect was shown.[35]

Clostridium hiranonis has garnered attention in its role in intestinal health, as well as the conversion of bile acids in the gut. This bacterial species has a strong negative correlation with an increase in the dysbiosis index in dogs.[4] In our study, *C. hiranonis* was below the canine reference range in 19 (70.4%) dogs, with a median value of 0.99. Our study population also exhibited significantly elevated pre- (median: 112.9, range 10.5-318.8) and post-prandial (median: 205.6, range 69-382.3) serum bile acid concentrations, consistent with what is typical for dogs with CPSS. The shunting of blood bypassing the normal hepatic recycling of bile acids in these dogs with CPSS is likely the main driving force behind this elevation in bile acids, but the prevalence of low abundances of *C. hiranonis* may play a role as well.

The abundance of *C. hiranonis* was also significantly positively correlated with preoperative serum albumin and cholesterol. While these findings may be incidental, they may also be associated with subclinical malabsorptive GI disease. It is the authors' observation that many dogs with CPSS are suspected to have concurrent GI disease at the time of presentation, in addition to reference of this concurrent process in a group of dogs with intrahepatic CPSS.[37] Several studies have elucidated the correlation between inflammatory enteropathies and dysbiosis in dogs, and the above findings may support this association.[5-7,38] Further study is required to determine the individual role of fecal *C. hiranonis* in dogs with CPSS prior to medical management.

We identified a significant negative correlation between the abundance of *E. coli* and both pre-operative lymphocyte and platelet counts. Several studies in human medicine have investigated a link between the GI microbiome, macronutrient environment, and various immune cells, including B lymphocytes present in the gut-associated lymphoid tissue (GALT).[39,40] Platelets, in addition to their role in primary hemostasis, have also been shown to be capable of immune defense against *E. coli*, while conversely the lipopolysaccharide associated with *E. coli* has been shown to be lethal to platelets.[41,42] Though these relationships have only been studied in humans, the negative correlation identified in our study between *E. coli* abundance and platelet counts may be the result of these effects. Further study focused on *E. coli* abundance and platelet and lymphocyte counts would be required to confirm this correlation in dogs.

In our study, no significant correlation was identified between the DI and the manifestation of clinical signs, either before or after medical management was initiated. This may be the case for several reasons; the clinical signs in dogs with CPSS may be more attributable to the shunting of blood bypassing the liver, increased production and absorption of ammonia, or alterations to the GI blood supply as opposed to intestinal

dysbiosis. Additionally, our characterization of the microbiome was limited to fecal samples. Fecal samples have been shown to be significantly different from small intestinal samples, and the bacterial populations exhibit significant differences even within individual dogs in transit from the stomach to the colon.[36] It may be the case that changes in the small intestinal microbiome are more clinically significant than those in the colon or the feces for dogs with CPSS. Finally, there are many protective mechanisms in place to prevent dysbiosis from cultivating clinical signs. These mechanisms include the intimate association between the intestines and the immune system, as well as the normal intestinal mucus layer that is present in healthy dogs and likely dogs with CPSS (as opposed to dogs suffering from chronic inflammatory enteropathies).[39,40] If these protective functions were intact in the dogs in our study population, this may have also contributed to the lack of significant association with the DI and the clinical signs listed.

Interpretation of the microbiome in our study was performed with 16s rRNA qPCR, as determined by previous studies investigating the DI in dogs.[4] This methodology is intended to look at the "functional core" of the microbiome in dogs, which is well preserved across differing individual dogs, dog breeds, and even between dogs and humans.[3] Hence, changes in other bacterial taxa or species not included in this "functional core" may not have been identified in this population. Despite this potential limitation, we elected to utilize the qPCR as a way of avoiding overinterpretation of changes in the microbiome that may not be clinically relevant.

Our study had several limitations. The sample size of 27 dogs is relatively small, increasing the likelihood for type II error for our statistical analyses, though it is the only study to-date to specifically evaluate the fecal microbiota in dogs with CPSS. Though fecal sample collection was performed prospectively, data collection on outcome was performed retrospectively, and interpretation of clinical signs was often based on owner observations or referring veterinarian medical records. Because medical management administration was performed at home, owner compliance was likely variable, and medical management strategies were diverse to start with as many referring veterinarians elected different protocols when initiating treatment for suspected CPSS. Unfortunately, there may be ethical implications to denying patients diagnosed with CPSS medical management to improve or mitigate clinical signs and side effects related to their diagnosis for study purposes.

In summary, our study is the first to describe the GI microbiome in dogs with CPSS. We identified that dysbiosis is common in dogs being medically managed for CPSS, despite some variability in the protocol being used. We did not observe a relationship between fecal dysbiosis and clinical signs or outcome in dogs diagnosed with CPSS. Areas of future study should include characterization of the microbiome and DI in dogs prior to the initiation of medical management, and isolating individual components of the medical management protocol with control groups to determine their individual contribution to the changes described here. These studies will need to be performed in such a way to avoid negative effects to patients which may require certain medications to maximize the

likelihood of an uncomplicated clinical course following CPSS diagnosis. The DI and microbiome should also be described in dogs after having undergone surgical attenuation of their CPSS and following cessation of medical management. Though the DI did not appear to have any obvious effect on clinical outcomes in our study, controlled, prospective studies are warranted to further investigate this relationship as this may hold relevance for the treatment plan for dogs with CPSS.

CHAPTER TWO

LITERATURE REVIEW

What is the normal canine GI flora/microbiota?

The microbiota of the mammalian gastrointestinal (GI) tract consists of trillions of bacterial organisms performing a variety of crucial roles both locally and systemically.[1-3] Characterization of the GI microbiome has evolved from culture-based models to high-throughput DNA sequencing techniques, which have allowed for the characterization of the microbiome on the genetic and species level in various species, including canines.[3-5]

The canine GI microbiota consists of a massive array of bacterial species in addition to archaea, fungi, protozoa, and viruses. In total, the estimated intestinal microbial load ranges between 10^{12} and 10^{14} organisms which equates to roughly 10 times the population of host cells.[6] In comparing the canine microbiome to the human GI microbiome, a "functional core" has been established that appears to result in a similar functional capacity despite considerable inter- and intraspecies differences in the GI microbial population.[3] This has important implications for physiologic function in the host, including polysaccharide degradation, synthesis of short-chain fatty acids, amino acids, and vitamins, immune regulation, nutrient metabolism, and other physiological processes.[3,6-8]

In general, in the dog, the bacterial concentrations in the GI tract increase in an aborad direction beginning at the stomach. Typical bacterial counts in the stomach of the dog range from 10^{1} to 10^{6} cfu/g.[9] Bacterial counts in the duodenum tend in general to be low ($<10^{3}$ cfu/g in duodenal aspirates), though considerable variation has been documented between dogs, whereas the ileum, in general, exhibits higher bacterial counts closer to 10^{7} cfu/g.[6,10] In the small intestine of cats and dogs, the dominant bacterial groups to have been cultured are *Bacteroides, Clostridium, Lactobacillus, Bifidobacterium, and Enterobacteriaceae*, though more recent molecular characterization techniques have revealed a larger diversity of bacterial species than previously recognized.[6,11] Bacterial counts in the colon of dogs have been documented between 10^{9} and 10^{11} cfu/g, with *Firmicutes, Bacteroides,* and *Fusobacteria* the predominant phyla.[11] Considerable investigation of the other components of the microbiome has also been performed, but is outside the scope of this project.

How is the microbiome evaluated in an individual dog?

Given the diversity and immense number of bacteria present in the canine microbiome, the techniques for determining the contributing organisms, and their relative abundance, have evolved over time. Each technique inherently comes with a unique set of benefits and drawbacks, which may impact their utility in research settings as opposed to clinical applications. In general, evaluation of the fecal microbiome (as compared to colonic,

small intestinal, gastric, etc.) is logistically the easiest and least invasive. Earlier techniques for fecal microbiome evaluation were culture-based, and initially aided in determining the most abundant taxa present in the microbiome of healthy dogs and cats: *Bacteroides*, *Clostridium*, *Lactobacillus*, *Bifidobacterium* spp., and *Enterobacteriaciae*.[9,11,12] Bacterial cultures are performed both aerobically and anaerobically, though anaerobic species are inherently more fastidious to grow in culture. Distinct bacterial species were previously identified based on "colonial and cellular morphologies, gram reactions, spore formation and anaerobic growth," though more recently genomic sequencing has made this process more streamlined. The bacterial count per gram of feces can be calculated based on growth in various culture media, though this may lead to underestimation of the true bacterial burden depending on the ease of growth in culture.[9] Proposed benefits of culture-based methods include the ability to identify an active infection and the ability to perform concurrent antibiotic sensitivity testing in a clinical setting.[13]

Culture-based microbiome analysis also has significant limitations. Because many of the bacterial species in the microbiome are anaerobic, growth in culture may be challenging or impossible, potentially leading to an underrepresentation of either bacterial diversity or estimations of abundance.[11,13] Additionally, performing bacterial cultures can be laborious and time consuming, which may be a significant disadvantage when applied in a clinical setting.

Subsequent studies sought to characterize the microbiome on a genomic level. Multiple studies in dogs have evaluated the bacteria of the microbiome based on sequencing of 16s ribosomal RNA (rRNA) genes utilizing polymerase chain reaction (PCR). Based on the genomic sequences that are generated (so-called "shotgun sequencing"), operational taxonomic units (OTUs) are determined, which aid in assigning these sequences to a specific bacterial taxon.[13,14] In doing so, the aim is to evaluate the bacterial diversity of the microbiome in cats and dogs by identifying nearly all species that are present and eliminating the need for organisms to be amenable to culture. These assays are not necessarily targeted to individual bacterial taxa, but rather would often employ "universal bacterial primers" during the PCR process.[15] Though these assays are able to identify many more bacterial species, there are still limitations to identification, particularly at the species and strain level, and thus the true bacterial diversity or abundance may still be underrepresented.[16]

With knowledge of the specific 16s rRNA sequences in these bacterial species, targeted PCR primers can be used to identify and quantify bacterial species of particular interest, such as those that exhibit abundance differences in healthy as compared to diseased canine microbiomes. After identification of many of the bacterial species in the canine microbiome and using their respective primers as part of PCR, it was possible to evaluate differences in these populations between control dogs and those with various systemic pathologies, including conditions such as inflammatory enteropathies.[4] By isolating the investigation of the microbiome to certain species, much of the "noise" is filtered out.

Given that the coinciding changes in bacterial populations for a specific disease process can be identified, this may lead to more clinical utility than culture-based methods or shotgun sequencing. For example, the concept of the dysbiosis index was developed after recognition that certain groups of bacteria were repeatably significantly different in dogs with chronic enteropathies as compared to healthy control dogs.[4,16] This index has been standardized and differences can be directly evaluated between dogs.[4,16]

Results of these analyses are currently likely of more use in a research setting to evaluate bacterial diversity in the microbiome, but their clinical application is limited given the lack of implicit functional significance associated with each bacterial species, and the fact that normal protective mechanisms (such as the mucosal mucus layer present in normal intestine) may prevent changes in the microbiome composition from leading to pathology or clinical signs.[16] The study of "metabolomics" is an emerging field, and seeks to clarify the function of the microbiome, as well as its contributions to various disease processes. Importantly, even significant alterations in the microbiome may not manifest as clinically identifiable disease states because of the presence of these protective mechanisms present in healthy canine intestinal tracts. Thus, it is clear that evaluation of the microbiome and its potential ramifications in a clinical setting should involve a multifaceted approach that evaluates not only the microbiota, but also the relevant metabolic pathways, gene expression, and interactions with the host.[16] This combined approach likely represents the bridge between laboratory research and clinical application of interpretation of microbiome shifts.

What disease processes have been shown to change canine GI microbiota?

Coinciding with a better understanding of the composition of the GI microbiome in healthy mammals, extensive research has been performed evaluating the GI microbiome characteristics in human and canine populations with various pathologies. Disease models evaluated in canines and humans include but are not limited to chronic inflammatory enteropathies, diabetes, obesity, and hepatopathies leading to hepatic encephalopathy (HE).[15,17-21] By using the previously identified control profiles of intestinal microbiota in comparison to the profiles from the diseased populations, the concept of a "dysbiosis index" was established. This has been postulated to be a useful adjunct for identifying disease states, or as a potential monitoring target for treatment efficacy.[4,5,15,17,18,21]

What findings have been reported regarding hepatopathies and microbiota?

The role of the GI microbiome in chronic hepatopathies and hepatic encephalopathy (HE) has been investigated in humans.[21,23,24] Ammonia has been considered as one of the primary causative agents of HE and is produced primarily from the process of urea breakdown by urease producing large intestinal bacteria, as well as in the kidneys and small intestine.[21,23,24] Significant differences have been shown in the microbiota of the colon between human patients with cirrhosis and/or HE and healthy control individuals,

and some suggest that the alterations in the microbiome in these patients may contribute to more significant clinical signs of HE or complications leading to mortality.[23,25,26] Specifically, dysbiosis has been shown to have an important role in late-stage cirrhosis in humans including intestinal bacterial overgrowth, small bowel dysmotility, increased gut permeability, and decreased immunological defenses, all of which can predispose the affected individual to bacterial translocation from the gut to the systemic circulation.[26] This may also lead to septic bacterial peritonitis in human patients. The most commonly isolated bacteria in these cases is *E. coli*, which has also been shown to be one of the main bacteria increased in dysbiosis of dogs.[4,26] Multifactorial immune suppression has been identified as an underlying cause for these bacterial infections, related to decreased activity of bactericidal phagocytic cells.[27,28]

Intestinal dysbiosis has also been linked specifically with HE in humans, in which the prevalence of certain bacteria is increased, resulting in metabolic effects that contribute to HE.[29] Reported bacteria associated with HE in humans with cirrhosis include *E. coli, Staphylococcus spp., Streptococcaceae, and Veillonellaceae*.[30,31]

Investigation of the GI microbiome in canines with hepatic pathology is more limited. A pilot study in 2015 conducted with 10 dogs revealed that one dog with chronic active hepatitis exhibited *Fusobacteria* as the dominant bacterial phyla in the distal gut, as opposed to healthy dogs or dogs with distinct systemic pathology not involving the liver, in which the dominant organisms were *Firmicutes* or *Proteobacteria*, though clinical significance could not be determined.[32] Given the sparsity of literature, the role of dysbiosis in relation to hepatic dysfunction or pathology in dogs has yet to be elucidated.

What effects does lactulose have in the GI tract? Does it change the microbiota?

In dogs, the most common cause of HE is related to congenital portosystemic shunts (CPSS) due to the high prevalence of this disease.[33] Congenital portosystemic shunts are defined as one or more aberrant blood vessels that allow portal venous blood to bypass the liver parenchyma. This leads to neurologic clinical signs such as ataxia, lethargy, head pressing, or seizures.[34] Typical medical management of dogs with PSS includes a restricted protein diet, lactulose administration, potential administration of antibiotics, anticonvulsant medications, or probiotics, all of which are intended to reduce ammonia production and absorption.[35,36] Additionally, dogs with intrahepatic portosystemic shunts are at an increased risk for gastric or duodenal ulceration, and current recommendations for therapy include proton pump inhibitors both in the short and long terms.[35] Provided these patients are deemed healthy enough to undergo general anesthesia, surgical attenuation of the CPSS is often recommended in order to restore more normal portal blood flow and liver function in the long term. Except for anticonvulsants, the effects that the individual components of medical management for CPSS have on the microbiome have been investigated individually in dogs.[22,37-39]

Lactulose is a synthetic disaccharide, an osmotic cathartic, and an ammonia reducer. Osmotic cathartics result in water being retained or attracted into the intestinal lumen, and may also cause enhanced mucosal secretion of fluid. Because no endogenous enzyme exists to digest lactulose, it passes through the small intestine undigested.[40] In the colon, lactulose interacts with flora that break down saccharides, resulting in the production of lactic, acetic, and other organic acids that decrease luminal pH. In patients with HE, this effect is desirable as acidification of the fecal contents enhances the production of ionized ammonium, which is nonabsorbable, as opposed to ammonia.[40]

The effect of lactulose on the GI microbiota has been studied in humans, mice, pigs, and in healthy dogs, with effects ranging from negligible to dramatic.[41-44] In one study of 18 healthy dogs, lactulose administration resulted in a decrease in bacterial diversity, characterized by significant increases in Firmicutes and Actinobacteria, and a decrease in Bacteroidetes and Fusobacteria.[41] The authors posited that the increased presence of the family *Veillonellaceae*, members of which convert lactate to acetate and butyrate, may be beneficial as acetate has been negatively associated with proinflammatory cytokines in cirrhosis, and butyrate has been shown to be protective against HE in humans.[45] These changes in the GI microbiota were found to be reversible with the cessation of lactulose administration in this population of dogs.[41] The effects of lactulose on the fecal microbiota in dogs with hepatopathies and/or HE remains to be studied.

What effects does diet have on canine GI microbiota?

Dietary modification is another important component of medical management for dogs with CPSS and/or HE. In general, restricted protein diets are recommended for dogs with CPSS; the goal of a restricted protein diet is to reduce the production and absorption of ammonia in the large intestine; this is accomplished, at least in part, by limiting the substrate (i.e. protein) available to produce ammonia.[36] Restricted protein diets with varying sources of protein have been shown to reduce the severity of HE scores in dogs with CPSS, and soy-based restricted protein diets may also lower the plasma ammonia concentration.[46]

The nearly endless array of dietary effects on the GI microbiota that have been investigated in humans are beyond the scope of this project. In dogs, several studies have been published investigating the effects of diet on the intestinal microbiota, though none have been performed specifically with a protein restricted diet.[47-49] As discussed, the role of the canine GI microbiota in hepatopathies, or more specifically CPSS/HE, has yet to be elucidated, though extrapolation from studies in humans is compelling for a meaningful relationship between the two.

As different diets lead to altered presence of nutrients and degradation products in the intestinal tract, it stands to reason that there would be an impact on the GI microbiota which have been shown to be closely involved in nutrient processing and absorption. In healthy beagle dogs that were fed cellobiose, dose-dependent significant increases in

Lactobacillaceae, Alloprevotella, Bacteroides, and Prevotella were observed.[47] The fecal pH in that study was unaffected by the dietary change, though the authors hypothesized that the increased fecal lactate concentration in the study may have acidified the colonic lumen (as opposed to the feces itself), which could be clinically relevant to dogs with CPSS/HE. Additionally, this theoretical change in colonic luminal pH may have been related to (or responsible for) the change in colonic microbiota.[47] Fat content of the diet showed no significant impact on fecal bacterial richness or diversity in one study, though there were minor, statistically significant changes in individual bacterial taxa.[48] Another study investigating the impact of a raw meat diet as compared to a commercial diet found that GI microbiota diversity was improved in the raw diet group, and that diet had a significant impact on the end products of fermentation (e.g. lactic acid, acetate, butyrate).[49] Yet another study investigating a "bones and raw food" (BARF) diet showed no significant increase in microbiota diversity, but did show an increase in prevalence of *E. coli* and *C. perfringens*.[50] It is apparent from these various studies that changes in the nutrient profile in the gut can have significant effects on the microbiome, and that the macronutrient composition of distinct diets can differ quite dramatically.

There is currently no literature detailing the effects of a restricted protein diet on the canine GI microbiota, but these other dietary studies, as well as those documented in human medicine, make it seem possible or even likely that such a relationship exists.

What effects do antibiotics have on canine GI microbiota?

As agents that inhibit bacterial growth or survival, antibiotics inherently have effects on the GI microbiota that have been documented in numerous species, including humans and canines. In humans, the alteration to the GI microbiota associated with antibiotic use may be so significant as to predispose to pathologic colonization of the GI tract with *Clostridium difficile*, which has been documented following use of several classes of antibiotics including cephalosporins, clindamycin, and fluoroquinolones.[51,52] The risk for dysbiosis appears to last for several weeks after antibiotic exposure. A review article published in 2017 details the multitude of effects of various antibiotics on the human GI microbiome.[53] The use of broad-spectrum antimicrobials has been shown to decimate microbiome diversity, and in some cases may even precede long-lasting states of dysbiosis that may predispose individuals to the development or exacerbation of other disease processes. For more information on the individual profiles of effects of specific antimicrobials on the human microbiome or individual bacterial taxa, the reader is referred to this review article.[53]

In human patients with HE, antibiotic therapy is often a key component of preventing or reducing the presence of clinical signs.[54] The underlying rationale for antibiotic therapy in these patients is the reduction in production and absorption of gut-derived neurotoxins, as well as minimizing endotoxemia and inflammation. The most commonly used antibiotics in these patients are neomycin, metronidazole, vancomycin, and rifaximin, with rifaximin being most efficacious.[54,55] An altered fecal microbiome in humans with

cirrhosis has also been linked directly to changes in cognition.[56] Several studies have elucidated the role of rifaximin in altering the microbiome, and indicate that it may actually have eubiotic properties as opposed to other antibiotics that cause or exacerbate dysbiosis; the authors posit that this may be due to targeted effects on pathogenic bacteria or indirect effects on the host, such as inhibition of bacterial attachment or reduction of mucosal inflammation.[57,58] Part of rifaximin's eubiotic effects are its promotion of growth of beneficial bacteria such as Bifidobacteria and Lactobacilli, even in patients with gastrointestinal or liver disease.[58]

Oral antibiotic therapy has classically been a component of medical management of dogs with CPSS and/or HE for the same reasons as listed above in humans, with metronidazole being among the most commonly used. Though the veterinary literature on changes in the GI microbiome is less robust, studies have examined antibiotic effects on GI flora in healthy dogs with multiple types of antibiotics. One such study on the effects of metronidazole found that there were significant decreases in fecal microbiome richness and in certain key bacteria such as Fusobacteria, and these changes were still in place four weeks after discontinuation of therapy.[59] Other changes to the intestinal microbiota, such as those seen with antibiotic responsive diarrhea, are less well understood.[14] The concept of metabolomics describes the translation of these alterations in bacterial populations into their functional capacity; until this can be investigated further in veterinary medicine, it will remain challenging to know the true relationship between the GI microbiome and various disease processes with which it has been linked.

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VITA

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