MICROBIAL CHARACTERIZATION, METABOLOMIC PROFILING, AND BILE ACID METABOLISM IN HEALTHY DOGS AND DOGS WITH CHRONIC ENTEROPATHY

A Dissertation

by

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ABSTRACT

Chronic gastrointestinal disease in dogs can manifest itself in many different ways including vomiting, diarrhea, and weight loss. Bile acid dysmetabolism has recently been recognized as an important component of chronic gastrointestinal disease (e.g., Crohn's disease, Ulcerative Colitis, Irritable Bowel Syndrome, and Inflammatory Bowel Disease) in humans. The aim of this research was to evaluate bile acid dysmetabolism in chronic enteropathy of dogs.

An assay for the measurement of unconjugated fecal bile acids using gas chromatography coupled with mass spectrometry was developed. The assay was accurate and reproducible. The percent of unconjugated secondary bile acids were significantly decreased in dogs with chronic enteropathy ($p=0.0161$), with approximately 60% of dogs having bile acid dysmetabolism. The percent of unconjugated secondary bile acids significantly increased in patients with chronic enteropathy after steroid therapy ($p=0.0183$). The effect of cholestyramine, a bile acid sequestrant, was evaluated for the ability to alter the fecal bile acid pool in healthy dogs. The concentration of secondary bile acids significantly increased in feces of healthy dogs administered cholestyramine ($p=0.0183$). These results demonstrate that a subset of dogs with chronic enteropathy show fecal bile acid dysmetabolism, and further studies are warranted to evaluate the use of bile acid sequestrants in clinical cases.

DEDICATION

To my wife Melissa and my son Wyatt. Wyatt – you are currently quite upset in the background as I write this dissertation. To my wife's family, your love, support, and Guadalupe getaway were just what we needed to make it through school. Also to Mr. Butters, while your stay with us was shorter than expected – that moment of time for our family was irreplaceable.

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My friends and colleagues in the Gastrointestinal Laboratory have become such a truly wonderful family. While I will be leaving the laboratory, I will only be a building away – this way, I can continue to seek their friendship.

My mother, father, and brother have been an enormity of support during my academic tenure. Please be sure to leave a light on for me when we move home, it's closer than you'd imagine. I would like to thank my wife for her sharp and witty personality, I'm truly blessed to be your husband and it's safe to say that I outkicked my coverage. And lastly, to my son Wyatt, I'm looking forward to a time when you're just a few years older so we can begin our outdoor adventures.

NOMENCLATURE

IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IBS-D	Irritable Bowel Syndrome-Diarrhea Predominant
GC/MS	Gas Chromatography/Mass Spectrometry
GI	Gastrointestinal
CA	Cholic Acid
CDCA	Chenodeoxycholic Acid
LCA	Lithocholic Acid
DCA	Deoxycholic Acid
UDCA	Ursodeoxycholic Acid
CE	Chronic Enteropathy
fUBA	fecal Unconjugated Bile Acids
CIBDAI	Canine Inflammatory Bowel Disease Activity Index

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Jan S. Suchodolski [advisor] and Jörg M. Steiner [co-advisor] of the Department of Small Animal Clinical Sciences in the College of Veterinary Medicine at Texas A&M University [Home Department]. Additional supervision was given by Dr. Albert E. Jergens, Dr. Craig B. Webb, and Dr. Arul Jayaraman of the following departments, respectively: Small Animal Medicine and Surgery in the College of Veterinary Medicine at Iowa State University, Department of Clinical Sciences in the College of Veterinary Medicine and Biomedical Sciences at Colorado State University, and the Department of Chemical Engineering at Texas A&M University [Outside Departments].

Technical and scientific contributions for metabolite identification was provided by Dr. Arul Jayaraman. Practical clinical applications for study design and implementation was provided by Dr. Albert Jergens. Development of the GC/MS assay was done with technical assistance and troubleshooting by Dr. Julia Honneffer. Michelle Jonika aided in data acquisition and raw data processing of bile acids generated by GC/MS. A subset of samples for Chapter 3 and 4 was provided by Dr. Albert Jergens and outside collaborators. The clinical study in Chapter 5 was conducted in part by Dr. Kelly Swanson and Celeste Alexander at the University of Illinois Urbana-Champaign. Anitha Isaiah performed initial sequencing analysis for samples in Chapter 5.

All other work conducted for the dissertation was completed by the student independently.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW*

Recent molecular studies have revealed a complex microbiota in the dog intestine. Convincing evidence has been reported linking changes in microbial communities to acute and chronic gastrointestinal inflammation, especially in canine inflammatory bowel disease (IBD). The most common microbial changes observed in intestinal inflammation are a decrease in the bacterial phyla Firmicutes (i.e., Lachnospiraceae, Ruminococcaceae, and *Faecalibacterium*) and Bacteroidetes, with a concurrent increase in Proteobacteria (i.e., *E. coli*). Due to the important role of microbial-derived metabolites for host health, it is important to elucidate the metabolic consequences of gastrointestinal dysbiosis. Metagenomic studies have utilized shotgun sequencing of DNA as well as PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) to characterize functional changes in the bacterial metagenome in GI disease. Untargeted measurements of metabolic products derived by the host and the microbiota continue to better describe functional alterations that occur in gastrointestinal disease. For example, changes in bile acid metabolism and tryptophan catabolism have recently been reported in humans and dogs with GI disease. Metabolites associated with the pentose phosphate pathway were significantly altered in chronic GI inflammation, indicating the presence of oxidative stress in dogs with IBD.

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* Part of the data reported in this chapter is reprinted with permission from the Journal of Animal Science by Guard, B. C., and J. S. Suchodolski. "HORSE SPECIES SYMPOSIUM: Canine intestinal microbiology and metagenomics: From phylogeny to function." Journal of animal science 94.6 (2016): 2247-2261.

Our understanding of the canine gastrointestinal microbiota during health and disease has increased drastically over the past decade, however, major hurdles, such as understanding the function of microbes, still exist. Metabolomics measures the collective activity of metabolites in the GI tract. Additionally, metabolomics can identify actual alterations in the GI ecosystem and can be reflective of real time bacterial and host by-products. This approach helps to answer the question of 'what are microbes actively doing?' This functional capacity may be similar between dogs and humans (i.e., functional core). The functional core is made up of important host-microbial end products, such as complex polysaccharide degradation, as well as the synthesis of short-chain fatty acids, amino acids, and vitamins (Turnbaugh et al., 2009; Qin et al., 2010; Swanson et al., 2011).

The microbiota in healthy dogs and dogs with GI disease

The complex interactions that exist between GI microbes and the canine host are critical for immune regulation, nutrient metabolism, and various other physiological processes (Amtsberg et al., 1978; Swanson et al., 2002; Satyaraj et al., 2013; Kainulainen et al., 2015). Over the past decade, a combination of culture-based methods coupled with next-generation sequencing has provided a thorough overview of the phylogenetic composition that makes up the intestinal microbial community in dogs (Suchodolski et al., 2009; Middelbos et al., 2010; Garcia-Mazcorro et al., 2011; Handl et al., 2011; Garcia-Mazcorro et al., 2012). The mammalian intestine is home to a total of 10^{10} to 10^{14} microbial cells, which is approximately 10 fold more than the number of host cells. The GI microbiota plays a critical role in host health. This is achieved by establishing a first line of defense against pathogens, aiding in energy harvest and digestion, supporting

enterocyte nutrition, and bolstering the immune system (Swanson et al., 2011). Nutritional benefits of gut microbes include the digestion of complex carbohydrates to produce short chain fatty acids (SCFA, i.e., acetate, propionate, and butyrate), which promote intestinal health (Roediger, 1982). It has become evident that the gastrointestinal microbiota also plays a role in disease. Alterations in the composition of the microbiota have now been well documented in dogs with chronic enteropathy (CE) and dogs with acute diarrhea (Suchodolski et al., 2012a; Suchodolski et al., 2012b; Minamoto et al., 2014b; Guard et al., 2015). Similar changes have been noted in humans with IBD, such as Crohn's disease and ulcerative colitis (Frank et al., 2007; Dethlefsen et al., 2008; Packey and Sartor, 2009). These studies suggest that the microbial response is conserved across mammalian species during inflammatory conditions. Therefore, dogs may serve as a useful model for inflammatory conditions of the gastrointestinal tract in humans.

In healthy dogs, initial studies using traditional bacterial culture methodology estimated that the bacterial load in the small intestine of dogs ranged from 10^2 to 10^9 cfu/g of small intestinal contents (German et al., 2003). In the colon the number of bacteria ranges from 10^8 to 10^{11} cfu/g (Mentula et al., 2005). High-throughput sequencing studies have shown that the predominant phyla in the feces of healthy dogs are Bacteroidetes, Firmicutes, Fusobacteria, and Proteobacteria (Handl et al., 2011; Igarashi et al., 2014; Guard et al., 2015). Less abundant phyla (less than 1%) are Spirochaetes, Tenericutes, Verrucomicrobia, and TM7 (Candidatus Saccharibacteria) in healthy dogs (Suchodolski, 2011). The GI microbiota of dogs is similar to humans, where Firmicutes, Bacteroidetes and Actinobacteria also constitute the major bacterial phyla (Consortium, 2012).

In contrast, the phylogenetic composition of the GI microbiota in dogs with GI disease is distinctly different from that of healthy dogs. Chronic enteropathies can be categorized by how a patient responds to treatment (e.g., antibiotic responsive, food responsive, or steroid responsive diarrhea). IBD in dogs is characterized by confirmed inflammation upon histopathological evaluation of GI tissue, similarly, this diagnosis can be categorized by how a patient response to treatment as mentioned previously. Microbiota alterations in patients with GI disease vary depending on the sampling site. In the duodenum of dogs with IBD, bacterial groups belonging to Fusobacteria, Bacteroidaceae, Prevotellaceae, and Clostridiales are underrepresented while the bacterial genera (within Proteobacteria) *Diaphorobacter* and *Acinetobacter* are overrepresented (Suchodolski et al., 2012a). The changes in fecal microbiota in dogs with IBD are, to some extent, similar to changes observed in the duodenal microbiota of dogs with IBD, with members of Proteobacteria being increased (i.e., Gammaproteobacteria) (Minamoto et al., 2014b). The same study identified a decrease in fecal bacterial groups belonging to Erysipelotrichia, Clostridia, and Bacteroidia. A decrease in the abundance of *Faecalibacterium* and the phylum Fusobacteria has also been identified in the fecal microbiota of dogs with IBD (Suchodolski et al., 2012b). Decreased abundances of *Faecalibacterium,* Clostridia, Erysipelotrichia in dogs with IBD may be detrimental to the production of SCFA, which as discussed previously, can significantly influence host health. Minamoto et al. (2014b) reported that compared with healthy dogs, microbial communities were altered in dogs with IBD before treatment (n = 12), but did not cluster with the microbial communities belonging to healthy dogs post-treatment. In patients with

Crohn's disease, the bacterial phyla Firmicutes and Bacteroidetes are consistently decreased with a concurrent increase in Proteobacteria (Sokol et al., 2008; Frank et al., 2011). Since the depletion of commensal bacterial groups seems to be apparent in dogs with chronic GI disease, it may be reasonable to counterbalance intestinal dysbiosis with beneficial bacterial species (i.e., probiotics) (Rossi et al., 2014).

Infectious organisms may cause acute diarrhea. (e.g., *Camplyobacter jejuni*, *Salmonella*, *Clostridium perfringens*) (Cave et al., 2002; Unterer et al., 2011). Dogs with acute diarrhea experience major shifts in their fecal microbial community compared with healthy dogs (Suchodolski et al., 2012b; Guard et al., 2015). This is characterized by a decrease in *Blautia*, *Faecalibacterium*, Ruminococcaceae, and *Turicibacter* coupled with an increased abundance of *Clostridium perfringens*. Many of the bacterial groups decreased during acute diarrhea are also thought to be producers of SCFA. Decreased abundances of Ruminococcaceae and *Faecalibacterium* were correlated with decreased fecal propionate and inversely correlated with fecal butyrate concentrations in dogs with acute diarrhea (Guard et al., 2015). A recent study found a correlation between increased abundance of *Faecalibacterium* and improvement of clinical activity index, which may indicate that this bacterium can be used to monitor recovery from GI disease (Rossi et al., 2014). Profound alterations in microbial communities have been noted in dogs with acute diarrhea, necessitating further investigation into the role these microbes play in the GI tract. Table 1 summarizes results from various studies highlighting microbial alterations in canine health and disease.

Table 1. Summary of microbial alterations in dog with various GI diseases.

Study	Sample site	Presentation (sample size)[1]	Sequencing method	Bacterial groups increased[2,4]	Bacterial groups decreased[2,3,4]
(Minamoto et al., 2014b)	Feces	Controls (n = 12), IBD (n = 12)	454-pyrosequencing/qPCR	Enterobacteriaceae, Gammaproteobacteria, Proteobacteria, *Escherichia,* *Enterococcus*	Coprobacillaceae, Lachnospiraceae, *Collinsella,* Erysipelotrichales, Coriobacteriales, *Prevotella,* Ruminococcaceae
(Guard et al., 2015)	Feces	Controls (n = 13), Acute Diarrhea (n = 13)	454-pyrosequencing/qPCR	*Clostridium perfringens*	Bacteroidetes (2.1), *Faecalibacterium* (15.0), Unclass. Ruminococcaceae, Unclass. Lachnospiraceae (2.9), *Eubacterium* (10.0), *Blautia* (3.9), *Prevotella*
(Rossi et al., 2014)	Feces	Controls (n = 10), IBD (n = 20)	qPCR	None Reported	*Faecalibacterium,* *Turicibacter*
(Xu et al., 2014)	Feces	Controls (n = 11), IBD (n = 23)	DGGE/qPCR	None Reported	*Lactobacillus*
(Minamoto et al., 2014a)	Feces	Controls (n = 95), GI disease (n = 104)	qPCR	*Bifidobacterium,* *Lactobacillus,* *E. coli*	Fusobacteria, Ruminococcaceae, *Blautia,* *Faecalibacterium*
(Markel, 2012)	Feces	Controls (n = 242), CE (n = 118), Acute Hemorrhagic Diarrhea (AHD) (n = 57)	qPCR	CE *Lactobacillus,* *Streptococcus* AHD *Clostridium perfringens,* *E. coli*	CE Bacteroidetes AHD *Lactobacillus,* *Streptococcus,* Firmicutes
(Jia et al., 2010)	Feces	Controls (n = 8), Chronic Diarrhea (n = 9)	FISH	*Bacteroides*	None Reported

Table 1. Continued.

Study	Sample site	Presentation (sample size)[1]	Sequencing method	Bacterial groups increased[2,4]	Bacterial groups decreased[2,3,4]
(Suchodolski et al., 2012a)	Duodenum	Controls (n = 6), IBD (n = 14)	454-pyrosequencing	Proteobacteria (2.3), *Diaphorobacter, Acinetobacter*	Fusobacteria (29.6), Bacteroidaceae, Prevotellaceae, Clostridiales
(Suchodolski et al., 2012b)	Feces	Controls (n = 32), Acute Non-Hemorrhagic Diarrhea (n = 12), Acute Hemorrhagic Diarrhea (n = 13), Active IBD (n = 9), Therapeutically Controlled IBD (n = 10)	454-pyrosequencing/qPCR	AHD *Sutterella, Clostridium perfringens*	AHD *Blautia* (49.5), Ruminococcaceae (20.0), *Faecalibacterium, Turicibacter* NHD *Blautia* (49.5), *Ruminococcaceae* (3.4), *Turicibacter* (5.0), *Faecalibacterium,* Bacteroidetes Active IBD *Faecalibacterium,* Fusobacteria
(Suchodolski et al., 2010)	Duodenum	Controls (n = 7), IBD (n = 7)	Gene Clone Libraries	Proteobacteria	Clostridia
(Allenspach et al., 2010)	Duodenum	Controls (n = 8), CE (n = 13)	Gene Clone Libraries	*Actinobacteria* (8.8), Lactobacillales, Erysipelotrichi	None Reported
(Xenoulis et al., 2008)	Duodenum	Controls (n = 9), IBD (n = 10)	Gene Clone Libraries	Enterobacteriaceae (29.9), *E. coli*	None Reported

[1]Alterations in bacterial groups are based on comparison to controls or baselines in the related study, unless otherwise noted.

[2]Fold changes were calculated and displayed in parenthesis for only studies that compared the percent of total bacteria between groups.

[3]In cases where bacterial abundance was decreased the inverse value is provided.

[4]Fold changes were not calculated between groups where a “zero” abundance was present and in the absence of raw data provided in the study.

Metabolomics in canine gastrointestinal disease

Metabolomics was introduced in the 1990s, formatted initially for evaluating drug toxicity (Nicholson et al., 1999). By definition, metabolomics is the "study of metabolites which employs non-biased identification and quantification of all metabolites in a biological system" (Ellis et al., 2007). The context of metabolomics in the GI tract includes investigating the role of metabolites in various GI disorders and potentially elucidating novel biomarkers for the etiology, progression, and treatment of such diseases (Walker et al., 2014). This approach is useful in that it can provide non-invasive strategies for the prognosis and diagnosis of GI diseases in addition to understanding complex metabolic pathways.

Several different techniques in metabolomics are used to create a metabolic profile. Untargeted metabolomics is a technique that unbiasedly profiles metabolites. Because inherent instrument limitations exist for the detection of all metabolites, a multitude of platforms needs to be used, such as gas chromatography (GC), liquid chromatography (LC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) to increase the number of qualitative and semi-quantitative results (Weckwerth, 2003). In untargeted metabolomics, high-mass accuracy instruments such a GC-Time-Of-Flight (TOF)-MS are capable of generating and matching mass spectra to libraries of compounds. Alternatively, in targeted metabolomics, unknown samples are compared with pure standard compounds for positive identification and quantitation.

Metabolomics is still in its infancy with little standardization across studies or platforms (Kanani et al., 2008). Feces can be a difficult matrix to work with compared

with serum and urine, given the large amount of impurities present in a sample. This proves to be especially true when working with highly sensitive machines (i.e, GC/MS and liquid chromatography/mass spectrometry). There is currently no gold standard for normalization of fecal metabolite concentrations (e.g., adjusting by fecal dry weight or starting with lyophilized fecal sample).

Minamoto et al. (2014b) described for the first time alterations in serum metabolites in healthy dogs and dogs with idiopathic IBD (Minamoto et al., 2014b). Multivariate analysis of all untargeted serum metabolite concentrations revealed tight clustering in healthy dogs while dogs with IBD before treatment were very scattered, indicating global variability among metabolite profiles in dogs with IBD. This study reported a decreased abundance of amino acids in dogs with IBD, whereas a wide variety of metabolites were increased in dogs with IBD according to the heatmap. Predictive metagenomics using 16S rRNA genes, indicated that the microbiota in dogs with IBD contribute to dysfunctional amino acid metabolism. These results are some of the first of its kind in dogs to describe the functional capacity of the metagenome and its function in vitro. A link is becoming clearer between amino acids and the pathogenesis of IBD. For example, a study in humans measured the plasma amino acid concentrations in 387 IBD patients and 210 healthy controls (Hisamatsu et al., 2012). That study reported decreased concentrations of histidine and tryptophan in patients with IBD (~72 μM and ~45 μM, respectively) compared with healthy controls (~83 μM and ~49 μM, respectively). The concentrations of histidine and tryptophan were inversely correlated with the inflammatory marker C-reactive protein ($r_2 = -0.460$ and -0.370, respectively; $p<0.001$

for both). Interestingly, the amino acid tryptophan is metabolized into kynurenine by indoleamine 2,3-dioxygenase and may act as an immunological regulator (O'connor et al., 2009). This may provide further evidence of a potential relationship between amino acids and inflammation.

Of the hundreds of serum metabolites measured in the study by Minamoto et al. (Minamoto et al., 2014b), only nine were significantly altered. Of those, four metabolites were named and five were unnamed. The four identified metabolites included gluconolactone, hexuronic acid, 3-hydroxybutanoic acid, and ribose; all which were significantly increased in dogs with IBD before treatment (approximate median peak intensity = 750, 2,100, 2,100, and 8,500; respectively) compared with healthy control dogs (approximate median peak intensity = 300, 1,000, 1,700, and 3,000; respectively). Gluconolactone was the only identifiable metabolite that decreased after treatment in dogs with IBD (approximate median peak intensity = 450). Gluconolactone is an oxidized derivative of glucose and is capable of scavenging free radicals. It may serve as a surrogate marker for inflammation given its ability to discriminate between dogs with IBD before and after treatment. Metaboanalyst (Xia et al., 2015) (a comprehensive and freely-available online (http://www.metaboanalyst.ca) tool suite for metabolomics data analysis) was used to compare metabolic pathways; the pentose phosphate pathway was more active in dogs with IBD, mainly due to an increase in gluconolactone and ribose. The pentose phosphate plays a role in redox balance, proliferation, and protection from oxidative stress. Alterations of metabolites involved in these pathways may be indicative of the presence of oxidative stress during periods of inflammation. Metabolomic profiling in the serum of

dogs with IBD is fairly novel and draws major parallels to what is reported in human GI disease. The pathogenesis of human IBD is highly complex; therefore, a multitude of causes may be at play. These studies further support an inappropriate inflammatory response and a variety of metabolic fluctuations that may play a role (Colombel, 2014). Reactive oxygen species may contribute to oxidative stress because, in humans, they have been found to be increased during active ulcerative colitis and decreased once patients are in remission. These changes are also accompanied by decreased antioxidant levels, which in a two-fold approach, likely contribute to major pathogenic mechanisms in IBD (Beltran et al., 2010; Bhattacharyya et al., 2014). Metabolite alterations in this study add new depth to understanding IBD in dogs given that studies in human IBD have largely focused on and reported altered lipid and amino acid metabolism (De Preter and Verbeke, 2013).

Therapeutic intervention in canine gastrointestinal disease

Studies in mice and humans have pioneered the use of metabolomics and metagenomics in characterizing GI disease (Schicho et al., 2012; Zhu and Li, 2012; Walker et al., 2014). One study in patients with ulcerative colitis and Crohn's disease used NMR in an untargeted metabolomics approach to evaluate urine metabolites (Stephens et al., 2013). That study reported the percent difference of succinate (−77%), trans-aconitate (−43%), and citrate (−35%) to be significantly decreased in patients with IBD. These metabolites are well-known to be crucial in the tricarboxylic acid (TCA) cycle. Decreased energy metabolites, such as those belonging to the TCA cycle, have been shown in another study involving the dextran sulfate sodium (DSS)-induced colitis mouse model (Shiomi et al., 2011); they found decreased abundances of TCA cycle intermediates as well as

glutamine, tryptophan, tyrosine, asparagine, and glycine compared with controls. Furthermore, they observed a positive correlation between glutamine and inflammation. In addition, that study reported that supplementation of glutamine could reduce colon tissue lesions in a dose-dependent fashion. These results indicate a potential usefulness for metabolomics as a novel approach to discover therapeutic targets against GI disease.

Due to the complexity of bacterial and host interactions in the GI tract, a multi 'omics' approach will likely be necessary in the future to fully understand various GI diseases. Studies from our laboratory have only recently begun to describe metabolomic changes on a large scale (Minamoto et al., 2014b; Guard et al., 2015). These studies have used an untargeted metabolomics approach whereby hundreds of metabolites were detected and quantified. These studies, however, have only investigated serum and urine metabolites while none have investigated fecal metabolites. Feces may contain surrogate markers that correspond to various types of GI disease and serve as a non-invasive alternative for sample collection. Some preliminary work indicates that feces from dogs with CE contain several hundred significantly altered metabolites (Honneffer et al., 2015a). Analysis of these metabolites has indicated alterations in bile acid metabolism, tryptophan metabolism, and the pentose phosphate pathway.

Tryptophan metabolism is another pathway that has garnered recent attention in canine GI disease (Guard et al., 2015; Honneffer et al., 2015a). Minamoto et al. (2014b) noted increased serum tryptophan in dogs with IBD (Minamoto et al., 2014b). The essential amino acid L-tryptophan is required for the biosynthesis of proteins and is a precursor for several biologically important compounds. It can be catabolized by several

different pathways; however, one of particular interest is breakdown of tryptophan to kynurenine by tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase (IDO). An increased kynurenine to tryptophan ratio can indicate increased IDO, which promotes immune activation and endogenous interferon-γ formation (Schröcksnadel et al., 2006). Thymus cells (T-cell) have before been implicated in the pathogenesis of IBD in humans (Rossi et al., 2014; Maeda et al., 2015), and it is known that IDO regulates cell proliferation and survival. Studies in human IBD have shown that IDO mRNA is markedly increased in colonic biopsies of IBD patients (Wolf et al., 2004). A study mentioned previously used an untargeted-targeted metabolomics approach to evaluate the serum and urine of dogs with acute diarrhea (Guard et al., 2015). Urine metabolomics in these dogs revealed relatively little changes, with 2-methyl indole and 5-methoxy-1H-indole-3-carbaldehyde being decreased in dogs with acute diarrhea (~median concentration = 25 and 75 fg/mg of creatinine, respectively) compared with healthy dogs (~median concentration = 125 and 125 fg/mg of creatinine, respectively). While little is known about these indole-like compounds, this study also found that serum kynurenic acid (a catabolite of kynurenine and tryptophan) was decreased in dogs with acute diarrhea compared with healthy dogs (~median concentration = 15 and 45 ng/mL, respectively) as well as the ratio of tryptophan to kynurenic acid (Guard et al., 2015). It is important to remember that these changes occurred in serum and urine metabolites. Tryptophan can be catabolized in a number of different ways; one example is enzymatic breakdown of tryptophan by IDO into kynurenine, kynurenic acid, quinolinic acid, and xanthurenic acid (Takikawa, 2005). During periods of inflammation, cytokine involvement such as interferon-γ, tumor

necrosis factor-α, IL-2, IL-6, IL-18, and IL-1β may shift the direction of tryptophan metabolism away from end products such as serotonin and indole derivatives towards kynurenine and its downstream products (Oxenkrug, 2007). Many studies performed in humans over the past decade have pinpointed significant increases in IDO in patients with Crohn's disease. However, the role of IDO overexpression in human IBD can be somewhat complicated. For instance, IDO has been shown to exert inhibitory effects on T-cell proliferation in vitro and in vivo (Fallarino et al., 2002). In addition, deprivation of tryptophan by upregulation of IDO may regulate T-cell survival (Fallarino et al., 2002; Mellor et al., 2002; Wolf et al., 2004). It is important to note that alterations in tryptophan metabolism may be different depending on biological site sampled. Recent research has demonstrated alterations in fecal tryptophan catabolism in dogs with IBD (Honneffer et al., 2015a). In this untargeted fecal metabolomics study, several pathways were investigated. As opposed to some findings in the human literature, there were no changes in kynurenine compounds, hinting at a seemingly normal IDO and tryptophan 2,3-dioxygenase expression. Fecal serotonin also remained unchanged in both healthy dogs and dogs with chronic GI disease. Alternative pathways of tryptophan metabolism, such as downstream indole products, may deserve further attention in dogs with IBD. Indolepropionate has been shown in models of rodent brain to act as a potent hydroxyl radical scavenger (Poeggeler et al., 1999). Therefore, it could be surmised that decreased indolepropionate may further exacerbate oxidative stress, which has been reported in dogs with chronic GI disease. While the tryptophan pathway in and of itself does not reflect dramatic alterations in fecal or serum metabolites in dogs with GI disease, the interplay

still remains to be fully understood. The notion that many metabolic disturbances in unison contribute to the etiology of the disease gives leverage to the idea of a complex and multifactorial process contributing to dogs with chronic GI disease, specifically dogs with IBD.

Bile acid metabolism

Overview

Bile is made up of primarily bile acids along with phosphatidylcholine, bilirubin, and cholesterol (Russell, 2009). In the human body, bile acids are well-known to play an important physiological role in the uptake of lipids and fat-soluble vitamins in the intestine (e.g., A, D, E, and K) (Hofmann, 1999; Trauner et al., 2010). The synthesis, circulation, and metabolism of bile acids is highly complex and varies depending on the mammalian species. At the core of bile acid metabolism, primary bile acids are synthesized from cholesterol and converted to CoA esters and then conjugated with the amino acids glycine and taurine (Russell and Setchell, 1992). In humans, glycine is conjugated preferentially to taurine in a 3:1 ratio (Sjövall, 1959). In dogs, however, bile acids are primarily conjugated with taurine. Primary bile acids generally consist of cholic and chenodeoxycholic acid. After synthesis and conjugation, bile acids are released into the proximal duodenum to the site of active absorption in the terminal ileum where approximately 95% of bile acids are reabsorbed (Scott et al., 1983); additionally they are comprised of a basic micellar structure, and it is with mixed micelles that the absorption of fat into mucosal cells is facilitated.

Bile acid metabolism is rate limited by the enzyme Cytochrome P450 7A (CYP7A1) in the traditional pathway for bile acid synthesis (Chiang, 1998). The gene that encodes the enzyme CYP7A1 is regulated in part by a variety of small lipophilic molecules that includes steroid and thyroid hormones, cholesterol, and bile acids. CYP7A1 gene expression occurs during influx or feeding of cholesterol and is consequentially repressed by bile acids as a negative feedback mechanism. Recently, bile acid receptors have garnered attention for their role in glucose and energy homeostasis, along with regulation of inflammation (Houten et al., 2006).

Nuclear receptors as well as liver receptors play an integral role in the regulation of bile acid metabolism (Russell, 1999). Most recently the liver X receptor alpha and farnesoid X receptor (LXRα and FXR, respectively), have been implicated in the feedback loops associated with bile acid synthesis (Forman et al., 1995). Both of these receptors are abundantly expressed in the liver and when LXRα is knocked out in mice, CYP7A1 expression is suppressed in response to cholesterol feeding and furthermore results in an accumulation of cholesterol (Peet et al., 1998).

Role of bile acids in diabetes

In type-2 diabetes the host is unable to utilize insulin properly. Recently, it has been suggested that bile acid metabolism is altered in patients with type 2 diabetes and that modification of the enterohepatic production of bile acids can improve glycemic control in some patients (Prawitt et al., 2011). The Farnesoid X Receptor (FXR) is a nuclear receptor necessary in the regulation of bile acid synthesis (Stanimirov et al., 2012). Studies in FXR deficient mice have shown developing signs of insulin resistance and

consequentially, further evidence suggests that FXR agonists may reduce blood glucose levels in murine models of obesity and diabetes (Zhang et al., 2006; Cipriani et al., 2010). Downstream targets of FXR such as the hormones Fibroblast Growth Factor 15 and 19 (FGF 15 and FGF 19, respectively) have also been implicated in diabetes mellitus. It has also been reported that FGF 15 and FGF 19 are capable of suppressing gluconeogenesis (Potthoff et al., 2011). Moreover, targeting bile acid metabolism using bile acid sequestrants (i.e., colesevelam) was also found to increase glycemic control (Beysen et al., 2012). Other promising targets involved in bile acid metabolism are the glucagon-like 1 peptide (GLP-1) from intestinal L-cells through the activation of membrane receptor TGR5 in addition to tauroursodeoxycholic acid which may help improve insulin resistance by attenuating endoplasmic reticulum stress (Katsuma et al., 2005; Özcan et al., 2006).

Secondary bile acids, the microbiota, and host health

Traditionally, it has been well known that the gut microbiota has profound effects on bile acid metabolism largely through the deconjugation, dehydrogenation, and dehydroxylation of primary bile acids in the distal small intestine and colon (Ridlon et al., 2006). This facilitates the production of secondary bile acids, namely lithocholic, deoxycholic, and ursodeoxycholic acid (UDCA). Interestingly, the gut microbiota have recently been linked to regulating bile acid metabolism through interactions with specific bile acid receptors (Sayin et al., 2013a). A study in germ free mice and conventionally raised mice identified that the presence of gut microbiota upregulated the expression of FXR and its molecular targets (i.e., Fibroblast Growth Factor 15 (FGF15)), compared to germ-free mice (Ridlon et al., 2006). In contrast, there was no effect on expression of FXR

or its molecular targets in the liver. These findings suggest that the gut microbiota is capable only of regulating the activity of FXR in the ileum but not the liver. The same study identified that when FXR was knocked out in mice, FGF15 was incapable of being expressed in the ileum highlighting the need for FXR in bile acid regulation.

Ideal composition of the bile acid profile in the gastrointestinal tract is critical for maintaining intestinal health. Pilot data suggests increased secondary bile acids in the feces of healthy dogs and decreased secondary bile acids in dogs with inflammatory bowel disease (Honneffer et al., 2015b). Studies in humans have described similar findings (Weingarden et al., 2014). Table 2 summarizes studies in mammalian systems investigating the different roles of bile acids in disease. Presence of secondary bile acids (i.e., lithocholic and deoxycholic acid (DCA)) has been linked to not only maintaining a healthy gut but to carcinogenesis as well. For example, the anti-inflammatory properties of secondary bile acids were described several decades ago when it was recognized that chronic cholestasis was associated with immune suppression. This understanding led to the discovery that bile acids helped to inhibit the secretion of TNF-α, IL-1β and IL-6 in macrophages (Greve et al., 1989). The G-protein-coupled receptor TGR5 was recognized as a bile acid specific membrane receptor capable of reducing proinflammatory cytokines such as IL-1α, Il-1β, IL-6 and TNF-α. In humans, the gut microbiota produced the strongest activator responsible for activating TGR5 and its anti-inflammatory capabilities.

Alternatively, secondary bile acids have been implicated as a causative role in colorectal carcinogenesis (Nagengast et al., 1995). Studies in humans with colonic adenomas have found increased DCA compared to healthy controls. Additionally, studies

in mice that were fed DCA reported inflammation, edema, and necrosis in tissue after DCA treatment (Wargovich et al., 1983). Lithocholic acid (LCA) has been identified as a tumor promoter through inhibition of DNA polymerases (Ogawa et al., 1998). There is also evidence that LCA downregulates NF-kB (a protein complex that has been linked to cancer, inflammatory, and autoimmune diseases) in colonic cancer cells (Sun et al., 2008). This study identified that LCA as a vitamin D receptor (VDR) ligand acted synonymously to the hormonal form of vitamin D which is involved with anti-inflammatory action through VDR. Colonic cell lines were used to illustrate these effects and it was shown specifically that LCA decreased IL-8 secretion induced by IL-1β. Patients with *Clostridium difficile* infection have increased fecal primary bile acids compared to healthy donors before fecal microbial transplant (Weingarden et al., 2014). It has been hypothesized that the pro-germination properties belonging to taurocholic acid (a primary bile acid) are essential to *C. difficile* growth.

Table 2. Summary of bile acid alterations in studies on intestinal inflammation.

Source	Species	Study Population	Experimental Design	Testing Method	Alterations in Bile Acids (Results)
(Lajczak et al., 2015)	human	T_{84} colonic epithelial cells	colonic epithelial cells wounded by scratching with a pipette tip, treated with either DCA (150 µM) or ursodeoxycholic acid (100 µM)	NA	In controls, after 48 hours wounds spontaneously closed to 37% wound size of baseline values. In presence of DCA, would healing was reduced (i.e., 76% wound size). In presence of UDCA and DCA, UDCA completely prevented inhibition of wound closure by DCA. FXR mimicked DCA effects on wound healing.
(Bazin et al., 2015)	human	Crohn's disease n=25 ulcerative colitis n=27 healthy controls n=28	fecal samples from patients with IBD	liquid chromatography coupled with tandem mass spectrometry	Isomerization of LCA (reported as Iso-LCA/LCA) was decreased compared with controls (10% vs. 20%, respectively) and was lower in flare vs. remission (8.5% vs 14%, respectively).
(Gothe et al., 2014)	human	Children with Crohn's disease (n=44) Children with ulcerative colitis (n=14)	measurement of bile acid malabsorption in children with inflammatory bowel diseases	7 alpha-hydroxy-4-cholesten-3-one (C4) by HPLC	C4 concentrations increased in 23% of CD patients but only one ulcerative colitis patient. Crohn's disease patients with diarrhea had significantly higher C4 values compared to those without (76.9 vs. 30.45 ng/mL).
(Degirolamo et al., 2014)	mouse	C57BL/6J	Mice were treated with VSL#3 probiotics and feces were collected to measure bile acid metabolism and receptor signaling	liquid chromatography coupled with tandem mass spectrometry	Increased BA deconjugation and fecal excretion in VSL#3 treated mice | increased hepatic synthesis and biliary output and repression of FXR/FGF15 which can be reversed by FXR agonist.
(Dossa et al., 2016)	rat	IEC-6 cells	EGFR an FXR pathway in cell proliferation	cell culture and qPCR	TCA induces intestinal cell proliferation and in contrast DCA inhibits proliferation.
(Rau et al., 2016)	mouse	C57-BL/6J-Fue and BL6-IL10tmlCgn ($IL10^{-/-}$)	analyzed the enterohepatic regulation of FGF15-mediated pathways in 2 different IBD mouse models	qPCR, Western Blot, Elisa, HPLC-MS/MS	DSS colitis mice had increased serum FGF15, while $IL10^{-/-}$ had a trend toward decreased FGF15 serum concentrations. Downregulation of FXR mRNA expression in both models.

Table 2. Continued.

Source	Species	Study Population	Experimental Design	Testing Method	Alterations in Bile Acids (Results)
(Jahnel et al., 2014)	human	mucosal biopsy specimens from Crohn's disease patients (n=21) ulcerative colitis patients (n=14) healthy controls (n=9)	BA transporters, detoxifying systems, and nuclear receptors that regulate BA transport and detoxification were targeted and assessed	qPCR	Main ileal BA uptake transporter (i.e., apical sodium dependent bile acid transporter) was downregulated in active CD and UC and CD in remission.
(Dwyer et al., 2015)	human	T_{84} colonic epithelial cells	Investigated the effect of the FXR agonist, GW4064 on miRNA expression in colonic epithelial cells	miRNA profiling by Nanostrint Tech. and Target Scan	Found to increase expression of epithelial miR-29a-3p and reduce expression of its target PTEN, deduced possibility of FXR agonists to help preserve intestinal barrier function by inhibiting apoptosis.

Bile acid receptors and inflammation

The Farnesoid X Receptor (FXR) has been identified as a prominent nuclear receptor that controls bile acid and glucose homeostasis (Ma et al., 2006). The pregnane X receptor (PXR), also expressed in the intestine and in the liver, can trigger phase I detoxification metabolism through induction of Cytochrome P450, subfamily A (CYP3A). Lastly, VDR, is activated in the intestine by bile acids and plays a role in inhibition of bile acid synthesis by upregulating CYP3A. FXR is activated by any one of the main primary and secondary bile acids (i.e., cholic, chenodeoxycholic, lithocholic, and DCA), while VDR is primarily activated by LCA and PXR is activated by UDCA and LCA (Pols et al., 2011).

A variety of membrane receptors are also preferentially activated by the bile acid pool, namely G-protein coupled receptors (GPCR) (activated by any one of the primary and secondary bile acids), muscarinic receptors (activated by lithocholic and DCA), and formyl-peptide receptors (FPRs) (activated by DCA and chenodeoxycholic acid (CDCA)) (Raufman et al., 2002). FPR receptors can be located in neutrophils and monocytes and when activated are believed to induce cell chemotaxis and aid in the identification of damaged tissue; moreover, this likely contributes to the anti-inflammatory properties of bile acids (Chen et al., 2000).

TGR5 is a G protein-coupled receptor specific for bile acids that is present on the cell-surface (Kawamata et al., 2003). Kupffer cells have an abundant expression and presence of TGR5 among resident macrophages (Keitel et al., 2008). Furthermore, cyclic adenosine monophosphate (cAMP) has been reported to inhibit lipopolysaccharide (LPS)-

induced cytokine secretion, which may be activated by bile acids given their capability to activate TGR5 and subsequently trigger increased cAMP production in alveolar macrophages. The same study in alveolar macrophages noted that bile acids reduced phagocytic activity in these cells and inhibited LPS-induced production of pro-inflammatory cytokines (i.e., tumor necrosis factor-α (TNF-α), Interleukin (IL)-1α, IL-1β, IL-6, and IL-8), (Kawamata et al., 2003). Kuppfer cells isolated from rat show that TGR5 agonists such as oleanolic acid, tauurolithocholic acid, as well as CAMP can result in reduced expression of proinflammatory cytokines (i.e., IL-1α, IL-1β, IL-6 and TNF- α) (Keitel et al., 2008).

Historically, IBD in humans was tentatively linked to bile acid malabsorption in some individuals. A recent study in humans that examined serum and fecal samples of 42 IBD patients and 29 healthy subjects aimed to connect microbial dysbiosis, bile acid dysmetabolism, and gut inflammation in patients with IBD (Duboc et al., 2013). Fecal conjugated bile acids were significantly increased in active IBD (Standard error of the mean (SEM): ~9 and ~3%, in active IBD compared with healthy controls, respectively). In contrast, fecal and serum unconjugated secondary bile acid concentrations were significantly decreased in IBD (SEM: ~50 and ~20%, respectively) compared with healthy controls (SEM: ~90 and ~32%, respectively). The recent abstract by Honneffer et al. (2015) described a similar pattern in fecal secondary bile acids in dogs with IBD. This may indicate that a so-called bile acid dysmetabolism may be present in humans and dogs, and may deserve further investigation in canine medicine.

Bile acid sequestrants

Bile acids sequestrants may be a potential therapeutic option as they have been used to help treat hyperlidemia, bile acid malabsorption, and primary sclerosing cholangitis among many other diseases (Scaldaferri et al., 2013). Cholestyramine is a bile acid sequestrant which is capable of binding bile acids in the gastrointestinal tract to prevent reabsorption and accumulation. It is most well-known for its capacity as a strong ion exchange resin working through the exchange of chloride anions with anionic bile acids which utilizes a resin matrix. Functionally, the exchange resin consists of a quaternary ammonium group (positively charged polyatomic ions) attached to an inert styrene-divinylbenzene copolymer. Cholestyramine has been marketed under the trade names Questran, Questran Light, Cholybar, and Olestyr (Pepper, 1986).

Cholestyramine has reportedly been used in several clinical instances regarding the treatment of cyanobacterial poisoning in dogs and anecdotally in the treatment of hypercholesterolemia as well as chronic diarrhea. In one case report, a two and a half year old spayed female Miniature Australian Shepherd presented with acute onset of anorexia, vomiting, and depression (Rankin et al., 2013). Feces from the affected dog were positive for cyanobacterial biotoxin, microcystin-LA (217ppb). After 8 days of hospitalization and supportive fluid therapy, and administration of mucosal protectants, vitamins, antibiotics, and supplements, the bile acid sequestrant cholestyramine was administered orally. Rapid clinical improvement was noted. In rats, cholestyramine has been show before to bind >99% of microcystin-LR in vitro and in vivo (Dahlem et al., 1989).

Another study used cholestyramine in dogs to investigate the effect on bile acids in feces and in serum (Jansen and Zanetti, 1965). This study only reported the sum of DCA, CDCA, and CA without identifying the concentrations of each individual bile acid. Nevertheless, the study identified the cholesterol lowering property of cholestyramine as part of the serological tests it reported. Cholestyramine increased bile acid concentrations in feces in a dose dependent fashion from 1 gram per day to 6 grams per day. While malabsorption of bile acids in people is relatively well described now, little has been described before in dogs. One study in 17 dogs with chronic diarrhea measured serum concentration of 7α-hydroxy-4-cholesten-3-one (C4) in dogs as a potential target for bile acid malabsorption (Kent et al., 2016). Results from this study indicated that 3 of the 17 dogs had a C4 concentration above the range for clinically healthy dogs and were also poorly responsive to conventional therapy. Serum C4 concentrations are indicative of major enzymes in the bile acid synthesis pathway and studies in humans show good correlation with the rate of bile acid synthesis (Gälman et al., 2003).

Idiopathic bile acid diarrhea was first described in 1975 and is now recognized a common cause of chronic diarrhea in human patients (Williams et al., 1991). The pathophysiology of the disease is somewhat unclear, however, it is thought to in part involve defective reabsorption of bile acids either in the ileum or in the colon. Mechanistically, diarrhea is thought to be caused by secretion of sodium and water into the colon due to increased colonic permeability (Mekhjian et al., 1971; AMIN, 1981). Alternatively, it has been hypothesized that diarrhea could be caused by accelerated colonic transit, increased secretion of mucus, and stimulation of defecation (Sadik et al.,

2004). Cholestyramine has been effectively shown to help treat patients with idiopathic bile acid diarrhea to help ameliorate aforementioned symptoms of the disease. For instance, in an open-label study using cholestyramine in patients with bile acid diarrhea, cholestyramine was shown to significantly prolong transit in the transverse part of the colon. There was also a trend for prolonged transit in the small bowel.

The gold standard test for bile acid malabsorption has traditionally been the SeHCAT (23-seleno-25-homotaurocholic acid) test. The procedure uses a capsule containing radiolabeled SeHCAT, which is taken orally and monitors recirculation of bile acids. Patients are scanned periodically by a gamma camera in the supine position and values above 15% are considered to be normal while values less than 15% are considered to be indicative of excess bile acid loss. Testing with SeHCAT is limited because only bile acid fluctuations in the small intestine are measured (Bajor et al., 2009).

Cholestyramine is suggested as a therapeutic agent is in the treatment of diarrhea-predominant irritable bowel syndrome (IBS-D) (Camilleri, 1999). However, IBS-D is primarily associated with small bowel and proximal colonic transit and, therefore, loperamide is generally prescribed to decrease intestinal transit, enhancing intestinal water and ion absorption (VASSALLO et al., 1992). It is estimated that about one-third of patients with IBS-D have evidence of increased bile acid synthesis or excretion. Bile acid sequestrants are commonly used and efficacious in improving stool consistency in many of these cases (Camilleri et al., 2015). There is mounting evidence that supports the intervention of bile acid metabolism in patients with GI disease, however, further studies will need to fully characterize the physiological effects as well as therapeutic targets

necessary to alter the disease state.

Hypothesis and objectives

The hypotheses of this research were that the fecal metabolome undergoes continuous alterations after therapy in dogs with chronic GI disease, also that bile acids may play a role in the disease process and that there may be value in manipulating bile acid pool in some canine patients.

The objectives of this study were to:

(1) develop a GC/MS assay for the quantification and identification of fecal bile acids in dogs,

(2) measure fecal bile acids in dogs with CE in addition to monitoring their long term outcome,

(3) characterize the fecal metabolome in dogs with IBD over time,

(4) and lastly, describe the effect of cholestyramine (a bile acid sequestrant) on the fecal bile acid pool and microbiome in healthy dogs as a preliminary understanding in its potential usefulness as an alternate or adjunct therapy to dogs with chronic GI disease.

CHAPTER II

DEVELOPMENT OF A GAS CHROMATOGRAPHY/MASS SPECTROMETRY ASSAY FOR THE MEASUREMENT OF FECAL UNCONJUGATED BILE ACIDS IN DOGS

Overview

Bile acids play an important role in dietary regulation. In the gastrointestinal tract, the primary bile acids are CA and CDCA (i.e., the primary bile acids), while LCA, DCA, and UDCA are the major secondary bile acids. The small intestine has a large proportion of primary bile acids that are conjugated to taurine or glycine, while the large intestine harbors a large proportion of secondary bile acids that have been deconjugated and dehydroxylated by colonic bacteria. There is mounting evidence in humans that bile acids play a role in maintaining gastrointestinal health and bile acid dysmetabolism may play a role in GI disease. Bile acid dysmetabolism may also be present in dogs with gastrointestinal disease. Therefore, an in-house assay was developed using gas-chromatography coupled with mass spectrometry to rapidly assess fecal bile acid profiles in dogs. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were as follows for each compound: CA (3.9 and 1000 µg/mL), CDCA (6.25 and 200 µg/mL), LCA (1.9 and 500 µg/mL), DCA (31.3 and 1000 µg/mL), and UDCA (0.78 and 50 µg/mL). For intra-assay variability, the average coefficient of variation (CV%s) were: 6.0, 5.6, 7.1, 7.3, and 8.8% for CA, CDCA, LCA, DCA, and UDCA, respectively. For inter-assay variability, the average CV%s were: 8.3, 8.0, 4.8, 8.6, and 13.2% for CA,

CDCA, LCA, DCA, and UDCA, respectively. The assay was found to be both precise and reproducible for the identification and quantification of unconjugated fecal bile acids in dogs.

Introduction

Bile acids are a product of cholesterol and are essential to the process of excreting bile lipids while also aiding in the absorption of dietary lipids and fat soluble vitamins (de Aguiar Vallim et al., 2013). Bile is mainly comprised of three classes of biliary lipids: bile acids, phospholipids, and cholesterol. Bile acids are responsible for the formation of mixed and simple micelles. Micelles act preventatively in the gall bladder to inhibit cholesterol crystallization as well as gall stone formation (Di Ciaula et al., 2013). While bile acids can be protective against some of the consequential effects of cholesterol, bile itself delivers somewhere between 500-2400 mg of cholesterol per day to the intestine which is nearly five times the dietary intake of western-diets consumed by humans (Mok et al., 1979).

Once bile acids are secreted into the duodenum, they are reabsorbed and returned to the liver via portal venous circulation. During recirculation they are prepared once again to be re-secreted in to the intestine. It is generally agreed that less than 5-10% of bile acids escape reabsorption and are later eliminated into the feces (Dawson et al., 2003). This is considered a highly efficient process of enterohepatic circulation and bile acids undergo several distinct types of absorption along the gastrointestinal tract. This reabsorption means that approximately less than 10% of bile acids are a product of *de novo* hepatic synthesis. Firstly, both unconjugated and conjugated bile acids undergo passive absorption via electrochemical gradients in the jejunum, then, active transport in the distal (i.e.,

terminal) ileum via the apical sodium bile acid cotransporter (ASBT, gene name: *Slc10a2*), and finally passive absorption in the colon (Schiff et al., 1972). Bile acids undergo a complex process of reabsorption whereby the co transporter (mechanistically, sodium and membrane potential driven) moves both unconjugated and conjugated bile acids from the lumen across the apical brush border membrane. Once this has been facilitated, bile acids are then moved to the basolateral membrane before being secreted into portal circulation where they can once again be transported across the sinusoidal membrane of the hepatocyte and lastly be re-secreted, and re-conjugated to then move across the canalicular membrane into bile (Dawson, 2011).

Bile acids have been suspected to be carcinogens in the gastrointestinal tract and are thought to act through the degradation of DNA (Bernstein et al., 2005). Increased bile acids have been implicated in the pathogenesis of irritable bowel syndrome (IBS) and may contribute to intraluminal and mucosal factors that may induce critical and detrimental changes in the gut (Camilleri, 2012). Previous reports have suggested that in as many as 32% of patients with IBS-diarrhea type symptoms a bile acid malabsorption (BAM) is present and in-fact when treated with bile acid sequestrants seem to improve (Wedlake et al., 2009).

In dogs, similar disease pathologies exist for patients with chronic diarrhea. Idiopathic inflammatory bowel disease (IBD), for instance, is a gastrointestinal tract disorder of unknown cause and ill-defined pathogenesis (Jergens et al., 2003). The clinical signs are highly variable but can include vomiting, diarrhea, and weight loss. A diagnosis of canine IBD is typified by confirmed intestinal inflammation and empirical treatment

(e.g., antibiotics, food, and steroids). To elucidate the role of bile acids in canine gastrointestinal disease, the goal of this study was to develop and validate an in-house gas chromatographic/mass spectrometry assay that could readily evaluate fecal unconjugated bile acids in dogs.

Materials and methods

For the identification and quantification of unconjugated bile acids, the protocol was adapted and modified from methods previously described (Batta et al., 1999; Batta et al., 2002). Unconjugated CA, CDCA, LCA, DCA, and UDCA were all purchased in powder form from a commercial supplier (Sigma-Aldrich, St. Louis, MO). Internal standards CA-d_4 and LCA-d_4 were purchased from CDN Isotopes (Quebec, Canada). All compounds were >95% pure as judged by thin-layer chromatography. Hydrochloric acid (37% American Chemical Society reagent), hexane (for high-performance liquid-chromatography (HPLC)), 1-butanol for HPLC, and derivitization agent (Supelco's® Sylon HTP HMDS + TCMS + Pyridine, 3:1:9 Kit) was used for preparation of trimethylsilylation (TMS) ether bile acid derivatives.

Naturally voided fecal samples were collected from healthy dogs and dogs with gastrointestinal disease. Approximately 0.5 g of wet feces was aliquoted into a tube (5 mL, 57x15.3 mm, polypropylene, Sarstedt, Nümbrecht, Germany) using a spatula (Smart Spatula. USA Scientific, Ocala, FL). Fecal samples were kept frozen at -80˚ C and then lyophilized overnight (Labconco FreeZone 2.5 Plus, Kansas City, MO). Samples were then removed from the lyophilizer and pulverized and aliquoted using a spatula (Smart Spatula. USA Scientific, Ocala, FL) into a disposable glass centrifuge tube (5 mL, Kimble-

Chase, Rockwood, TN). Approximately 10-15 mg of lyophilized feces were used for downstream extraction. The amount of bile acid was later back calculated according to the original weight of the aliquot to normalize for varying starting fecal weights. A total volume of 200 µL of butanol containing the internal standards CA-d_4 and LCA-d_4 was added to each fecal sample. Twenty microliters of HCl was then added for a final volume of 220 µL and vortexed for 30 seconds. Samples were then capped and incubated at 65˚ C for 4 hours. Next, samples were evaporated under nitrogen gas until dryness at 65˚ C for approximately 25 minutes. Two-hundred microliters of derivitization agent were then added to the sample and incubated at 65˚ C for 30 minutes. Following incubation, samples were again evaporated under nitrogen gas until dryness at 65˚ C (approximately 25 minutes). Samples were then re-suspended in 200 µL of hexane, vortexed briefly then centrifuged for 10 minutes at 3200 rcf. A 100 µL aliquot was then transferred to a GC/MS vial insert (250 µL glass with polymer feet, Agilent, Santa Clara, CA) and the vial was capped for further downstream analysis.

Gas chromatography (GC) and mass spectrometry (MS) was used (6890N and 5975 inert Mass Selective Detector, Agilent, Santa Clara, CA). The instrument was equipped with an auto sampler (7683 Series, Agilent, Santa Clara, CA). A capillary column (DB-1ms Ultra Inert, Agilent, Santa Clara, CA) was used with the following dimensions: length: 30 m, diameter: 0.250 mm, film: 0.25 µm. A 20:1 split ratio was utilized after a 1 µL sample injection with an inlet temperature of 250˚ C. After injection, oven temperature was kept at 150˚ C for 1 minute, then ramped at 21˚ C per minute to a final temperature of 276˚ C then held at that temperature for 21 minutes. At 28 minutes

the oven was ramped to 325° C for 3 minutes for post run column cleaning. Helium was used as the carrier gas at an approximate flow rate of 1 mL/min varying slightly with pressure to account for retention time locking to the compound cholestane-d_4 set to elute at 11.4 minutes. Mass spectral data was analyzed using ChemStation (Agilent's Enhanced Data Analysis in MSD version D.02.002.275).

The panel for fecal unconjugated bile acids was analytically validated by determination of accuracy and reproducibility by evaluating intra- and inter-assay variability, respectively. Calibration curve recovery was calculated as observed value (µg/mL)/expected value (µg/mL) x 100%. Accuracy was evaluated by assaying 6 aliquots taken from a single fecal sample from 4 dogs on the same run/day followed by calculating the intra-assay coefficients of variation (CV = [SD/mean] x 100%). Reproducibility of the assay was determined by assaying 6 aliquots taken from a single fecal sample from 4 dogs on 6 consecutive days followed by calculating inter-assay CV. Upper limit of quantification and LLOQ were established by standard curve development that spanned the working range of the assay useful in detecting a variety of fecal bile acid concentrations from a variety of dogs that belonged to students at Texas A&M University.

Results

Each individual bile acid underwent individual extraction as per the materials and methods section. Retention time was logged and used to further develop an acquisition protocol using ChemStation by Agilent. Target ions were selected via ion chromatograms for each individual bile acid being CA, CDCA, LCA, DCA, and UDCA as follows: 253.3, 412.4, 215.3, 255.3, and 502.5, respectively. Qualifier ions along with their relative

percent response were also selected to ensure proper compound identification and were as follows for CA, CDCA, LCA, DCA, and UDCA: 410.4, 255.3, 257.3, 256.3, and 503.5, respectively. Their relative response ratios for qualifier ions belonging to CA, CDCA, LCA, DCA, and UDCA were: 66.3%, 45.7%, 88.1%, 21.4%, and 46.5%, respectively.

Deuterated internal standards were used to confirm identity of primary and secondary bile acids while also serving as a control for extraction efficiency during the extraction and butyl esterification process of fecal bile acids. D4-CA was used as a surrogate internal standard marker for all primary bile acids (i.e., CA and CDCA), while D4-LCA was used as a surrogate internal standard marker for all secondary bile acids (i.e., LCA, DCA, and UDCA). The target ion, qualifier ion, and relative response ratio for D4-CA was 257.3, 414.5, and 77.7%, respectively. The target ion, qualifier ion, and relative response ratio for D4-LCA was 219.3, 261.3, and 85.8%, respectively. These results are available in Table 3.

Intra-assay validation and precision testing for fecal samples F1-F4 yielded an average CV of 7% for all compounds and all samples analyzed. More specifically, for intra-assay variability, the average CV%s were: 6.0, 5.6, 7.1, 7.3, and 8.8% for CA, CDCA, LCA, DCA, and UDCA, respectively. Inter-assay validation and reproducibility testing for samples F5-F8 yielded an average CV of 8.62% for all compounds and all samples analyzed. More specifically, for inter-assay variability, the average CV%s were: 8.3, 8.0, 4.8, 8.6, and 13.2% for CA, CDCA, LCA, DCA, and UDCA, respectively. These results are summarized in Table 4. The LLOQ and ULOQ for

Table 3. Ions profile for GC/MS Single Ion Monitoring

	RT (min)	Target Ion	Qualifier Ion	Relative Response %
D4-CA-n-butyl ester	25.56	257.30	414.50	77.70
CA-n-butyl ester	25.71	253.30	410.40	66.30
CDCA-n-butyl ester	25.38	412.40	255.30	45.70
D4-LCA-n-butyl ester	22.49	219.30	261.30	85.80
LCA-n-butyl ester	22.48	215.30	257.30	88.10
DCA-n-butyl ester	24.56	255.30	256.30	21.40
UDCA-n-butyl ester	26.82	502.50	503.50	46.50

Table 4. Precision and reproducibility of the GC/MS assay for unconjugated bile acids.

Fecal Sample	mean (µg/mg) ± standard deviation (µg/mg)					coefficiant of variation (%)				
Intra-Assay Variability	CA	CDCA	LCA	DCA	UDCA	CA	CDCA	LCA	DCA	UDCA
F1	3.01±0.464	0.90±0.093	0.61±0.084	5.69±0.699	0.03±0.004	15.43	10.32	13.71	12.30	16.01
F2	0.34±0.005	0.24±0.010	0.13±0.005	0.41±0.021	0.26±0.014	1.60	4.21	3.64	5.17	5.44
F3	2.63±0.105	0.45±0.017	0.20±0.016	4.57±0.284	0.07±0.003	3.99	3.83	8.02	6.20	4.92
F4	0.45±0.014	0.23±0.009	0.63±0.020	4.40±0.254	0.18±0.016	3.19	4.08	3.12	5.78	9.01
Inter-Assay Variability										
F5	0.22±0.012	0.23±0.024	1.28±0.040	3.51±0.440	0.05±0.007	5.36	10.42	3.13	12.55	15.14
F6	3.10±0.214	1.75±0.085	2.62±0.188	13.36±0.800	0.22±0.024	6.90	4.83	7.18	5.99	10.58
F7	0.21±0.020	0.26±0.012	0.51±0.021	2.42±0.204	0.02±0.003	11.22	12.05	5.13	7.44	12.23
F8	0.19±0.021	0.20±0.024	1.58±0.081	7.89±0.587	0.02±0.002	9.77	4.87	4.12	8.42	14.96

each compound were: CA (3.9 and 1000 μg/mL), CDCA (6.25 and 200 μg/mL), LCA (1.9 and 500 μg/mL), DCA (31.3 and 1000 μg/mL), and UDCA (0.78 and 50 μg/mL). OE ratios for all compounds were between 77% and 123%.

Discussion

Gas chromatography coupled with mass spectrometry or variants of compound detection such as flame ionization are commonly used methods for the identification and quantification of fecal bile acids in stool and serum (Batta et al., 1999). High-performance liquid chromatography (HPLC) coupled with mass spectrometry is also used (Kakiyama et al., 2014). The objective of this study was to develop and analytically validate an assay for the measurement and identification of unconjugated fecal bile acids in canine feces. Results from this study indicate that the adapted method from Batta et al. was sufficient in extracting and measuring fecal bile acids in dogs. The technique itself is was found to be highly efficient since there was no step requiring prior isolation and separation of bile acids from feces, a process that can significantly slow sample data acquisition times.

Several parameters were examined to confirm the precision and reproducibility of the assay. This assay does not use a fecal extraction process producing a liquid extract that can then be normalized by volume for sample injection. Instead, feces undergo direct isolation and derivatization prior to being reconstituted in a hexane solution to be analyzed. For the intra-assay variability a CV < 16.5% was achieved among all compounds (i.e., CA, CDCA, LCA, DCA, and UDCA) for the four fecal samples measured. For the inter-assay variability a CV < 15.5% was achieved among all compounds (i.e., CA, CDCA, LCA, DCA, and UDCA). While this is slightly above

standards in the field (Bansal and DeStefano, 2007) that support an acceptable CV of < 15%, this is likely due to heterogeneity in a fecal compound matrix and not a fluctuation in the machine. Furthermore, observed to expected ratios for calibration curves were found to be acceptable for bile acids measured.

A complete and thorough stability study is needed to fully assess the integrity of fecal bile acids during storage. Samples that contained fecal bile acids at amounts well within the calibration curve for each respective bile acid CA, CDCA, LCA, DCA, and UDCA were used for this pilot study. In some instances, only one compound was used for stability assessment since the others did not fall well within the calibration curve for the other compounds. However, pilot data suggests that once feces are lyophilized and stored at -80° C, the results are reproducible over two months later. Table 5 shows that a CV of < 11% was found for each compound. This suggests that fecal samples, once stored, are stable at -80° C conditions. Further studies are needed to assess the stability of compounds under room temperature and refrigerator conditions. Additionally, this study did not evaluate to see whether or not there was an effect of freezing and thawing samples before bile acid extraction. Inherently, due to protocol limitations, all samples must be frozen at least once to undergo lyophilization, which makes it difficult to run truly fresh fecal samples. Some anecdotal evidence and studies suggest that feces be frozen immediately as bile acids do not remain stable at room temperature (Camilleri et al., 2009).

Drawbacks of this assay are that it cannot be used for analysis of conjugated fecal bile acids (Batta et al., 1999). This could be especially limiting when needing to assess pathological and bile acid functional conditions in germ-free mice (Sayin et al., 2013a).

Table 5. Stability of fecal bile acids after 2 months storage at -80° C.

Fecal Sample	Time point	Compound	ug/mg	CV%
F1	Baseline	cholic acid	2.9353	1.18
F1	2 months	cholic acid	2.8666	
F2	Baseline	chenodeoxycholic acid	1.6640	9.37
F2	2 months	chenodeoxycholic acid	1.3788	
F3	Baseline	lithocholic acid	1.4160	6.87
F3	2 months	lithocholic acid	1.2338	
F4	Baseline	deoxycholic acid	9.3218	10.53
F4	2 months	deoxycholic acid	7.5456	
F5	Baseline	ursodeoxycholic acid	0.0151	1.17
F5	2 months	ursodeoxycholic acid	0.0147	

CHAPTER III

MEASUREMENT OF FECAL BILE ACIDS IN DOGS WITH CHRONIC ENTEROPATHY

Overview

Chronic enteropathy in dogs is poorly defined and diagnostic tools are limited. It is often a diagnosis of exclusion. Nevertheless, genetic predisposition, nutritional influence, and intestinal microbiota and their metabolites are likely to be involved. Mounting evidence in humans suggests that bile acid dysmetabolism may play a role in a variety of chronic GI diseases (i.e., Crohn's disease, bile acid malabsorption, IBS-D, IBD, and Ulcerative Colitis). In fact, it is thought that approximately one third of patients with IBS-D have a bile acid dysmetabolism. These are generally diagnosed by either bile acid malabsorption tests (i.e., SeHCAT or C4) and typified by a presence of increased primary bile acids in large intestine. Therefore, the purpose of this study was to characterize the fecal unconjugated bile acid profile in dogs with CE.

Cholic acid was significantly increased in dogs with CE compared to healthy dogs ($p=0.0425$). LCA and DCA were significantly decreased in dogs with CE ($p=0.0006$ and 0.0098, respectively). A reference interval (i.e., 54-96%) was established for the percent of secondary bile acids in the feces of healthy dogs. Twenty out of 34 patients with CE had a fecal bile acid profile outside of the healthy reference interval. Further studies are needed to understand how this may effect therapeutic strategies in treating CE in dogs.

Introduction

Canine CE in dogs is characterized by a poorly defined pathogenesis and highly variable clinical signs (Jergens et al., 2003). Typical presentation of CE in dogs may include watery diarrhea, vomiting, and anorexia (Schreiner et al., 2008). While pathogenesis of CE in dogs is not well understood, it is generally thought to involve microbial dysbiosis, functional alterations of microbiota (dysmetabolism), an underlying host genetic susceptibility, and environmental factors (Simpson and Jergens, 2011; Minamoto et al., 2014b). Ultimately, the interworking between intestinal homeostasis (e.g., impact of dysbiosis and dysmetabolism) and clinical signs in patients with CE still lacks understanding.

Mounting evidence suggests that the microbiota and their metabolites play a regulatory role throughout the intestine (Suchodolski et al., 2012a; Suchodolski et al., 2012b). Furthermore, the microbiota is responsible for multiple metabolic functions, one of which includes metabolism and regulation of bile acids (Sayin et al., 2013b). Bile acid production first occurs in the liver from cholesterol where the primarily bile acids CA and chenodeoxycholic are synthesized. Primary bile acids are then conjugated with either taurine or glycine in the liver and stored in the gall bladder (Ridlon et al., 2016). Upon release of bile acids into the small intestine following a meal, their role includes nutrient digestion and solubilizing vitamins (Dawson and Karpen, 2015). While the majority of enterohepatic absorption and recirculation of bile acids occurs at the terminal ileum, approximately 5% are excreted into the colon and deconjugated and dehydroxylated by colonic bacteria forming the secondary bile acids DCA, LCA, and UDCA (Hill and

Drasar, 1968; Ridlon et al., 2016). Thus, microbial dysbiosis identified in patients with CE may negatively affect gut homeostasis through bile acid dysmetabolism.

Studies in humans have suggested a role for bile acid dysmetabolism in gut inflammation and in inflammatory bowel diseases (Kruis et al., 1986; Duboc et al., 2013). Patients with IBD have a decreased percentage of secondary bile acids in feces. Duboc et al. (2013) also described a concurrent dysbiosis in patients with IBD and have shown *in vitro* experiments, which reflect decreased IL-8 response in the presence of DCA and LCA. A collection of work in humans with diarrhea predominant irritable bowel syndrome (IBS-D) has identified an increase in primary bile acids which affects approximately one third of patients and symptoms can often times be managed by bile acid sequestrants (e.g., cholestyramine) (Wedlake et al., 2009; Duboc et al., 2012; Shin et al., 2013). Despite a growing body of information describing fecal bile acids in humans with chronic GI disease, studies investigating the role of bile acids in the feces of dogs is limited.

Increased serum C4 has been reported in a subset of dogs with chronic diarrhea (Kent et al., 2016). Untargeted metabolomics conducted in feces of canine patients with inflammatory bowel disease have also described bile acid dysmetabolism (Honneffer et al., 2015b). Therefore, the objective of this study was measure fecal bile acids in healthy dogs and dogs with CE.

Materials and methods

Fecal samples from healthy dogs (n=24) were collected from pets belonging to personnel at Texas A&M University's College of Veterinary Medicine and immediately stored at -80° C until further analysis. Dogs with CE (n=34) were prospectively enrolled

from multiple centers including the Evidensia Specialist Animal Hospital in Helsingborg, Sweden, Iowa State University (ISU), Colorado State University (CSU) , San Diego Specialty Hospital (SDSH), and private clinics around the United States which submitted fecal samples to the Inflammatory Bowel Disease study at Texas A&M University (http://vetmed.tamu.edu/gilab/research/canine-ibd). Similarly, fecal samples were collected and immediately stored at -80° C until further analysis. Patients that were enrolled into the CE group had gastrointestinal clinical signs lasting three weeks or more in duration. A subset of those patients were followed-up over the period of three and 8 weeks after initial diagnosis and then over a year later. For the majority of patients, standard therapy involved sequential implementation of a food trial, antimicrobial intervention, followed by an immunosuppressive drug if they failed to respond to either. Dietary intervention generally consisted of a hydrolyzed diet or a diet consisting of a novel protein source as is typical with a workup for dogs with chronic signs of gastrointestinal disease.

Unconjugated fecal bile acids were measured using a gas chromatographer coupled with a mass spectrometer targeting CA, CDCA, LCA, DCA, and UDCA as described previously. Bile acid data was described in µg/mg of lyophilized fecal content in addition to being expressed as a percent of total fUBA measured. Furthermore, the unconjugated primary bile acids CA and CDCA were combined to represent total primary fecal unconjugated bile acids (fUBA) measured and LCA, DCA, and UDCA were combined to represent total secondary fUBA. Data was tested for normality using a Shaprio Wilk's test and followed by a Mann Whitney test or Friedman's test where appropriate followed by

Dunn's post testing to identify significant differences between groups. A reference interval for healthy dogs was constructed by first removing outliers by means of identifying those values that were 1.5 times the length of the box away from either the lower or the upper quartiles. The 97.5% percentile was then calculated to represent serve as a standard set of values for healthy dogs. A p-value of less than 0.05 was considered significant.

Results

The following represent the changes in fecal bile acids when measuring concentration alone. The primary bile acid CA was significantly increased in patients with CE (med [min-max]: 1.301 µg/mg [0.018-47.340 µg/mg]) compared with healthy dogs (med [min-max]: 0.234 µg/mg [0.068-12.040 µg/mg]; p=0.0425). There was no significant difference in concentration of the primary bile acid CDCA between patients with CE (med [min-max]: 0.320 µg/mg [0.000-2.959 µg/mg]) compared to healthy dogs (med [min-max]: 0.157 µg/mg [0.080-1.301 µg/mg]; p=0.1874). The secondary bile acid, LCA, was significantly decreased in patients with CE (med [min-max]: 0.033 µg/mg [0.000-5.318 µg/mg]) compared with healthy dogs (med [min-max]: 0.8182 µg/mg [0.000-2.388 µg/mg]; p=0.0006). The secondary bile acid, DCA, was significantly decreased in patients with CE (med [min-max]: 0.300 µg/mg [0.177-12.020 µg/mg]) compared with healthy dogs (med [min-max]: 1.931 µg/mg [0.202-9.101 µg/mg]; p=0.0098). There was no significant difference in concentration of the secondary bile acid, UDCA, between patients with CE (med [min-max]: 0.010 µg/mg [0.000-3.777 µg/mg]) compared with healthy dogs (med [min-max]: 0.022 µg/mg [0.002-0.330 µg/mg]; p=0.3556). There was a trend in the total amount of primary bile acids between patients

with CE (med [min-max]: 1.873 μg/mg [0.018-50.010 μg/mg]) compared with healthy dogs (med [min-max]: 0.415 μg/mg [0.147-13.340 μg/mg]; p=0.0614), however this comparison did not reach statistical significance. The total amount of secondary bile acids, however, were significantly decreased in patients with CE (med [min-max]: 0.571 μg/mg [0.177-15.670 μg/mg]) compared with healthy dogs (med [min-max]: 3.290 μg/mg [0.213-11.570 μg/mg]; p=0.0118). There was no significant difference in total fUBA between patients with CE (med [min-max]: 4.458 μg/mg [0.336-50.280 μg/mg]) compared to healthy dogs (med [min-max]: 3.964 μg/mg [1.507-13.930 μg/mg]; p=0.3724). These results are shown in Figure 1**.**

The following represent the changes in fecal bile acids as a percent of total fUBA measured. The fecal bile acid profile in healthy dogs for CA, CDCA, LCA, DCA, and UDCA was (median % values expressed): 5.54%, 5.08%, 21.21%, 61.54%, and 0.51%, respectively. The fecal bile acid profile in patients with CE for CA, CDCA, LCA, DCA, and UDCA was (median % values expressed) 49.49%, 10.55%, 0.30%, 14.96%, and 0.23%, respectively. The percent of secondary fUBA were significantly decreased in canine patients with CE (median [min-max]: 25.21% [0.53-99.62%]) compared with healthy dogs (median [min-max]: 87.98% [4.24-96.50%]; p=0.0161). These results are shown in Figure 2.

In a subset of canine patients with CE (n=16), fecal samples were collected over several time points (i.e., baseline or first enrollment, 1 month later, then 2-3 months later). The same parameters were measured as described earlier for the entire group where just a baseline fecal samples in diseased canine patients were compared to healthy dogs.

Lithocholic acid significantly increased in canine patients with CE from baseline (median [min-max]: 0.075 µg/mg [0.000-1.071 µg/mg]) to 1 month (median [min-max]: 0.802 µg/mg [0.000-1.615 µg/mg]) to 2-3 months (median [min-max]: 1.169 µg/mg [0.000-2.839 µg/mg]) post therapeutic intervention (p-value for Friedman's test and Dunn's post-test (baseline vs 1 month and baseline vs 2-3 months): 0.0005 and <0.05, respectively). Deoxycholic acid significantly increased in canine patients with CE from baseline (median [min-max]: 0.375 µg/mg [0.204-3.707 µg/mg]) to 1 month (median [min-max]: 3.587 µg/mg [0.183-11.25 µg/mg]) to 2-3 months (median [min-max]: 5.352 µg/mg [0.192-16.590 µg/mg]) post therapeutic intervention (p-value for Friedman's test and Dunn's post-test (baseline vs 1 month and baseline vs 2-3 months)): 0.0004 and <0.05, respectively. As such, secondary fUBA significantly increased overall from baseline (median [min-max]: 0.5707 µg/mg [0.215-6.680 µg/mg]) compared with 2-3 months (median [min-max]: 0.571 µg/mg [0.227-18.080 µg/mg]) post therapeutic intervention (p-value for Friedman's test and Dunn's post-test (baseline vs 2-3 months): 0.0034 and <0.05, respectively). Total fUBA increased from baseline (median [min-max]: 3.401 µg/mg [0.336-50.280 µg/mg]) compared with 2-3 months (median [min-max]: 8.780 µg/mg [2.386-35.680 µg/mg]) post therapeutic intervention (p-value for Friedman's test and Dunn's post-test (baseline vs 2-3 months): 0.0010 and <0.05, respectively). These results are shown in Figure 3.

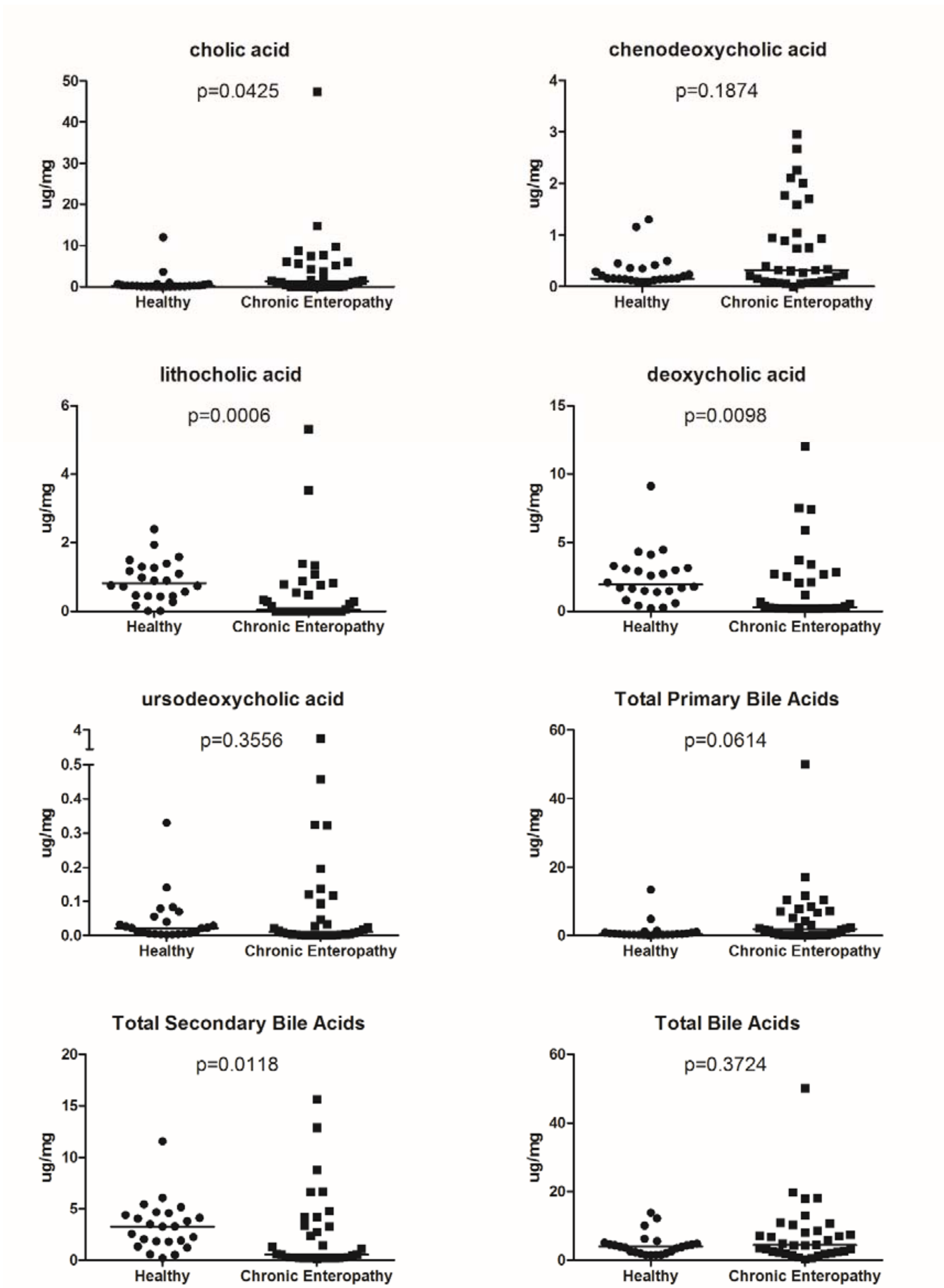

Figure 1. Fecal bile acids in µg/mg and corresponding parameters as measured by GC/MS in healthy dogs and dogs with CE.

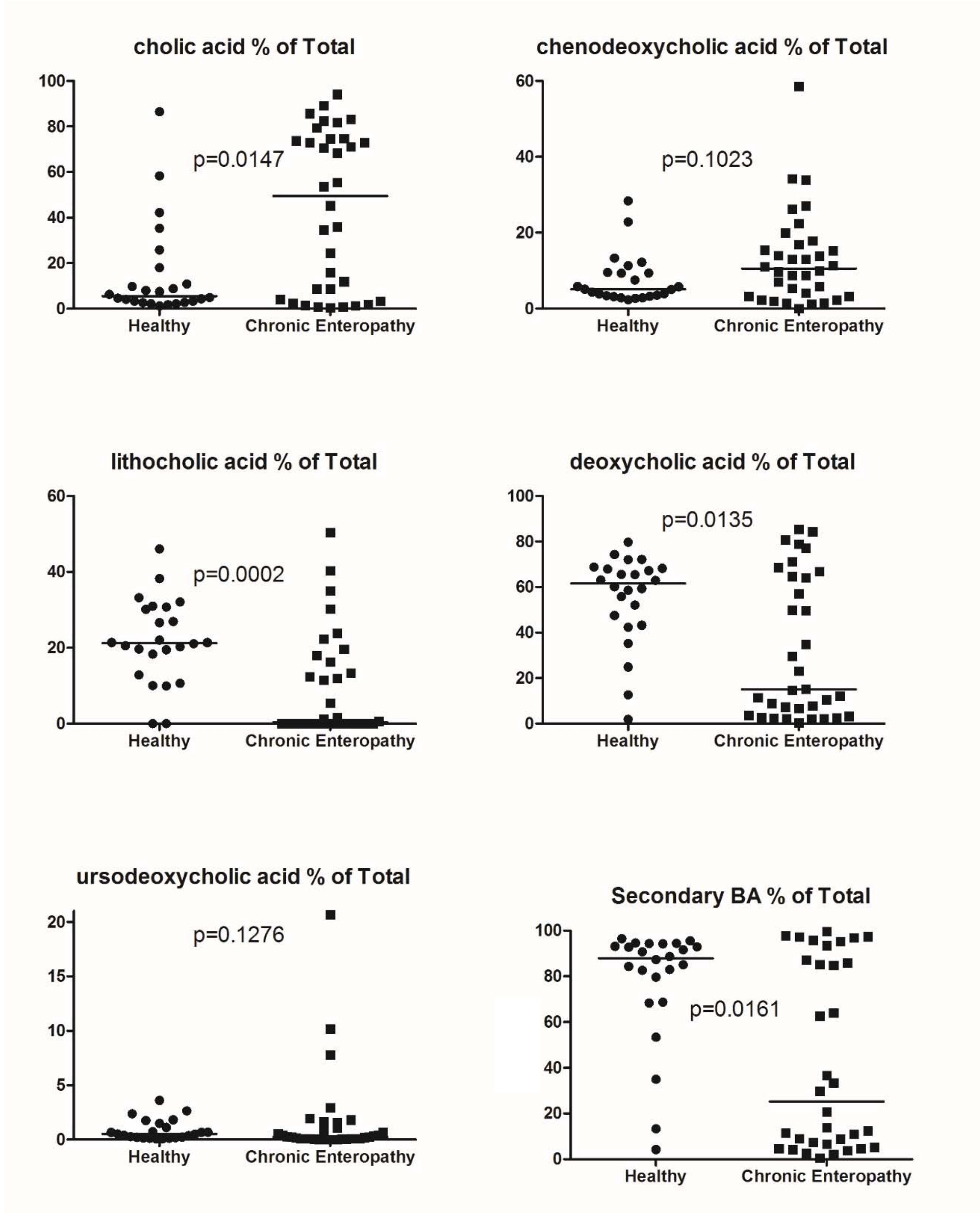

Figure 2. Percent of fecal bile acids and secondary bile acids as a percent of total in healthy dogs and dogs with CE.

The percent of secondary fUBA measured were significantly increased overall from baseline (median [min-max]: 28.65% [0.53-99.62%]) compared with 2-3 months (median [min-max]: 94.53% [1.12-99.27%]) post therapeutic intervention (p-value for Friedman's test and Dunn's post-test (baseline vs 2-3 months): 0.0183 and <0.05, respectively). Chenodeoxycholic acid expressed as a percent of total fUBA measured significantly decreased overall from baseline (median [min-max]: 9.23% [0.00-58.59%]) compared with 2-3 months (median [min-max]: 2.93% [0.45-15.39%]) post therapeutic intervention (p-value for Friedman's test and Dunn's post-test (baseline vs 2-3 months): 0.0152 and <0.05, respectively). There were no other significant changes in the percent of total fUBA measured over time. These results are shown in Figure 4.

Discussion

In this study, fecal samples from 24 healthy dogs and 34 dogs with CE were evaluated to describe their bile acid profile. In terms of total concentration, the secondary bile acids LCA and DCA were both significantly decreased in the CE group. The primary bile acid CA was significantly increased in dogs with CE. Anecdotal evidence suggests that fecal bile acid profiles are highly variable and therefore, it is also useful to express and analyze these data in percent of total bile acids measured or by a simple ratio of primary to secondary bile acids (Kamano et al., 1999; Duboc et al., 2013). When utilizing this strategy, the same significant findings were noted for CA, CDCA, and LCA. Dogs with CE had significantly decreased secondary fUBA when expressed as a percent of total fUBA compared to healthy dogs.

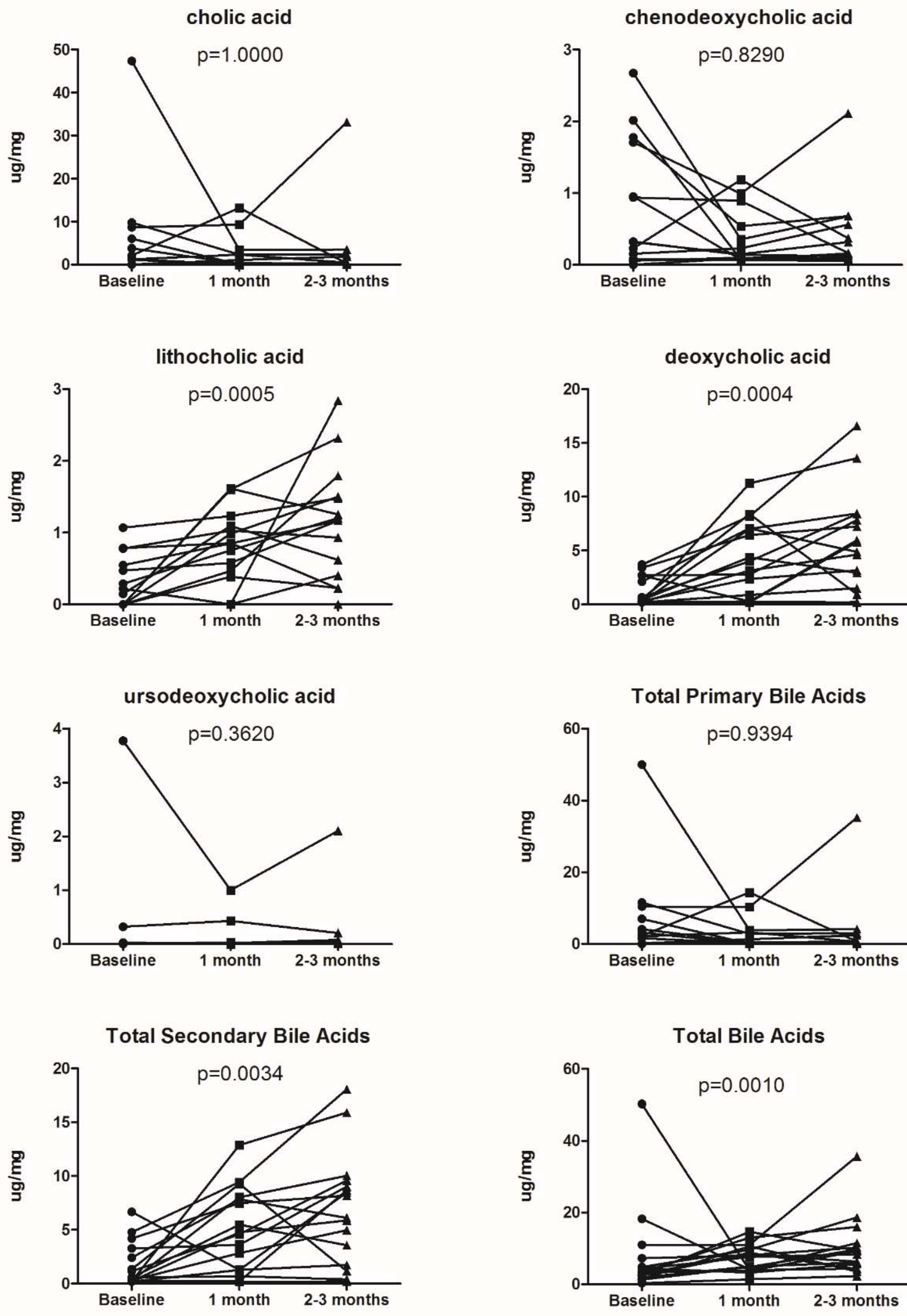

Figure 3. Fecal bile acids in µg/mg and corresponding parameters as measured by GC/MS in dogs with CE from baseline to 2-3 months post therapy.

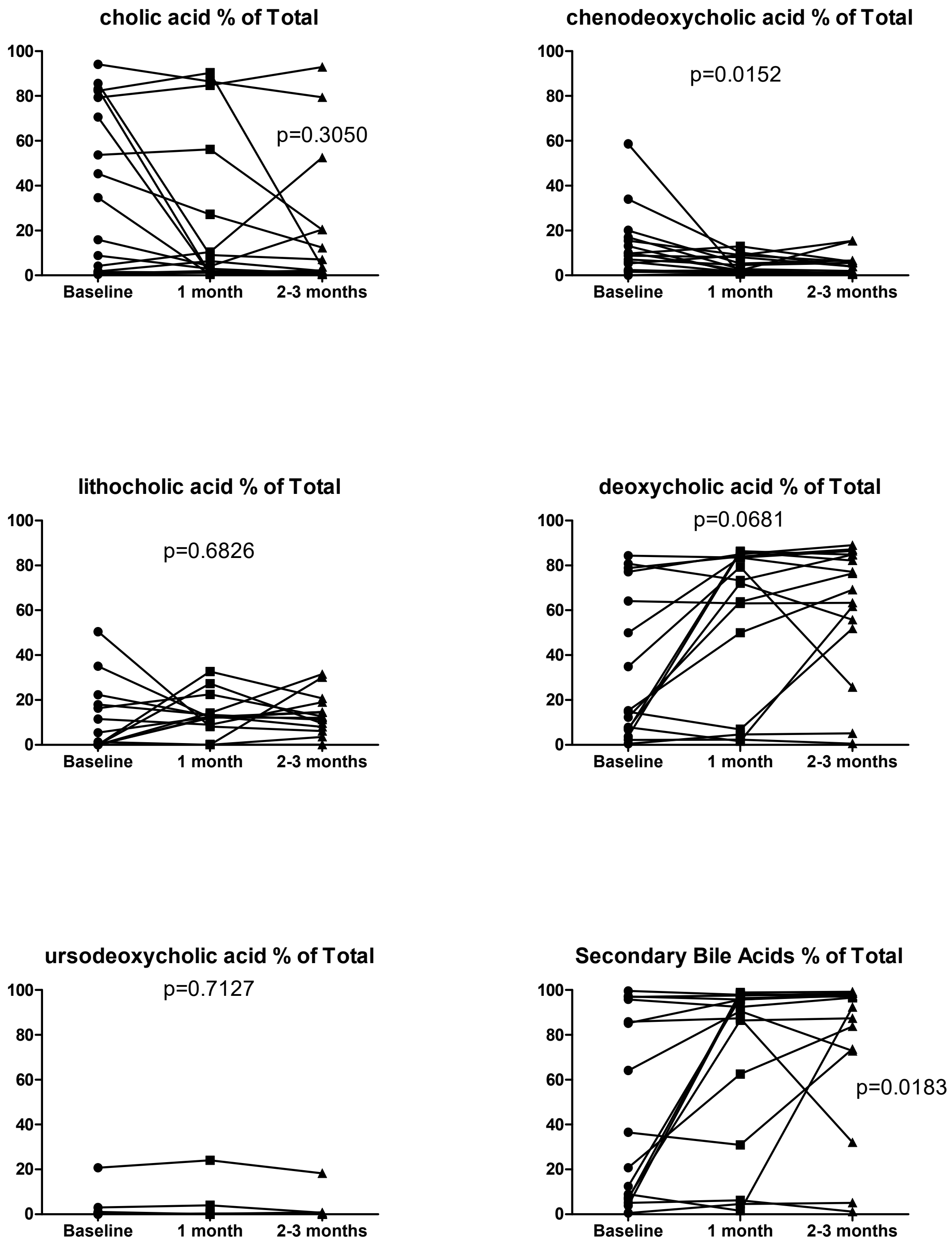

Figure 4. Bile acids as percent of total fUBA in dogs with CE from baseline to 2-3 months post therapy.

To capture global changes in fecal bile acid profiles a reference interval was calculated for the percent of secondary fUBA (Figure 5). A proposed reference interval for healthy dogs was constructed by first removing outliers by means of identifying those values that were 1.5 times the length of the box away from either the lower or the upper quartiles. The 97.5% percentile was then calculated to represent serve as a standard set of values for healthy dogs. The upper and lower limits of the reference interval for healthy dogs pertaining to the percent of secondary fUBA were 53.57% and 96.47%, respectively.

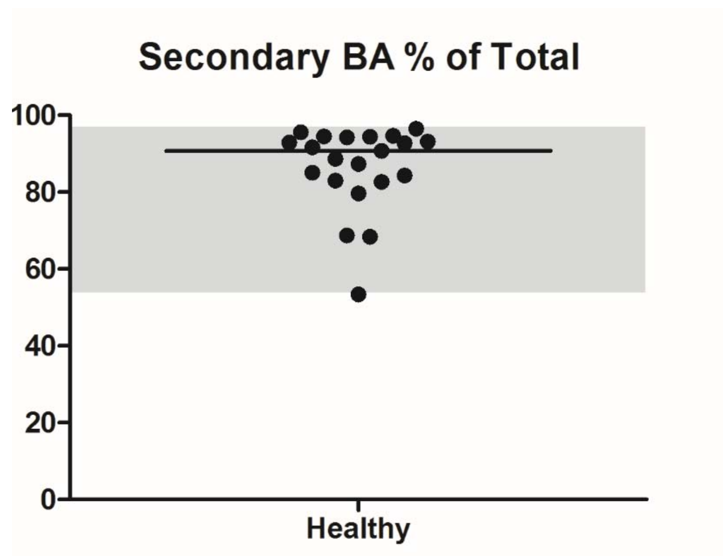

Figure 5. Reference interval for the percent of secondary fUBA in healthy dogs.

A subset of dogs with CE were followed up over the period of eight weeks. These dogs were treated with immunosuppressive therapy after baseline fecal samples were

collected. Concentrations of LCA and DCA significantly increased over time. When measured as a percent of total fUBA, DCA significantly increased over time, while CDCA decreased over time. The percent of secondary fUBA significantly increased over time and began to more closely resemble healthy dog profiles. The majority of these patients with CE were within the reference interval proposed for healthy dogs by the end of eight weeks.

Fecal bile acid dysmetabolism in canine patients with CE is somewhat of a renewed and understudied area in internal medicine. Anecdotal reports of clinicians using cholestyramine in the past to treat chronic diarrhea are available, but there is little literature to support the evidentiary need or usefulness. In humans, bile acid malabsorption can be prevalent on its own or as part of a differential diagnosis as it is coupled with several diseases (Klimova et al., 2015). It is often characterized by a defect in the enterohepatic circulation of bile acids where increased bile acids are not reabsorbed in the ileum and reach the colon. Another commonly used marker for bile acid malabsorption is the serum C4 test (7α-hydroxycholest-4-en-3-one). In patients with Crohn's disease this is typically used as a surrogate marker of bile acid malabsorption and it has been reported that up to 50% of adult patients with Crohn's disease have bile acid malabsorption (Lenicek et al., 2011). In humans, bile acid malabsorption occurs in patients with ileal resection, can be idiopathic or have a "Type 2" malabsorption with unknown etiology, or be secondary to various other primary diseases (e.g., chronic pancreatitis, celiac disease, small intestinal bacterial overgrowth and radiation enteritis) (Gothe et al., 2014).

In dogs with chronic diarrhea, clinical signs and therapeutic options can manifest themselves in numerous ways. Inflammatory bowel disease is typically recognized as a disease of exclusionary efforts coupled with finally immunosuppressive drugs as a final course of therapeutics. Generally, however, clinicians must also provide evidence of histological evidence to support an etiology with inflammation present (Jergens et al., 2003; Allenspach et al., 2007). Common therapeutic approaches include dietary trials as well as antimicrobial use to empirically treat disease and characterize it (Westermarck et al., 2005). Unfortunately, finding a causative agent to chronic diarrhea in dogs is not always simple nor is definitive. Given the prevalence of bile acid malabsorption in human chronic diarrhea patients, Kent et al. hypothesized that bile acid malabsorption may be a relevant disorder in dogs (Kent et al., 2016). Their study analyzed C4 concentrations in 17 dogs with chronic diarrhea and 20 healthy control dogs, however, they found no significant difference between control dogs (serum C4 median [min-max]: 80.9 nmol/l [15.1-180.1 nmol/l]) and dogs with chronic diarrhea (serum C4 median [min-max]: 59.9 nmol/l [21.3-518.6]; $p=0.8$) when evaluating C4 as a parameter for bile acid malabsorption. Three of the patients in this study had C4 concentrations above their reference interval and were noted to only partially respond to varying types of therapy. Our study indicated that almost 60% of dogs with CE had secondary bile acids as a percent of total that were below the reference interval established for healthy dogs. In the future this reference interval may serve as a useful tool in diagnosing bile acid dysmetabolism.

As mentioned previously, many of these patients were treated with immunosuppressive drugs. Collagenous colitis in humans is characterized by

inflammation in the large bowel with long-standing watery diarrhea and budesonide has been suggested as efficacious in its treatment (Bajor et al., 2006). The proposed mechanism of action supported by corticosteroids used in animal models is the upregulation of the Apical Sodium-dependent Bile Acid Transporter (ASBT), the main transporter responsible for uptake of bile acids in the terminal ileum (Nowicki et al., 1997). Corticosteroids may stimulate the reuptake of bile acids by promoting ASBT in the ileum and could improve gastrointestinal health in dogs with IBD that have downregulated ASBT gene expression.

Limitations of this study are that the assay used to investigate the fecal bile acid profile was unable to measure sulfated bile acids and conjugated bile acids. Also, studies in humans with IBD (Duboc et al., 2012; Duboc et al., 2013) have suggested that these may also play a role in the inflammatory loop. Furthermore, serum bile acids may further provide a systemic view into the regulation of bile acids. Currently efforts are underway to adapt this assay to measure bile acids in serum. One of the advantages to the assay used in this study is the ability to acquire results in only a couple days. This assay could be used as a first line of diagnostics when investigating chronic diarrhea in dogs as to assess the likelihood of bile acid dysmetabolism being a contributing factor in disease. If a fecal sample were to arrive in the morning at the laboratory, it can then be frozen at -80° C for several hours, lyophilized overnight, and then extracted and analyzed the following day. Furthermore, fecal samples offer a non-invasive diagnostic approach contributing to a positive experience for the patient. In addition, this assay can be performed on a simple gas chromatographer coupled with a mass spectrometer which comes at a fraction of the

price of a high mass accuracy instrument, making it a potentially affordable option for clients. Further, research is needed to correlate clinical outcome with fecal bile acid profile and the potential usefulness of sequestrants or predictive power of bile acid profiles and the use of corticosteroids.

CHAPTER IV

LONGITUDINAL CHARACTERIZATION OF UNTARGETED FECAL METABOLOMICS IN DOGS WITH INFLAMMATORY BOWEL DISEASE

Overview

The fecal metabolomic profile in dogs with IBD over time has not been previously described. IBD in dogs follows a general diagnostic workflow, where patients are sequentially trialed on novel diets, antimicrobials, and, when these fail, immunosuppressive therapy. Furthermore, upon biopsy, these patients must show evidence of intestinal inflammation. The aim of this study was to globally assess metabolites over time. In this study, fecal samples from patients were collected at baseline (prior to immunosuppressive therapy), 3 weeks, 8 weeks, and more than one year later. Gas chromatography coupled with mass spectrometry was used to identify and measure metabolites. Principal component analysis revealed separation between healthy dogs and dogs with IBD at baseline. Univariate analysis revealed that the most significantly altered metabolites were those belonging to amino acids isoleucine, proline, valine, leucine, threonine, serine, glycine, aspartic acid, oxoproline, alanine, and methionine ($q<0.05$ for all). Untargeted metabolomics shows differences between healthy dogs and dogs with IBD up to a year after initial diagnosis.

Introduction

Idiopathic inflammatory bowel disease (IBD) is thought to involve the inappropriate activation of the mucosal immune system with inflammatory cell infiltrates in the intestine (Allenspach et al., 2007). Studies have shown that there is also a microbial dysbiosis present in dogs with IBD along with disturbances in the serum metabolite profile (Suchodolski et al., 2012a; Suchodolski et al., 2012b; Minamoto et al., 2014b).

Untargeted metabolomics can describe biological systems and allows understanding of the relationship between the host and the microbiota along the gastrointestinal tract (Guard and Suchodolski, 2016). The benefit of untargeted metabolomics is the ability to, without bias, sample hundreds of metabolites and then to systematically create networks of pathways and associations to further better understand an active disease or healthy state (Xia et al., 2015). It is clear that there still remains much to be delineated from biological systems regarding the pathogenesis of IBD in dogs. Furthermore, up until this point in time, few studies have looked at fecal metabolites using an untargeted metabolomics platform (Honneffer et al., 2015a). Some studies have described serological and urinary metabolomics changes, but these may inherently miss the most active biological sample of disease and healthy states in the gastrointestinal tract of dogs (Minamoto et al., 2014b; Guard et al., 2015).

To date, there is no literature that describes the fecal metabolome in dogs with IBD over time after initial diagnosis. Therefore, the aims of this study were to evaluate canine patients with IBD at initial treatment, 3 weeks post treatment, 8 weeks post treatment, and then more than one year after initial enrollment. Gastrointestinal function was assessed by

gas chromatography coupled with mass spectrometry. The goal of this study was to better describe and understand the pathogenesis and therapeutic effects on the fecal metabolome of dogs treated for IBD.

Materials and methods

Fecal samples from healthy dogs (n=13) were collected from pets belonging to personnel at Texas A&M University's College of Veterinary Medicine and immediately stored at -80° C until further analysis. Dogs with inflammatory bowel disease (IBD) (n=9) were prospectively enrolled from Iowa State University (ISU). Similarly, fecal samples were collected and immediately stored at -80° C until further analysis. Patients were diagnosed with IBD by a board certified veterinary internist based on the World Small Animal Veterinary Association (WSAVA) criteria: chronic GI signs (>3 weeks), histopathologic evidence of mucosal infiltration with inflammatory cells, inability to document other causes of GI inflammation, inadequate response to dietary, antibiotic, and anthelmintic therapies, and clinical response to anti-inflammatory or immunosuppressive agents. Fecal samples were collected from these dogs at baseline enrollment, 3 weeks post therapeutic intervention, 8 weeks post therapeutic intervention, and then a subset of samples (n=5) were followed up more than a year later.

Fecal samples were lyophilized and approximately 10 mg was sent to the West Coast Metabolomics Center (WCMC) at University of California at Davis (http://metabolomics.ucdavis.edu/). Samples were analyzed on a gas chromatography/mass spectrometry platform.

Statistical analysis was carried out using MetaboAnalyst 3.0. Peak intensity tables were uploaded and underwent autoscaling for normalization. MetaboAnalyst 3.0 was used for multivariate analysis and data reduction. JMP Pro 12 (Cary, NC, USA) was used to test for normality using the Shaprio-Wilks test, and to test between multiple time points using the Kruskal-Wallis test with blocking where appropriate to account for repeated measures. The Benjamini and Hochberg False Discovery Rate was used to adjust for multiple comparisons.

Results

Of the 664 metabolites identified, 233 metabolites were named compounds.

Figure 6 is a PCA score plot of all unnamed and named metabolites in the feces of healthy dogs and dogs with IBD at baseline, 3 weeks, 8 weeks, and more than 1 year later. Ellipses represent the 95% confidence interval of metabolite profiles for each group. While none of these groups have clear separation from one another, it can be appreciated that dogs with IBD at baseline, 3 weeks, and 8 weeks do not share considerable overlap with healthy dogs. More than 1 year later after therapy several dogs with IBD still remain outside of the 95% confidence interval of healthy dogs.

A heatmap of all named and unnamed compounds is represented in Figure 7. More than 1 year later after immunosuppressive treatment dogs with IBD still had concentrations of compounds that were unlike those of healthy dogs.

For univariate analysis a Kruskal-Wallis test was used to compare all named compounds for each group in all combinations (i.e., healthy vs. baseline vs. 3 week vs. 8 week vs. >1 year later [LT]). Q-values represent adjusted p-values based on the Benjamini

and Hochberg False Discovery Rate. Eight-five named compounds were significantly different between groups after adjusting for multiple comparisons. These results are displayed in Table 6.

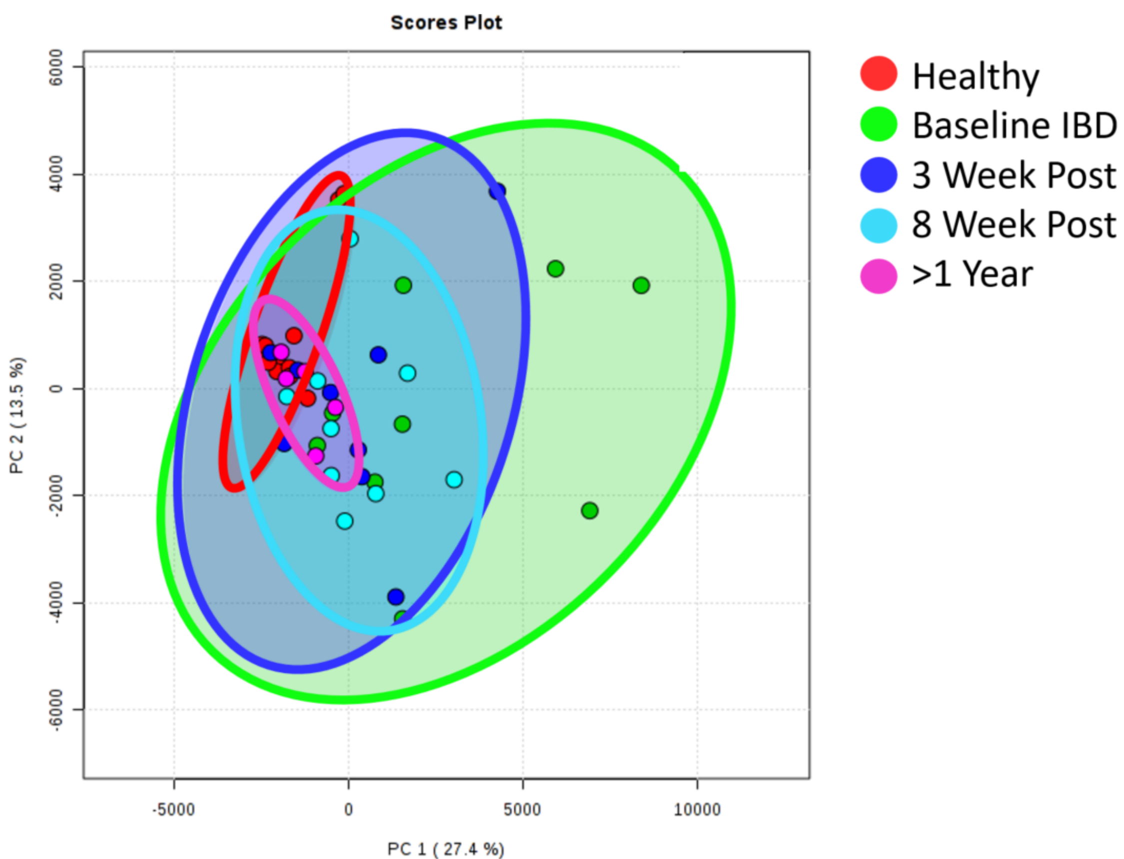

Figure 6. Principal component analysis of all metabolites and patient groups. Shaded areas of color indicate 95% confidence intervals.

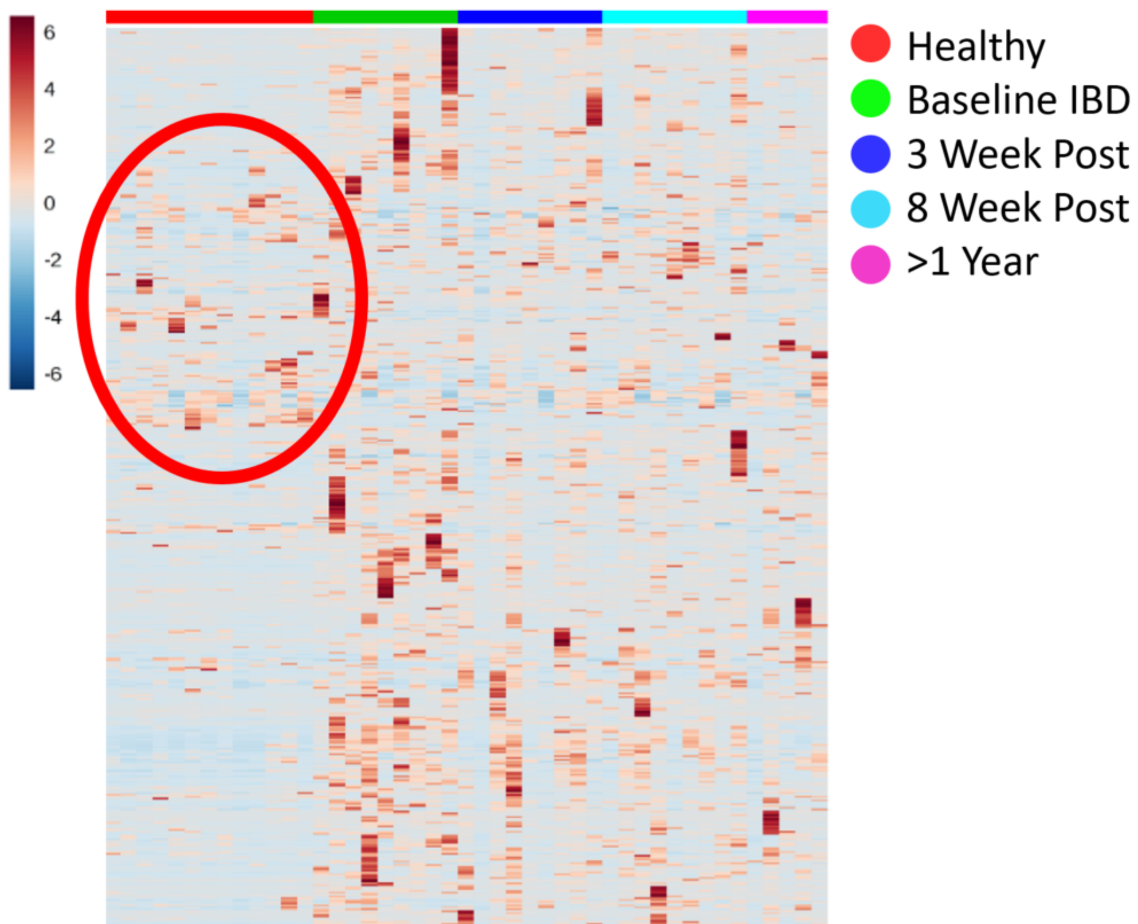

Figure 7. Heatmap of all named and unnamed metabolites in healthy dogs and dogs with IBD over time.

Table 6. Named compounds identified by untargeted metabolomics approach.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
isoleucine	26577(10376-289686)	556836(294264-1690615)	438367(67537-924792)	494757(79849-958678)	90876(36000-336552)	<0.0001	0.0008
N-acetyl-D-mannosamine	882(331-2818)	31739(3431-164573)	15297(1684-44299)	13339(3996-48955)	1757(1163-30923)	<0.0001	0.0008
proline	23221(11811-142383)	515475(47021-698587)	128358(51736-280586)	184146(24832-547090)	40940(19356-189587)	<0.0001	0.0008
glycerol	7539(5223-29787)	64110(32140-1765705)	65627(5708-219343)	72492(30732-470719)	39131(10634-47787)	<0.0001	0.0013
glycerol-3-galactoside	413(343-879)	3827(1037-53291)	2581(379-8372)	2441(916-64972)	1181(264-2117)	<0.0001	0.0015
valine	55326(20761-536562)	749451(337020-2792415)	461472(80038-895556)	611083(78449-992207)	164748(75384-441410)	<0.0001	0.0016
leucine	43494(13811-609130)	1038002(396641-2712966)	494919(109327-1506572)	812199(60812-1202834)	153784(59713-490046)	<0.0001	0.0016
threonine	7027(2033-17178)	82789(5234-400823)	78011(16232-194723)	59227(5690-147818)	14148(8082-57445)	0.0001	0.0017
serine	5405(3326-14758)	70278(11063-486844)	55806(6596-256332)	88199(4086-182978)	17150(7164-71159)	0.0001	0.0018
glycine	11080(5897-120523)	172487(29837-501579)	51108(18225-130575)	50104(19538-242928)	33255(11443-45668)	0.0001	0.0018
aspartic acid	2928(1326-5528)	35358(7619-279780)	22165(2730-97491)	17802(5776-68706)	12267(2900-30772)	0.0001	0.0018
hypoxanthine	955(444-2940)	22179(1342-80813)	16713(1073-82680)	24983(1997-42927)	2497(849-8800)	0.0001	0.0019
phosphate	1568(120-3107)	7192(2589-31210)	3107(829-10316)	4371(1392-11848)	1453(356-3351)	0.0001	0.0020
hexuronic acid	682(226-3622)	15788(747-244698)	3231(863-24216)	2108(1374-11672)	6028(1708-26564)	0.0001	0.0020
oxoproline	11680(5196-44065)	162102(34840-748606)	59130(12016-245911)	83169(11113-161594)	96628(13565-278660)	0.0001	0.0022
alanine	208236(48686-522481)	774432(390159-2900922)	918172(153011-2129205)	1036280(214667-2886082)	450399(187458-948840)	0.0002	0.0025
methionine	2196(795-5921)	27886(3286-49761)	33553(843-158744)	55474(3170-114884)	10590(6382-62943)	0.0002	0.0026
ethanolamine	8733(1657-26138)	44208(31857-214050)	42835(5301-103047)	36105(16667-379372)	20975(5473-93690)	0.0002	0.0026
4-hydroxybenzoate	16925(7637-29168)	6127(1194-48606)	2758(1220-11802)	4395(1519-12754)	2617(990-3841)	0.0002	0.0026
1-monostearin	618(55-1482)	3100(1240-22869)	1984(412-3983)	1257(282-13565)	1604(1422-5013)	0.0002	0.0027
lauric acid	5560(1206-124088)	34502(3692-3481516)	207554(6922-2069588)	203923(10968-873995)	59206(5864-152745)	0.0003	0.0028
2-ketoisocaproic acid	2791(1430-4146)	6611(3283-13668)	5025(3097-13537)	3802(2288-9867)	3801(2585-4849)	0.0003	0.0028
glutamic acid	12600(1932-36918)	87004(18902-671376)	38378(9905-195461)	51121(7453-226163)	28380(15696-36007)	0.0003	0.0035
myo-inositol	725(151-1394)	31877(463-151492)	3854(477-5752)	5274(432-9667)	780(149-182031)	0.0005	0.0045

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
glycyl tyrosine	348(133-1080)	2482(389-5428)	591(311-5720)	1382(304-3565)	631(251-1724)	0.0005	0.0046
galacturonic acid	460(148-4876)	11424(576-204182)	7153(366-74290)	1960(570-13181)	5299(1578-29560)	0.0006	0.0051
xanthine	897(193-10486)	3665(474-43306)	15814(436-72799)	15028(4569-44507)	6756(1379-32620)	0.0006	0.0051
glucose	1373(433-26551)	43808(879-897934)	39501(1210-1258868)	56316(7990-1278392)	22022(1394-392146)	0.0006	0.0052
glyceric acid	1393(557-5357)	4375(3323-21877)	5700(846-14547)	2415(1330-41876)	4108(1527-20551)	0.0007	0.0052
3-ureidopropionate	380(168-2338)	1536(378-29342)	1952(686-19985)	2530(1439-5968)	1696(1106-3021)	0.0007	0.0052
fructose	619(238-2354)	10814(611-70881)	2350(179-45190)	5699(1636-20497)	1127(455-11651)	0.0007	0.0055
tocopherol gamma-	3259(1288-6006)	3226(679-43695)	9970(5341-27474)	13945(2675-30244)	14056(6223-39758)	0.0008	0.0056
ribose	4279(1477-49192)	18391(5613-552964)	73667(1836-361182)	43664(25332-96132)	29162(4163-55537)	0.0008	0.0057
stearic acid	223504(103989-354253)	977125(322242-4372844)	659264(193132-2455396)	566598(200772-1292419)	306946(233655-861578)	0.0008	0.0057
indole-3-lactate	53140(10285-100744)	4920(857-185932)	4092(652-40125)	5636(521-11746)	6704(255-14597)	0.0009	0.0057
6-deoxyglucose	2906(1828-30658)	27467(5401-632255)	75744(5177-490158)	43034(16408-290371)	25606(5869-102650)	0.0009	0.0057
arachidonic acid	4875(481-24715)	145437(8608-608539)	23434(2421-308858)	49092(4581-610110)	23732(18841-42727)	0.0009	0.0057
cystine	120(81-845)	1672(278-9927)	408(95-2248)	355(143-7192)	513(177-1509)	0.0010	0.0063
arachidic acid	3807(857-6403)	14601(2943-255769)	9415(3422-56311)	6735(1533-16836)	5001(1049-6960)	0.0012	0.0069
fucose	7380(2795-37415)	52545(5814-895532)	42144(10371-1002140)	66594(13939-329459)	7615(3863-1262224)	0.0013	0.0073
N-acetyl-D-galactosamine	1241(631-3121)	25145(293-86721)	5887(520-178798)	15116(5987-130321)	2228(910-385457)	0.0013	0.0075
dehydroabietic acid	727(315-1328)	1344(972-4654)	1144(373-6460)	628(204-1813)	429(370-692)	0.0015	0.0084
phenylalanine	45415(2748-375944)	262770(106143-756259)	265733(29745-617906)	281273(77957-575192)	92060(58615-219988)	0.0016	0.0084
tryptophan	33488(2989-164489)	308541(44962-389516)	101110(22429-547341)	156554(20369-587750)	165374(33302-264007)	0.0016	0.0084
isomaltose	390(229-870)	1250(394-16385)	649(254-2984)	1241(429-7275)	866(284-42612)	0.0018	0.0093
caprylic acid	995(740-1623)	1427(1127-2030)	1576(1094-2947)	1331(902-2024)	1183(991-1614)	0.0027	0.0134
ribonic acid	179(91-798)	977(187-2834)	744(141-1596)	340(110-1620)	944(293-1077)	0.0027	0.0134
aminomalonate	577(297-5082)	7323(1104-40505)	1670(270-4745)	2229(270-7073)	2061(502-3139)	0.0029	0.0141

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
maltose	4086(1260-14014)	5672(3213-92323)	36684(852-305114)	18781(2004-103462)	27706(10763-368735)	0.0033	0.0156
urocanic acid	317(112-653)	1828(191-5828)	1957(142-9255)	2149(388-18966)	566(344-2457)	0.0035	0.0164
pseudo uridine	1598(330-2846)	3202(398-7836)	4391(1056-8884)	3224(2031-12055)	1816(465-4129)	0.0037	0.0167
3-(4-hydroxyphenyl)propionic acid	27330(12628-95206)	4909(193-94230)	17485(932-30945)	22353(915-57023)	11254(621-12763)	0.0038	0.0170
nicotinic acid	8187(1182-22197)	12122(1352-40303)	23604(8009-71569)	43627(12742-65764)	17631(4723-44494)	0.0044	0.0190
inositol-4-monophosphate	179(66-469)	740(274-1233)	302(58-3865)	207(127-1282)	206(92-676)	0.0044	0.0190
xylulose NIST	258(89-12652)	1737(443-140913)	2212(581-41182)	3373(958-5791)	3458(584-6148)	0.0046	0.0195
sinapinic acid	554(107-5910)	534(216-1453)	284(118-1170)	224(61-347)	251(109-274)	0.0050	0.0206
nonadecanoic acid	962(425-1889)	2520(861-11053)	1963(896-21054)	1579(874-42875)	1524(1027-3390)	0.0053	0.0217
gluconic acid	102(47-407)	270(92-6789)	216(89-540)	260(101-13408)	181(113-335)	0.0055	0.0219
pinitol	140(81-654)	1596(166-24757)	272(76-9589)	185(115-1137)	2683(136-6475)	0.0057	0.0225
lignoceric acid	708(252-1646)	3147(639-18549)	1111(440-5123)	1350(614-2244)	1114(682-1319)	0.0059	0.0228
lyxitol	1115(428-2883)	10974(648-37592)	4168(619-10370)	3131(1403-18653)	5431(875-9730)	0.0060	0.0228
tyramine	69361(1580-314004)	533616(63017-2792734)	286305(29660-1521298)	290170(23469-660553)	99311(4259-326182)	0.0061	0.0228
isoheptadecanoic acid NIST	1684(628-6892)	3801(2235-12719)	3190(494-7580)	4280(2218-9118)	1539(533-9439)	0.0064	0.0237
beta-gentiobiose	580(276-5671)	4366(735-104767)	1141(292-34083)	1174(648-34232)	1541(549-36953)	0.0067	0.0245
pentitol	224(152-500)	729(133-1801)	470(189-2404)	623(282-914)	690(432-1947)	0.0070	0.0249
methionine sulfoxide	8442(4675-93493)	61977(19978-154439)	49847(5055-83688)	35710(5353-47437)	11242(4504-43917)	0.0071	0.0249
adenosine	1633(293-5364)	441(205-8151)	461(81-14435)	363(167-1194)	979(282-1413)	0.0074	0.0258
oxamic acid	198(111-445)	901(307-1858)	286(165-2547)	300(115-1220)	477(181-694)	0.0078	0.0268
glucoheptulose	348(224-1311)	1537(506-11886)	471(133-1732)	470(155-1122)	371(212-2059)	0.0080	0.0268
malic acid	147(98-307)	772(61-3472)	830(60-3004)	349(78-558)	430(176-1986)	0.0081	0.0268
2-hydroxyglutaric acid	320(117-565)	1207(115-6574)	509(192-2042)	581(247-43173)	554(309-820)	0.0102	0.0326
threonic acid	150(62-322)	790(99-1718)	334(108-1145)	248(99-567)	429(91-3052)	0.0102	0.0326
tagatose	135(73-703)	678(157-5063)	380(91-1720)	283(155-2210)	180(98-645)	0.0103	0.0326

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
lysine	24523(5879-90749)	121540(26306-461633)	152916(13443-778380)	146398(7737-957242)	41522(4552-268191)	0.0104	0.0326
asparagine	1026(625-5414)	8328(1185-29660)	1623(562-7690)	2443(624-13117)	3141(1073-4127)	0.0119	0.0370
hexitol	241(111-446)	1053(349-1833)	328(67-3686)	260(117-3527)	229(163-815)	0.0121	0.0372
diglycerol	2192(514-7187)	7350(2205-24931)	6018(246-17619)	1526(506-6493)	3743(837-9240)	0.0123	0.0372
5,6-dihydrouracil	225(157-3126)	972(211-6333)	285(134-1520)	308(113-2962)	1857(338-2699)	0.0134	0.0399
n-acetyl-d-hexosamine	700(231-1877)	1581(316-4482)	1382(391-2286)	1045(719-5229)	998(294-2506)	0.0135	0.0400
tocopherol delta- NIST	1089(625-2109)	1026(434-13065)	5363(919-12271)	3820(993-17238)	2145(1464-9718)	0.0138	0.0402
catechol	812(93-2846)	162(77-1914)	134(67-639)	545(81-1877)	150(87-409)	0.0142	0.0409
3,4-dihydroxyphenylacetic acid	1599(593-4633)	582(319-2067)	512(101-5080)	443(165-1931)	398(157-2496)	0.0149	0.0420
3,4-dihydroxyhydrocinnamic acid NIST	221409(80063-677635)	30154(1756-355869)	80861(1137-273783)	36676(4273-481152)	64810(9744-235721)	0.0150	0.0420
indole-3-acetate	8205(4345-14470)	3371(335-20169)	2492(664-4459)	3483(1119-10497)	2531(736-9412)	0.0152	0.0422
isothreonic acid	232(67-1218)	957(306-6941)	949(224-3263)	465(121-741)	424(280-3836)	0.0165	0.0454
dihydrocholesterol	3340(91-16341)	7696(5181-14043)	4055(211-7939)	2918(311-8502)	3376(1351-5031)	0.0189	0.0512
myristic acid	2205(291-6739)	6148(1652-630293)	5101(1436-207152)	9743(1021-114200)	2821(2011-18287)	0.0196	0.0526
trans-4-hydroxyproline	4894(1463-102989)	12260(3496-99723)	6137(723-10146)	3409(1109-16460)	7362(1248-26102)	0.0203	0.0537
pipecolinic acid	9241(5077-20941)	3851(718-31419)	4491(1478-16133)	7968(1659-16159)	3671(2736-23968)	0.0215	0.0559
pyruvic acid	951(617-3313)	1495(533-3768)	1131(570-14870)	2864(1385-4288)	2122(484-5185)	0.0216	0.0559
alpha-ketoglutarate	103(64-188)	179(82-923)	173(70-1819)	197(101-410)	164(125-352)	0.0240	0.0615
sorbitol	1236(521-103007)	5976(1819-85961)	4027(1015-152927)	4237(532-14196)	6884(770-19606)	0.0260	0.0658
allantoic acid	144(57-3731)	1543(140-4438)	502(112-20140)	346(161-13606)	338(97-15528)	0.0273	0.0683
glycyl-proline	2573(650-21760)	19938(1580-99872)	10607(1518-33310)	11310(2222-36831)	6786(1344-13944)	0.0293	0.0720
homocystine	579(124-1315)	1829(523-6761)	1012(203-6055)	1151(943-2243)	2818(323-9392)	0.0294	0.0720
adenine	1971(613-5329)	11827(1257-154122)	4694(1157-38419)	4756(476-76981)	2302(486-6297)	0.0309	0.0751
uric acid	497(99-3729)	1294(397-15419)	1497(254-6040)	2746(472-21520)	882(472-4603)	0.0323	0.0775
homoserine	1760(524-5279)	3716(1434-386718)	3157(1463-11620)	4063(940-71895)	7478(1943-11920)	0.0338	0.0798

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
cysteine	1061(477-6222)	7854(355-31103)	1786(733-7382)	1639(417-8775)	5225(1208-11107)	0.0339	0.0798
orotic acid	119(41-297)	410(131-2249)	280(53-4711)	219(55-1734)	179(83-260)	0.0356	0.0826
pelargonic acid	10763(7384-15788)	10561(5685-16297)	9726(4348-13112)	7061(4336-11873)	7320(6645-8963)	0.0358	0.0826
glycerol-alpha-phosphate	552(123-3542)	2049(451-11468)	735(161-2724)	324(128-2943)	1222(407-2095)	0.0389	0.0889
phenylpyruvate	658(251-4696)	4116(458-93952)	2562(1069-14066)	3944(1021-29587)	2026(631-11970)	0.0401	0.0907
octadecylglycerol	7015(1838-50345)	22552(4660-73404)	11363(746-36579)	27007(9116-42427)	10906(7242-13237)	0.0415	0.0930
citrulline	831(376-2446)	1586(866-15996)	1408(404-13847)	1998(1022-7261)	3006(179-5507)	0.0433	0.0961
tyrosine	90206(8682-639148)	375490(9478-595405)	278472(58750-1117954)	416541(50748-798207)	183471(126642-613622)	0.0451	0.0992
2-methylglyceric acid NIST	126(64-4996)	5562(93-45059)	589(77-1733)	192(99-2227)	2155(75-19369)	0.0458	0.0992
squalene	867(346-2503)	1688(1095-4182)	1306(791-6627)	1597(582-2813)	1366(788-2019)	0.0460	0.0992
succinic acid	633(361-246026)	251359(371-2131387)	14708(830-62713)	1332(968-44182)	79604(971-935926)	0.0465	0.0994
hydroquinone	1826(882-6201)	1519(406-5350)	1007(294-2320)	1704(666-3705)	751(441-1817)	0.0474	0.1005
behenic acid	8412(1162-74752)	13862(9932-184154)	12544(998-58346)	17673(9448-35544)	15293(8314-54846)	0.0487	0.1022
uridine	919(309-2124)	2478(856-21609)	1597(475-7200)	1098(508-5346)	1301(538-2467)	0.0512	0.1059
capric acid	562(108-860)	737(492-5580)	862(495-9912)	797(185-2432)	528(321-1522)	0.0514	0.1059
thymine	8572(1066-25719)	8099(1212-46784)	21682(1870-42343)	30348(10109-61035)	10790(5469-26657)	0.0523	0.1068
guanine	656(177-1369)	1112(291-8060)	845(194-5783)	1083(231-1463)	527(170-690)	0.0544	0.1101
docosahexaenoic acid	2731(1188-17090)	15522(1519-101773)	11159(1071-182212)	8699(2939-356759)	7892(2197-17284)	0.0570	0.1144
hexadecylglycerol NIST	2312(562-10838)	6802(1391-23311)	6379(603-7524)	6353(2372-9078)	4281(1469-12380)	0.0592	0.1179
3-phenyllactic acid	761(166-7193)	6070(259-190888)	3351(1019-21682)	5279(1324-45729)	2826(389-18694)	0.0600	0.1184
2-monopalmitin	1375(803-10336)	5504(1042-24422)	1997(1084-27083)	2722(805-47042)	3700(2431-9271)	0.0607	0.1184
alanine-alanine	8598(1738-31045)	30954(13689-95906)	15925(1258-121588)	12939(899-117776)	25104(15419-33897)	0.0610	0.1184
2-hydroxyhexanoic acid	3303(282-17248)	26496(837-118992)	6736(2142-12327)	12544(2067-43134)	5712(614-26052)	0.0615	0.1184
galactinol	202(115-473)	351(160-26777)	262(131-1097)	278(128-1853)	330(254-9217)	0.0627	0.1197
uracil	52469(1829-116654)	33484(1693-131318)	75439(4988-327791)	61895(41193-232327)	23492(8693-80296)	0.0650	0.1231

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
heptadecanoic acid	7231(1083-21615)	21830(4014-89154)	12385(2640-17983)	7544(948-19582)	4759(2605-29480)	0.0656	0.1233
parabanic acid NIST	987(833-5629)	2945(1230-5334)	2000(725-6473)	2056(1005-4184)	3226(892-4903)	0.0682	0.1272
palmitic acid	55348(26867-86999)	311370(41365-859769)	157830(23974-646985)	117410(32479-363361)	95953(34089-173961)	0.0724	0.1339
conduritol-beta-epoxide	182(76-868)	1077(139-14224)	226(69-2487)	174(84-18049)	435(129-1168)	0.0753	0.1382
lactic acid	3621(1815-759342)	150283(5940-1911888)	39431(4403-384602)	14429(2413-1253987)	47534(2086-791246)	0.0790	0.1438
pentadecanoic acid	12887(2780-72216)	35899(9780-59969)	31302(2151-42439)	29796(10968-60885)	8108(5544-43266)	0.0801	0.1447
benzoic acid	4823(1241-11550)	2907(1586-11394)	2897(905-5075)	4004(1868-7058)	3200(1633-5620)	0.0811	0.1454
maltotriose	103(53-963)	123(10-10663)	232(81-1487)	485(99-2732)	480(141-12341)	0.0858	0.1526
4-hydroxyphenylacetic acid	34153(19306-175023)	23973(432-128726)	19461(900-120375)	19597(1911-80201)	8122(2047-20551)	0.0874	0.1543
fumaric acid	595(456-7608)	1629(818-3550)	749(619-5836)	1296(529-6171)	1258(264-2952)	0.0885	0.1550
lactamide	124(84-432)	464(88-9514)	155(79-647)	123(96-1260)	270(157-438)	0.0905	0.1573
guanosine	218(70-385)	762(99-5500)	216(50-8578)	173(84-1183)	450(203-548)	0.0911	0.1573
3-hydroxypalmitic acid	2407(553-9762)	1536(235-4617)	1150(97-14567)	1367(186-4145)	791(194-5854)	0.0938	0.1606
ferulic acid	332(201-1870)	819(224-14534)	184(104-1157)	321(122-791)	264(74-458)	0.0945	0.1606
trehalose	936(234-7861)	3344(651-18886)	6108(378-67691)	4375(533-18067)	25343(163-79178)	0.0957	0.1616
maleimide	897(513-1755)	2298(575-8173)	1576(786-4768)	1102(727-4672)	1326(641-4119)	0.1020	0.1709
UDP-glucuronic acid	1691(531-5580)	6595(1613-25425)	2476(260-4419)	1995(772-4994)	2556(164-4877)	0.1056	0.1749
xylitol	352(180-6807)	1615(476-16179)	717(305-4115)	757(314-1529)	694(189-3838)	0.1058	0.1749
erythritol	323(111-11143)	6300(186-81480)	606(212-8446)	637(321-5912)	5085(165-18796)	0.1087	0.1783
ornithine	13523(5455-46997)	38334(7382-193909)	19505(1109-144493)	44221(7440-167807)	30026(8451-75091)	0.1118	0.1821
lithocholic acid	4950(142-19980)	283(112-23542)	10860(241-42445)	5033(1065-25907)	3085(136-31867)	0.1138	0.1841
kynurenic acid	413(80-3678)	1740(214-646033)	835(68-30041)	1281(131-12673)	875(145-40649)	0.1180	0.1895
sucrose	221(38-3197)	671(168-9943)	252(46-2991)	327(118-6792)	291(186-1762)	0.1187	0.1895
shikimic acid	1203(364-6416)	2575(876-4511)	1567(573-5153)	1332(969-2982)	1402(202-3322)	0.1259	0.1996
xylose	14438(3729-1122928)	47923(6712-4196861)	52045(18292-537964)	115290(18607-223497)	125649(11094-215332)	0.1298	0.2044

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
isopentadecanoic acid	32048(4977-174366)	52160(15672-128146)	49218(1761-142854)	53098(10527-154577)	13936(11693-43465)	0.1325	0.2062
linolenic acid	10465(1504-48697)	59097(4579-272928)	5217(527-413705)	21940(806-151720)	4999(1210-26144)	0.1327	0.2062
lactitol	230(57-2947)	1220(268-7637)	668(92-3495)	609(208-4362)	2067(130-31104)	0.1354	0.2090
3,4-dihydroxycinnamic acid	588(228-1980)	918(365-15809)	468(156-1172)	364(162-755)	525(159-913)	0.1369	0.2099
4-hydroxybutyric acid	902(237-1484)	1846(169-8253)	1368(443-2822)	1603(460-3735)	776(159-1481)	0.1435	0.2174
malonic acid	206(68-567)	401(132-1246)	165(52-1679)	129(67-663)	239(114-934)	0.1437	0.2174
2'-deoxyguanosine	208(80-759)	1177(127-3711)	536(111-7882)	426(70-1931)	278(162-1036)	0.1458	0.2192
1,5-anhydroglucitol	440(108-15640)	4550(209-23703)	1222(286-11246)	1055(156-9916)	1955(265-87571)	0.1477	0.2207
1-monopalmitin	1654(434-16253)	8544(1542-67121)	2576(1141-57106)	3258(382-23995)	2933(2004-12950)	0.1550	0.2300
norvaline	6691(736-109845)	2761(526-78353)	18704(1962-81718)	30154(871-85722)	13450(10948-44730)	0.1662	0.2452
p-hydroxylphenyllactic acid	388(116-2610)	579(326-61612)	722(249-9777)	914(343-8940)	1154(239-3545)	0.1923	0.2817
3-aminoisobutyric acid	1804(1042-10023)	13673(715-27112)	5508(813-40305)	6070(564-11009)	2224(1216-6673)	0.2055	0.2993
2,6-diaminopimelic acid	645(371-933)	792(283-2009)	437(90-1318)	403(190-1855)	321(174-820)	0.2109	0.3052
butane-2,3-diol NIST	2412(885-11694)	5770(586-148809)	5700(1203-40742)	7994(1960-106684)	8086(374-512201)	0.2145	0.3079
cis-gondoic acid	365(149-524)	742(160-33863)	753(97-28818)	471(146-9141)	500(217-1663)	0.2171	0.3079
4-aminobutyric acid	1721(324-4741)	19960(163-462782)	6402(336-132005)	4989(738-27162)	14473(609-49059)	0.2178	0.3079
1-monoolein	16510(6765-97660)	17268(4206-356627)	8145(1375-47160)	11273(1444-675160)	26760(21535-479094)	0.2180	0.3079
deoxycholic acid	45851(4504-451181)	6761(536-419961)	13244(187-969129)	127946(2868-1109441)	22337(1949-451190)	0.2374	0.3333
levoglucosan	214(130-1949)	473(134-7382)	559(172-2918)	471(178-1457)	1088(197-21351)	0.2389	0.3333
glutamine	1592(587-10279)	2898(1128-26204)	2942(410-10476)	3859(479-44652)	1235(546-2099)	0.2441	0.3374
5-methoxytryptamine	960(263-3243)	1736(409-6255)	1673(501-4925)	1635(909-5807)	2134(599-9904)	0.2447	0.3374
D-erythro-sphingosine	12822(2395-155273)	17906(875-135976)	37456(3108-96390)	53042(3774-118992)	23178(2633-45934)	0.2563	0.3513
phytanic acid	829(169-8551)	663(252-5031)	837(194-3399)	515(297-3559)	347(140-980)	0.2671	0.3622
thymidine	334(145-1020)	547(241-2565)	596(173-5889)	397(206-1849)	393(303-2857)	0.2677	0.3622
digalacturonic acid	192(74-1836)	440(164-12098)	231(116-6765)	193(118-2740)	431(139-838)	0.2703	0.3622

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
3,6-anhydro-D-glucose	884(131-3797)	1964(1552-3898)	1782(304-25110)	1495(134-14212)	2536(140-6359)	0.2705	0.3622
3,6-anhydro-D-galactose	977(338-4212)	2127(992-3954)	1965(402-9738)	2079(293-6890)	2539(615-6606)	0.2731	0.3636
sophorose	125(104-1105)	268(179-8652)	282(117-994)	251(85-637)	246(82-652)	0.2784	0.3685
N-acetylputrescine	2328(906-29801)	7471(1525-62787)	3826(271-43047)	15260(909-24994)	5828(3410-11448)	0.2815	0.3705
cholesterol	203208(2112-825533)	442353(159558-1501585)	306624(105229-662212)	476337(143419-631965)	308575(102889-507030)	0.2872	0.3759
biphenyl	1212(617-4296)	1373(318-2859)	529(284-2193)	501(349-3191)	993(507-2888)	0.2907	0.3783
2,5-dihydroxypyrazine NIST	394(206-801)	488(286-1170)	442(170-597)	262(172-580)	329(160-746)	0.2932	0.3795
glycolic acid	3609(1865-7605)	2801(860-20791)	5969(1865-9251)	5631(1830-7534)	4259(2389-10490)	0.2966	0.3819
phenylacetic acid	8667(912-107857)	23121(444-109914)	18969(560-63991)	18271(2077-80648)	3228(1380-7571)	0.2985	0.3821
lyxose	1489(352-62372)	2767(1548-212753)	4816(1088-34247)	2923(729-9070)	6570(854-8628)	0.3055	0.3889
1-methylhydantoin	7340(3996-16700)	3144(1125-76475)	4429(2419-277343)	6215(1416-116234)	6704(1659-7543)	0.3225	0.4062
inosine	1063(261-3447)	1480(96-6354)	227(103-13708)	279(135-4867)	569(239-845)	0.3225	0.4062
tyrosol	1642(716-5574)	1574(235-3303)	1474(258-3678)	2034(858-4397)	1522(583-1638)	0.3244	0.4064
phytol	614(307-2002)	951(182-10136)	532(316-2328)	498(275-1720)	466(313-893)	0.3263	0.4066
oleamide NIST	16397(3723-58280)	13510(384-29668)	5280(657-36934)	11434(344-19938)	6680(3957-33341)	0.3306	0.4097
taurine	1014(129-4841)	2144(40-16782)	2220(197-12032)	2884(88-37413)	3057(506-23154)	0.3483	0.4277
N-methylalanine	45310(14918-231749)	35366(1759-350653)	115841(12004-248669)	208916(14713-627338)	111799(28211-287734)	0.3488	0.4277
2-monoolein	5595(1492-32705)	8162(2563-482697)	2848(1920-8422)	5215(537-177100)	7387(1630-50786)	0.3542	0.4320
spermidine	4547(1353-13775)	6814(1418-14105)	5872(1049-24315)	6227(967-39089)	1989(556-13874)	0.3621	0.4394
2,4-diaminobutyric acid	9739(1179-23020)	3822(128-23069)	8589(1244-22530)	5904(1317-23187)	4856(822-36419)	0.3675	0.4436
pantothenic acid	2760(268-10092)	2291(633-68122)	6993(1148-42839)	4423(211-50687)	7227(1580-42662)	0.3840	0.4612
putrescine	259448(159312-4685827)	796818(23403-3129062)	386381(3636-3449841)	338754(9622-2096637)	242749(38216-1436002)	0.3890	0.4648
butyrolactam NIST	2630(1970-14143)	13718(1273-126618)	4793(848-40635)	4242(1623-14037)	7577(849-18997)	0.3944	0.4689
linoleic acid	11048(4100-51073)	31057(4748-333947)	14862(1637-510828)	18067(2165-196151)	15340(6230-185614)	0.4311	0.5099
cytosin	328(152-564)	507(280-3006)	423(126-709)	436(127-5434)	442(122-895)	0.4509	0.5306

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
2-hydroxybutanoic acid	5113(665-150324)	20364(863-305963)	7180(2450-76307)	9627(2079-164137)	13323(591-182282)	0.4841	0.5666
beta-alanine	5766(2959-73895)	5363(800-71313)	10805(2580-93715)	6066(1374-73401)	4214(612-31828)	0.4863	0.5666
vanillic acid	255(174-8563)	329(144-1463)	231(84-2545)	307(253-1458)	381(135-3592)	0.4903	0.5683
threitol	619(160-3116)	1061(337-3509)	887(201-2470)	927(333-1765)	1226(933-5187)	0.5037	0.5810
4-hydroxymandelic acid	518(225-1375)	805(309-44458)	605(201-1601)	723(220-1368)	978(222-3813)	0.5455	0.6262
phenylethylamine	5177(1162-117411)	37367(882-453129)	12233(1278-58583)	14176(2193-61974)	16636(2557-85168)	0.5560	0.6336
piperidone	89938(4990-486822)	36629(1317-160094)	105906(7609-218749)	98525(4707-233442)	33001(1844-356059)	0.5575	0.6336
palmitoleic acid	1167(644-3686)	6750(263-28814)	1525(303-30105)	1917(381-20837)	2513(236-4136)	0.5632	0.6370
creatinine	6070(238-76418)	4936(348-90867)	2534(850-65756)	9911(326-29523)	9358(7313-216444)	0.5920	0.6663
citramalic acid	425(217-763)	554(83-2069)	267(108-848)	335(151-474)	728(268-2225)	0.5957	0.6673
3-hydroxybutyric acid	2731(1472-15939)	6071(2082-11734)	4515(736-12390)	3880(1502-53108)	6046(1518-75219)	0.6153	0.6836
octadecanol	305(149-678)	359(264-605)	454(218-568)	430(238-939)	325(141-647)	0.6162	0.6836
lanosterol	130(77-1000)	150(74-2001)	377(84-2165)	594(89-1379)	450(186-1221)	0.6601	0.7290
propane-1,3-diol NIST	921(466-6897)	1769(356-24322)	1710(558-7343)	1713(677-44685)	1957(410-2794)	0.6640	0.7298
alpha-aminoadipic acid	536(138-1061)	859(102-2867)	773(241-1983)	585(108-1569)	715(340-941)	0.6713	0.7344
beta-sitosterol	73794(2170-171886)	25509(3764-250414)	22354(3394-148996)	63939(4842-204588)	60178(2120-96991)	0.6879	0.7490
oleic acid	20380(4751-37796)	200715(4270-538722)	46634(1339-765518)	23921(4817-411134)	39548(2758-471628)	0.6968	0.7552
lactose	411(239-4623)	685(300-4591)	435(234-6179)	726(279-3730)	789(275-13175)	0.7143	0.7705
N-acetylornithine	1568(607-5923)	3168(144-156625)	2292(328-4857)	2203(1191-7260)	1154(497-6803)	0.7200	0.7731
7-methylguanine NIST	1349(370-3156)	1297(585-4114)	1247(270-2479)	1008(702-2218)	1468(283-2822)	0.7253	0.7751
O-acetylserine	997(482-2402)	1161(290-3246)	1243(458-4570)	954(172-3239)	1025(496-2516)	0.7285	0.7751
beta-glutamic acid	189(58-1417)	114(86-6485)	165(121-434)	119(106-4278)	81(48-1181)	0.7542	0.7988
monomyristin	222(165-374)	265(118-52086)	248(125-618)	218(115-5146)	362(166-535)	0.7595	0.8007
glutaric acid	403(252-649)	304(85-2983)	393(198-908)	371(153-2191)	644(186-948)	0.7814	0.8154
5-aminovaleric acid	1190039(307367-2587727)	1046659(5666-2380281)	1022910(86535-2372618)	1057768(199389-2429102)	869805(252895-2883574)	0.7818	0.8154

Table 6. Continued.

	median (minimum-maximum)						
Compound Name	**Healthy**	**Baseline**	**3WK**	**8WK**	**LT**	**p-value**	**q-value**
2-deoxytetronic acid	1166(394-9120)	977(409-2969)	1606(420-4391)	1131(702-2900)	1797(525-3872)	0.7839	0.8154
3-(3-hydroxyphenyl)propionic acid	217(121-79508)	313(102-3365)	218(65-15794)	294(120-59887)	166(129-2489)	0.8432	0.8732
daidzein	543(94-18663)	547(89-13632)	3343(79-27648)	1831(150-30093)	1452(84-5067)	0.8549	0.8776
phosphoethanolamine	253(66-686)	252(70-1322)	206(106-876)	183(101-701)	279(148-811)	0.8550	0.8776
3-hydroxypropionic acid	2054(1307-13212)	3421(942-110349)	2648(969-12390)	2826(1168-23771)	7484(823-54041)	0.8695	0.8853
hydroxylamine	44272(25032-76223)	31807(13652-84391)	49033(14514-131442)	54890(14108-96170)	41548(13110-83278)	0.8701	0.8853
tocopherol acetate	7631(137-57866)	6790(378-18747)	6658(923-20013)	4394(203-17357)	5559(1011-8403)	0.9143	0.9263
hydroxycarbamate NIST	10701(2448-20817)	7834(2188-17813)	8496(1645-13676)	9771(3207-19724)	10383(1903-19071)	0.9222	0.9301
tocopherol alpha-	69464(1276-406178)	60018(1746-210418)	89716(15114-228288)	122859(18719-194586)	57494(19902-177958)	0.9785	0.9811
3,4-dihydroxybenzoic acid	4009(394-17828)	3830(445-12743)	4289(350-11269)	4676(923-11401)	1782(367-15662)	0.9811	0.9811

Discussion

The purpose of this study was two-fold, with one goal being to characterize the fecal metabolome in dogs with inflammatory bowel disease and the second goal being to characterize the changes in the metabolome longitudinally. Currently there are no published studies that have evaluated the fecal metabolome in dogs using an untargeted approach, moreover, any articles that have followed up patients over the period of 1 year. Untargeted metabolomics may help to uncover roles of the microbiome and metabolome and the mechanisms involved in the onset of inflammatory bowel disease, the alterations during active treatment, and potential restoration during long term recovery.

In this study, PCA of fecal metabolites showed limited overlapping of 95% confidence intervals among the groups analyzed. Untargeted serum metabolite analysis in dogs with IBD before and after treatment (3 weeks of therapy) failed to show any significant changes in the global profile (Minamoto et al., 2014b). Metabolite profiles from the current study may hint at the idea that these metabolites are slowly starting to shift back to that of healthy dogs and this is evident by 8 weeks post treatment. After one year, multivariate analysis revealed that there were still several dogs with IBD that did not fall within the 95% confidence interval of healthy dogs.

Of the compounds identified, amino acids and derivatives thereof were significantly different between groups. Furthermore, many followed a similar pattern in that the peak abundance in healthy dogs were less than dogs with IBD at any one time point measured. Some of these compounds were isoleucine, proline, valine, leucine, threonine, serine, glycine, aspartic acid, oxoproline, alanine, and methionine. Recent

studies in *Winnie* mouse models of colitis have demonstrated amino acid dysregulation where many are either up- or down-regulated in the colitis model compared to controls (Robinson et al., 2016). These models are considered reflective of human IBD. Amino acids continue to be of interest in chronic inflammation, however, their up or down regulation seem to be dependent on the study and model. For instance, Robinson et al., reported decreased amino acids in their model of colitis, specifically reporting branched-chain amino acids. The current study found those type of metabolites (e.g., leucine and isoleucine) to be significantly decreased in healthy patients compared to dogs with IBD. Alternatively, these fecal metabolites have been reportedly increased in human patients with Crohn's disease and Ulcerative Colitis which may be indicative, similar to this study, of a malabsorption of amino acids in patients with IBD (Marchesi et al., 2007). Amino acids play a critical role in gut health. Supplementation with amino acids (e.g., arginine, glutamine, glutamate, leucine, and proline) can modulate gene expression, enhance integrity and growth of the small intestine, and can reduce body fat (Wu, 2013).

When PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) was used to predict functional aspects of genes from bacteria in Minamoto et. al., 2014, KEGG (Kyoto Encylopedia for Genes and Genomes) (Kanehisa et al., 2014) orthologues were found to be underrepresented in dogs with IBD for those belonging to amino acid metabolism. While these results are slightly difficult to interpret, it could be that a lack in specific bacteria essential in amino acid breakdown and metabolism are responsible for build-up of amino acids. Since amino acids can affect gene expression it could also be that amino acid dysregulation may further

exacerbate the host response to inflammation. Alternatively, it may be that amino acid metabolism refers to the production of amino acids, in which case this hypothesis could not be validated since this dataset set analyzed serum, not feces.

Another hypothesis for amino acids may be that increased systemic amino acid prevalence in fecal metabolites is part of the anti-inflammatory response. For instance, certain amino acids have been reported to increase mucin production and colonic protection in DSS (dextran sulfate sodium) treated rats (Faure et al., 2006). Nutritional supplementation of specific amino acids may possibly be considered to further encourage the anti-inflammatory host response in the event that naturally it cannot sustain the anti-inflammatory response necessary to keep the gut in a healthy state. Arginine, glutamine, and cysteine may serve as useful amino acid candidates to regulate since they have well-defined roles and their effect on host physiology is well understood. Arginine promotes the secretion of insulin, growth hormone, prolactin, and insulin-like growth factor-I. Glutamine provides energy for enterocytes. Cysteine is a precursor of glutathione and its metabolism can be greatly altered in response to infection.

This study provides into the long term metabolic outcome of dogs successfully treated for IBD. Unfortunately, it proved very difficult to follow up these patients more than a year later. This was mostly due to owner involvement and compliance. Future studies should measure fecal amino acids in healthy dogs and dogs with IBD and verify whether a predictive or causative link exists with modification of these metabolites and their pathways.

CHAPTER V

THE EFFECT OF THE BILE ACID SEQUESTRANT CHOLESTYRAMINE ON THE MICROBIOTA AND FECAL BILE ACIDS IN HEALTHY DOGS

Overview

Cholestyramine is a bile acid sequestrant that acts in the gastrointestinal tract by binding bile acids, thus preventing their reabsorption. In human patients with inflammatory bowel disease (IBD), cholestyramine is often used to treat individuals with symptoms of diarrhea. Preliminary studies in canine patients with IBD suggest that their fecal bile acid concentrations are altered, typified by an increase in primary bile acids and a decrease in secondary bile acids compared to healthy controls. The purpose of this study was to determine the effect of cholestyramine on the fecal bile acids profile in healthy dogs.

Baseline fecal samples were collected from healthy Beagle dogs (n = 12) two weeks before administration of cholestyramine. Additional fecal samples were collected after two weeks of daily cholestyramine administration and again two weeks later after a washout period. All dogs were maintained on the same maintenance diet during the study and were fed 11.4 g/day of cholestyramine powder (8 g active ingredient) suspended in 75 mL of water during the cholestyramine administration period. Fecal concentrations of primary bile acids (i.e., CA and CDCA) and secondary bile acids (i.e., LCA, DCA, UDCA) were evaluated using gas chromatography coupled with mass spectrometry.

Microbial communities were assessed using Quantitative Insights into Microbial Ecology (QIIME). Data were assessed for normality using the Shapiro-Wilk test and differences in bile acid concentrations were compared using the Friedman's test. The Dunn's post-test was used where appropriate. Statistical significance was set at $p < 0.05$.

Total fUBA significantly increased after two weeks of cholestyramine administration (median [min-max]: 14.2 µg/mg [5.7-25.5 µg/mg]) when compared to baseline values (median [min-max]: 6.6 µg/mg [5.6-17.7 µg/mg]; $p = 0.0062$). Secondary fUBA were significantly increased after two weeks of cholestyramine administration (median [min-max]: 13.1 µg/mg [5.5-23.3 µg/mg]) when compared to baseline values (median [min-max]: 6.2 µg/mg [5.4-17.1 µg/mg]; $p = 0.0183$). There were significant changes in fecal microbial communities (i.e., for unweighted and weighted unifrac distances) of dogs administered cholestyramine according to principal coordinate analysis plots (PCoA), compared statistically using ANOSIM ($p<0.05$).

In conclusion, the fecal bile acids profile and microbiome is altered in healthy dogs after cholestyramine administration. Further studies are needed to understand the potential clinical utility of cholestyramine as a therapeutic option in canine patients with gastrointestinal disease.

Introduction

Bile acid malabsorption is gaining interest as studies are beginning to find that it may play a role in diarrhea or loose stool conditions in humans (Watson et al., 2014). Bile acid malabsorption is diagnosed by the gold standard SeHCAT test. A retention of less than 10-15% of the tracer is indicative of bile acid malabsorption (Gothe et al., 2014).

Cholestyramine and colestipol are generally effective treatments of gastrointestinal symptoms occurring from bile acid malabsorption (Wilcox et al., 2014). Cholestyramine is a positively charged non-digestible resins that bind to bile acids in the form of an insoluble complex, that is later excreted into the feces (Scaldaferri et al., 2013). Clinically, in humans bile acid sequestrants have been suggested for use in treatment of primary hypercholesterolemia and cholestatic pruritus given their ability to lower cholesterol. Furthermore, there are indications for use of bile acid sequestrants in the treatment of type 2 diabetes mellitus, metabolic syndrome, and insulin resistance (Staels and Kuipers, 2007).

Cholestyramine may help treat bile acid dysmetabolism in canine patients with chronic diarrhea. Cholestyramine is effective in increasing bile acid concentration in feces and reducing plasma cholesterol in dogs (Jansen and Zanetti, 1965). Jansen and Zanetti reported that plasma cholesterol could be decreased in a dose dependent fashion (i.e., 1, 3, 6, and 10 g/dog/day) using cholestyramine. That study did not differentiate between the bile acids measured. There is a bile acid dysmetabolism in approximately 60% of dogs with CE. It is unclear whether or not restoration of the fecal bile acid profile may be beneficial to the overall health of the canine patients with CE. Therefore, the purpose of this study was to characterize the effect of cholestyramine on the fecal microbiota and bile acids of healthy dogs using high-throughput sequencing and gas chromatography coupled with mass spectrometry, respectively.

Materials and methods

Healthy research colony beagle dogs (n=12) were used in this study. The mean and standard deviation for the age of dogs (years) was 3.17 ± 0.82, respectively. The mean and standard deviation for the weight of dogs (kg) was 10.35 ± 0.88, respectively. Cholestyramine was administered for two weeks duration at a dose of 11.4 g/day cholestyramine powder (8 g/day active ingredient; Cholestyramine for Oral Suspension, Generic Questran, Sandoz, Holzkirchen, Germany). During this study, all dogs were fed the same experimental diet that was formulated to meet nutritional needs recommended by the Association of American Feed Control Officials. Once a day feeding took place at approximately 8 AM to maintain body weight. The study was a randomized crossover design that consisted of a baseline period and two 14 day experimental periods separated by a 14 day washout period.

Unconjugated fecal bile acids were measured using a gas chromatographer coupled with a mass spectrometer targeting CA, CDCA, LCA, DCA, and UDCA as described previously in Chapter II.

For DNA extraction and Illumina high-throughput sequencing, approximately 100 mg of feces was first used in the downstream process of DNA isolation according to the manufacturer's instructions (PowerSoil®, Mo Bio, Carlsbad, CA, USA). Amplification of the 16S rRNA genes and sequencing was performed at Molecular Research DNA (MR DNA: http://www.mrdnalab.com, Shallowwater, TX, USA). Samples were barcoded and targeted using the forward primer 515F and the reverse primer 806R to amplify the V4 region of the 16S rRNA gene. Quantitative Insights Into Microbial Ecology (QIIME,

v1.8) was used for sequence processing and analysis. Raw sequences were de-multiplexed, de-noised, chimera removed (USEARCH 6.1) using reference based chimera checking, chloroplast and mitochondrial operational taxonomic unit (OTU) removed, and screened for quality control eliminating low quality reads using the default parameters in QIIME. Open OTU picking was utilized, then picked genes against the Greengenes database (v 13.8). Unweighted unifrac distances were used to visualize microbial communities for before during and after cholestyramine administration. The statistical analysis method Analysis of Similarities (ANOSIM) was used to test for statistical differences in microbial communities using the software package Primer 6 (Auckland, New Zealand). Species richness was compared between before, during, and after cholestyramine treatment through the observed species, chao1, and Shannon index matrices. Furthermore, univariate analysis was used to compare individual bacterial groups utilizing JMP Pro 12 (Cary, NC, USA) to use non-parametric, repeated measures testing through implication of a blocking variable and a Kruskal-Wallis test (Friedman's test equivalent). For those that were significantly different after adjusting for multiple comparisons using the Benjamini and Hochberg false discovery rate, a Dunn's post-test was then used to identify significant differences ($p<0.05$) between individual groups.

Results

For fecal bile acids, there was no significant change in concentration between baseline, cholestyramine, and washout in CA (median [min-max]: 0.080 µg/mg [0.040-0.690 µg/mg], 0.100 µg/mg [0.010-1.290 µg/mg], and 0.100 µg/mg [0.020-0.240 µg/mg], respectively; p=0.9785), CDCA (median [min-max]: 0.260 µg/mg [0.180-0.870 µg/mg],

0.350 µg/mg [0.130-1.170 µg/mg], and 0.365 µg/mg [0.130-0.650 µg/mg], respectively; p=0.3679), LCA (median [min-max]: 3.100 µg/mg [2.390-5.930 µg/mg], 3.900 µg/mg [1.790-6.260 µg/mg], and 2.915 µg/mg [1.740-4.870 µg/mg], respectively; p=0.1245), and UDCA (median [min-max]: 0.070 µg/mg [0.010-0.250 µg/mg], 0.060 µg/mg [0.010-0.500 µg/mg], and 0.055 µg/mg [0.020-0.310 µg/mg], respectively; p=0.9770). Deoxycholic acid, however, was significantly increased from baseline to after cholestyramine administration and after washout returned to baseline concentrations (median [min-max]: 3.315 µg/mg [2.630-11.02 µg/mg], 9.500 µg/mg [3.420-18.440 µg/mg], and 4.955 µg/mg [1.980-11.660 µg/mg], respectively; p=0.0014).

While there was no significant difference between baseline, cholestyramine, and washout groups in primary fUBA (median [min-max]: 0.3550 µg/mg [0.220-1.570 µg/mg], 0.4200 µg/mg [0.150-2.210 µg/mg], and 0.465 µg/mg [0.160-0.820 µg/mg], respectively; p=0.7165), there was an increase in total fUBA (median [min-max]: 6.635 µg/mg [5.600-17.700 µg/mg], 14.250 µg/mg [5.690-25.540 µg/mg], and 8.475 µg/mg [3.890-17.520 µg/mg], respectively; p=0.0062) and secondary fUBA (median [min-max]: 6.220 µg/mg [5.370-17.070] µg/mg, 13.050 µg/mg [5.540-23.330 µg/mg], and 8.025 µg/mg [3.740-16.700 µg/mg], respectively; p=0.0183) once again increasing significantly from baseline to after cholestyramine administration and after washout returning to baseline concentrations. These results are shown in Figure 8.

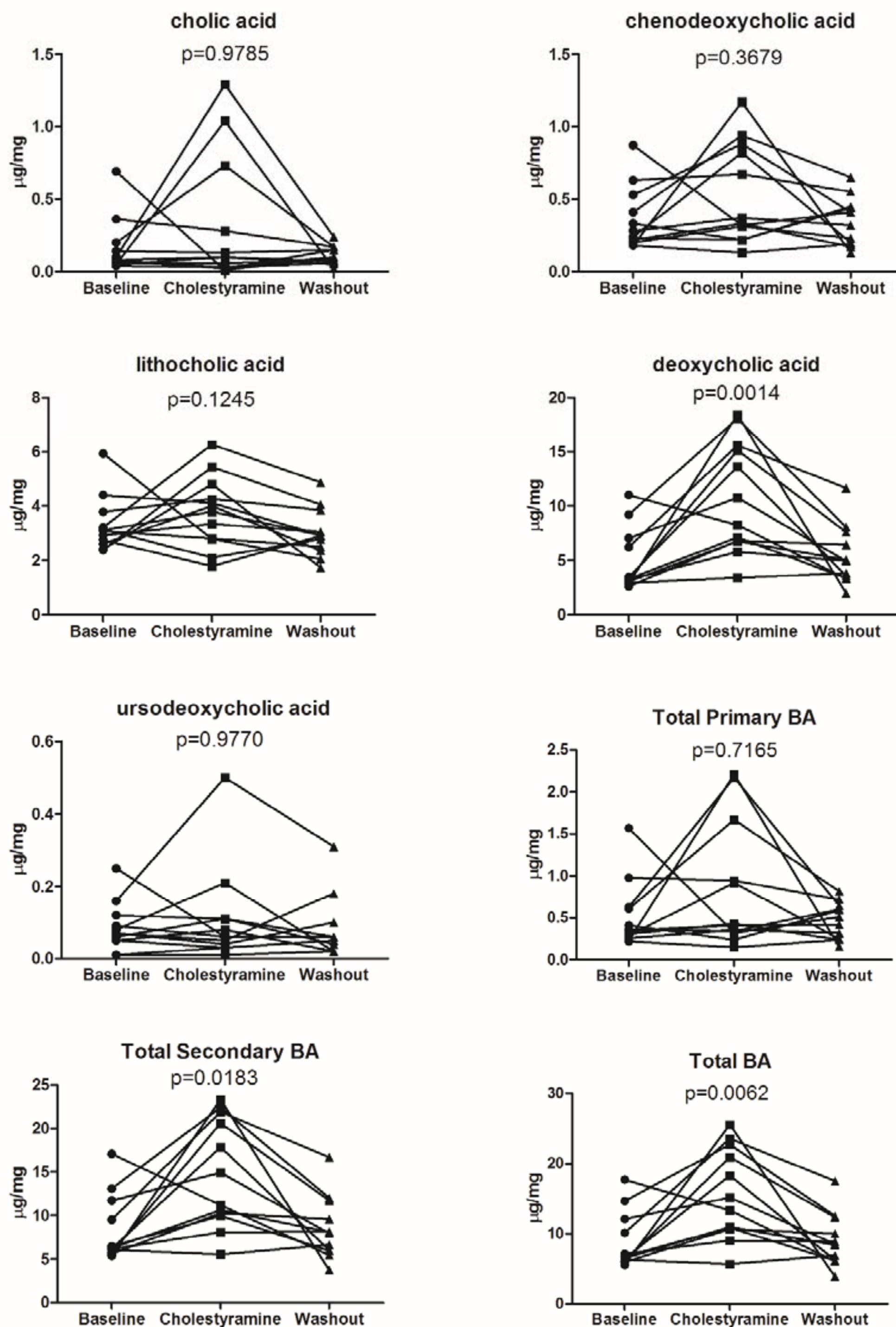

Figure 8. Fecal bile acid concentrations and additional parameters for healthy control dogs before, during, and after cholestyramine administration.

When expressed as a percent of total bile acids measured, there was no significant difference between the baseline, cholestyramine, and washout in CA (median [min-max]: 1.05% [0.57-4.99%], 0.58% [0.03-9.67%], and 0.99% [0.61-3.93%], respectively; p=0.4724), CDCA (median [min-max]: 3.47% [2.74-8.76%], 3.16% [1.03-7.53%], and 3.37% [2.70-6.70%], respectively; p=0.1054), and UDCA (median [min-max]: 0.83% [0.11-2.19%], 0.32% [0.15-5.56%], and 0.61 [0.26-3.44], respectively; p=0.3679). However, LCA, as a percent of total fUBA, was significantly decreased after cholestyramine administration (median [min-max]: 25.87% [18.73-37.03%]) compared with baseline (median [min-max]: 39.88% [25.81-49.75%]; p=0.0002). Deoxycholic acid, as a percent of total fUBA, was significantly increased after cholestyramine administration (median [min-max]: 52.77% [44.01-62.87%]) compared with baseline (median [min-max]: 65.83% [60.05-79.58%]; p=0.0023). There was no significant difference in secondary fUBA between baseline, cholestyramine, and washout (median [min-max]: 95.52% [86.25-96.59%], 96.45% [83.76-98.58%], and 95.87% [89.37-96.47%], respectively; p=0.1054). These results are displayed in Figure 9.

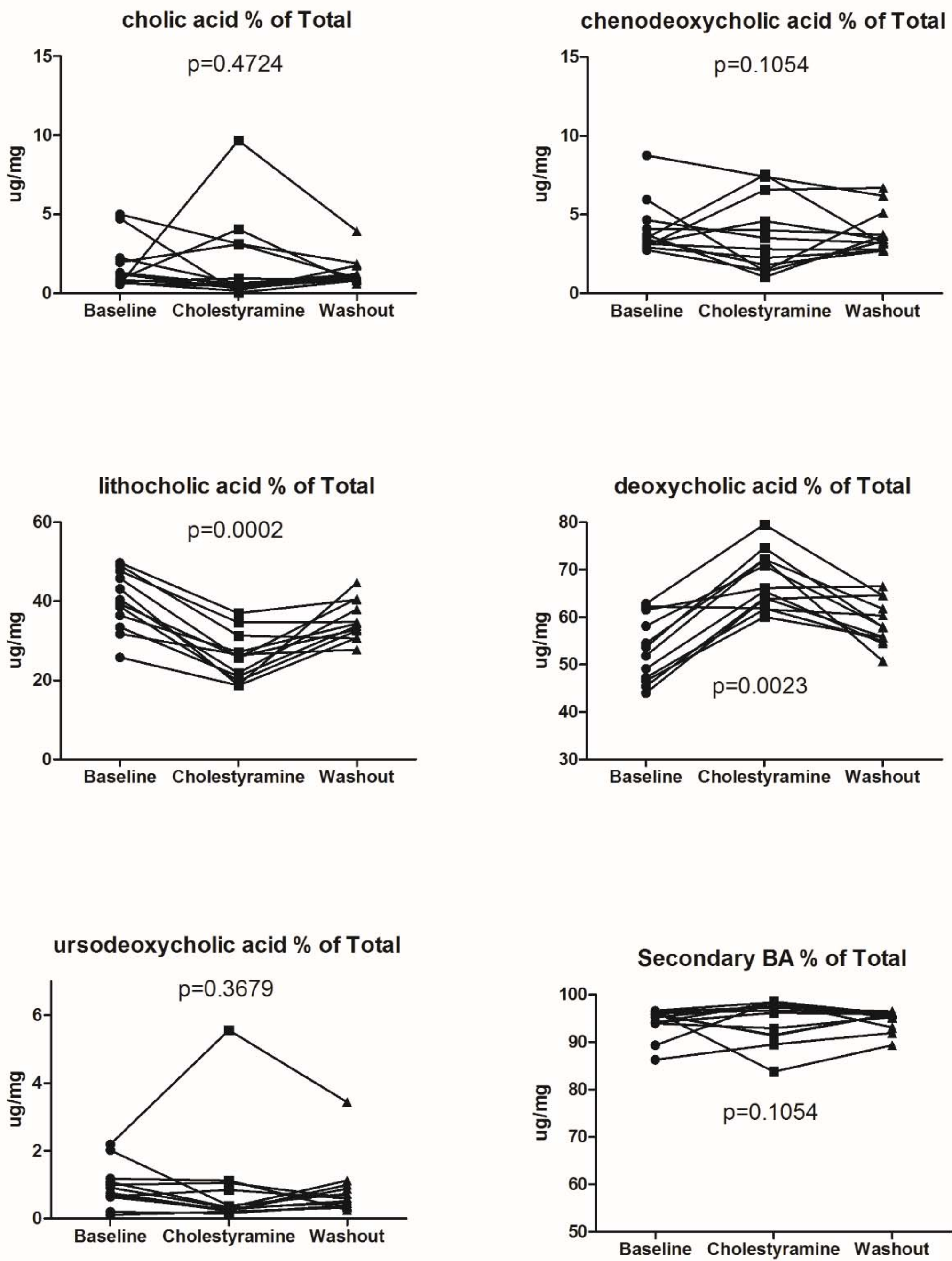

Figure 9. Percent of fecal bile acids and additional parameters for healthy control dogs before, during, and after cholestyramine administration.

Sequences belonging to fecal samples were rarified to 52,250 for equal sampling depth across all samples. When comparing dogs at baseline, during cholestyramine administration, and after the washout, clustered separately based on unweighted (ANOSIM p-value and R-statistic: 0.032 and 0.082, respectively; Figure 10) and weighted unifrac distances (ANOSIM p-value and R-statistic: 0.001 and 0.188, respectively; Figure 11). Pairwise comparisons using a post-test identified that in both weighted and unweighted unifrac distances there was a significant difference between microbial communities at baseline and after cholestyramine administration ($p<0.05$). There was no statistical evidence that cholestyramine administration altered species richness regardless of the metric that was analyzed (Figures 12-14).

The following are the most significant individual changes in taxonomic proportions. On the phylum level, the percentage of Proteobacteria was significantly increased in dogs after cholestyramine (median [min-max]: 6.09 [2.42-9.35]) administration compared to baseline (median [min-max]; q-value: 6.09 [2.42-9.35]; p=0.0005). On the class level, the percentage of Clostridia was signifcaintly decreased in dogs after cholestyramine (median [min-max]: 40.74 [0.17-52.98]) administration compared to baseline (median [min-max]; q-value: 33.80 [15.31-44.77]; p=0.0003). A complete list of taxonomic comparisons is in Table 7.

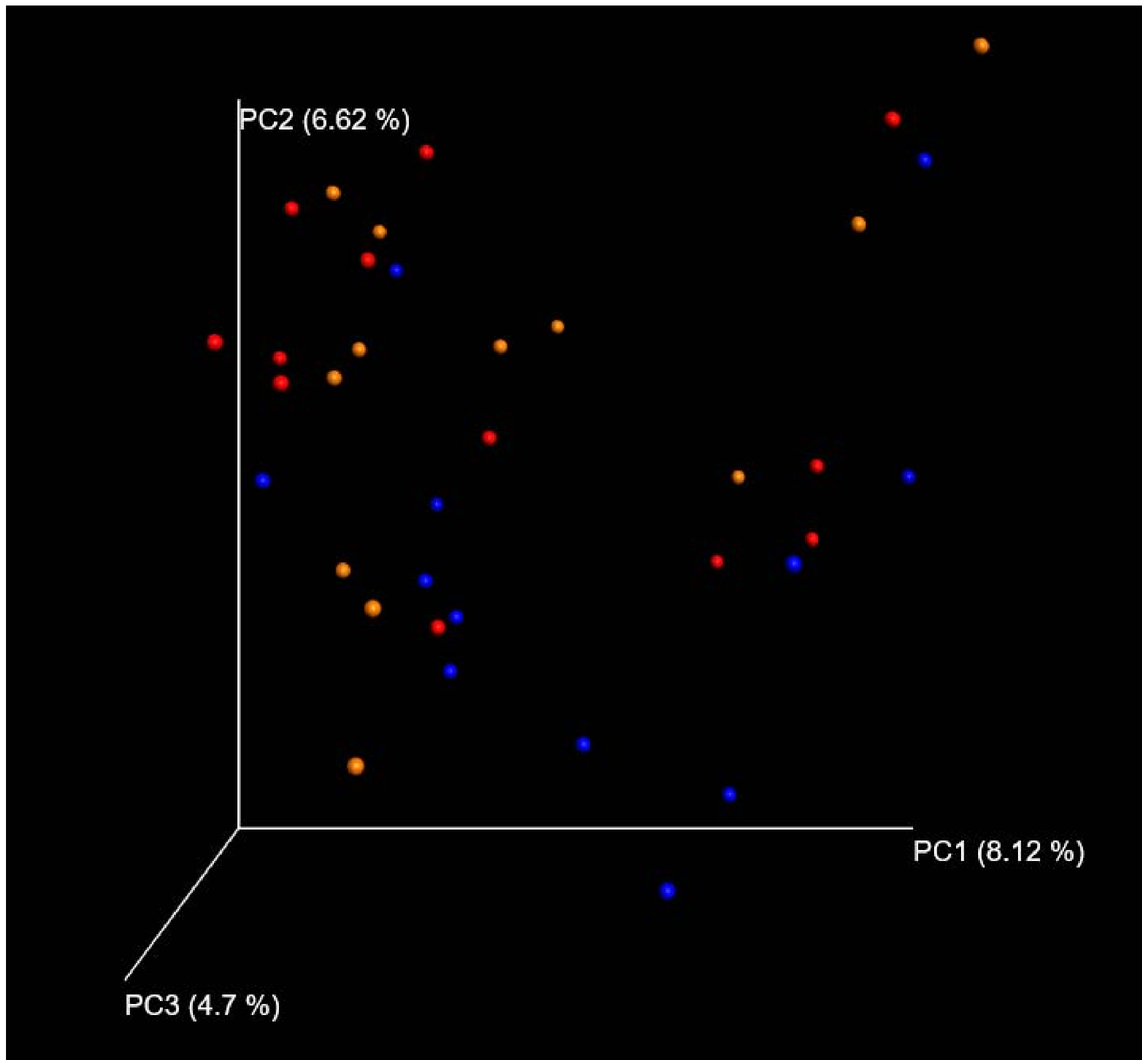

Figure 10. Principal coordinate analysis plot of unweighted unifrac distances. Red, blue, and orange dogs represent the microbial communities of dogs before, during, and after cholestyramine administration, respectively.

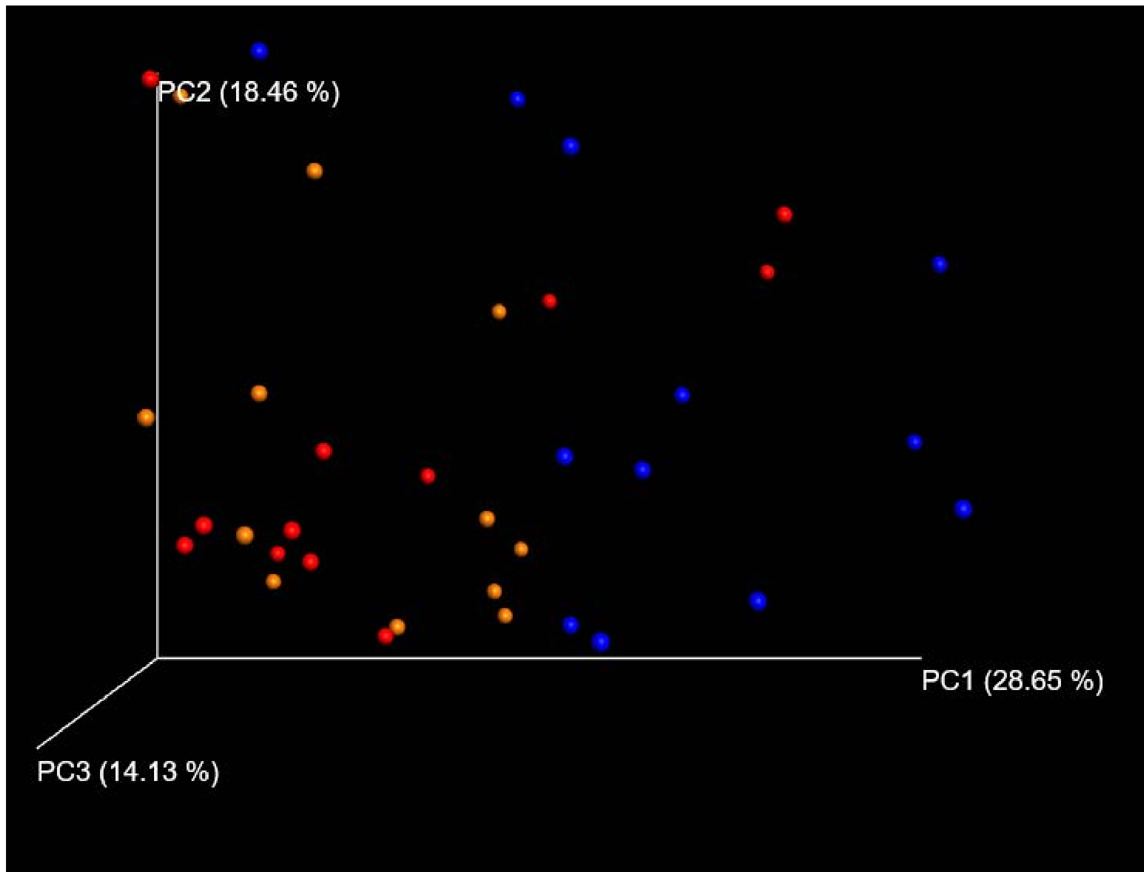

Figure 11. Principal coordinate analysis plot of weighted unifrac distances. Red, blue, and orange dogs represent the microbial communities of dogs before, during, and after cholestyramine administration, respectively.

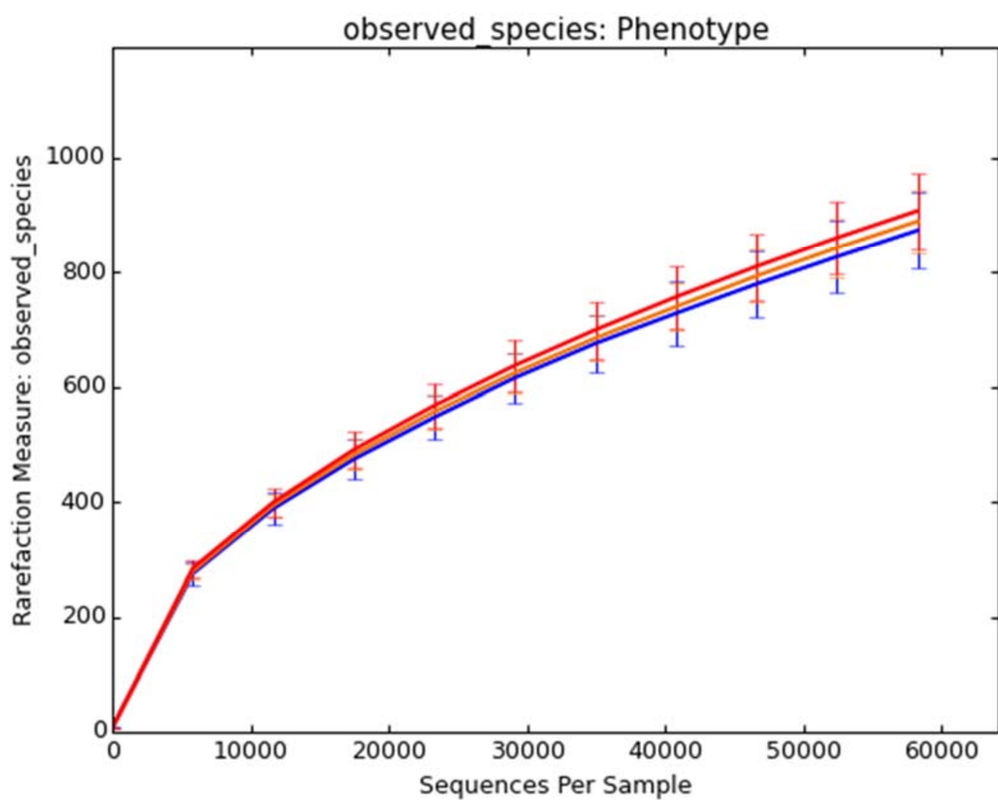

Figure 12. Rarefaction curve of the alpha diversity measure observed species. Red, blue, and orange lines represent the species richness of dogs before, during, and after cholestyramine administration, respectively.

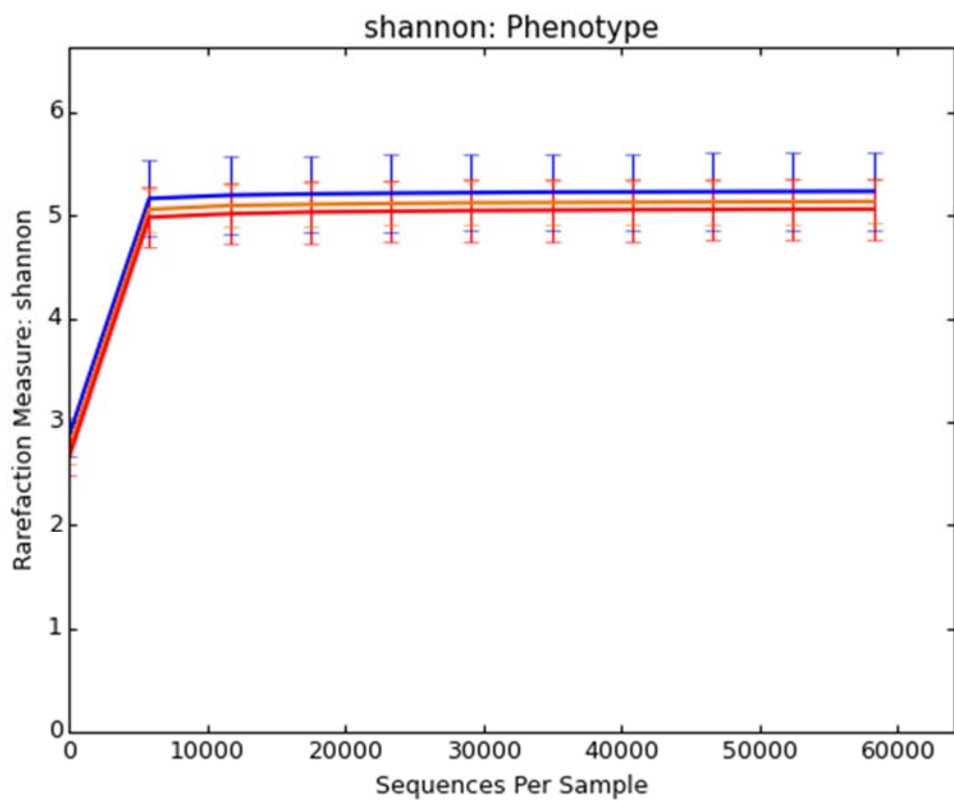

Figure 13. Rarefaction curve of the alpha diversity measure Shannon Index. Red, blue, and orange lines represent the species richness of dogs before, during, and after cholestyramine administration, respectively.

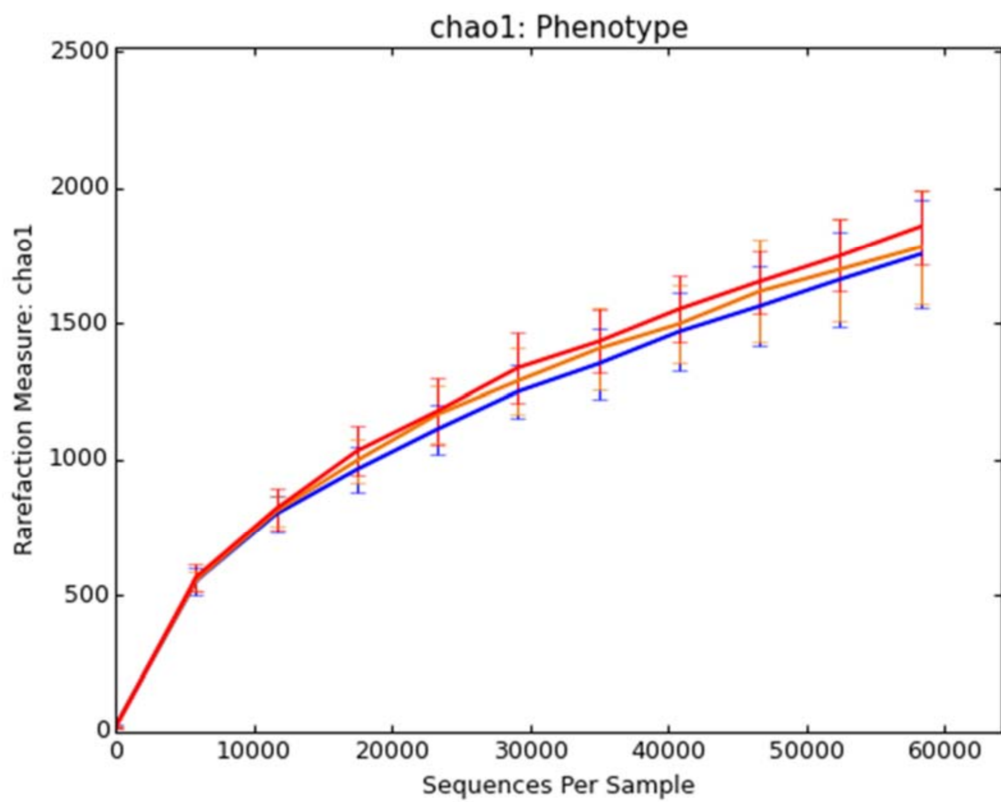

Figure 14. Rarefaction curve of the alpha diversity measure chao1. Red, blue, and orange lines represent the species richness of dogs before, during, and after cholestyramine administration, respectively.

Table 7. Taxonomic changes before, during, and after cholestyramine administration.

	median (minumum-maximum)				
Taxa	**Baseline**	**Cholestyramine**	**Washout**	**p-value**	**q-value**
p__Proteobacteria	2.75(0.03-7.36)	6.09(2.42-9.35)	3.19(1.64-12.25)	0.0001	0.0005
p__Bacteroidetes	11.76(0.21-20.81)	14.54(8.43-35.87)	9.26(3.70-15.92)	0.0081	0.0284
p__Actinobacteria	2.05(0.12-11.59)	1.55(0.60-6.76)	2.15(0.90-14.65)	0.0835	0.1949
p__Fusobacteria	22.69(0.22-32.78)	15.60(12.39-30.58)	21.81(11.23-38.15)	0.2056	0.3598
p__Firmicutes	51.95(0.43-75.92)	57.67(41.94-65.01)	63.50(37.70-74.02)	0.7290	0.8788
p__Tenericutes	0.00(0.00-0.01)	0.00(0.00-0.01)	0.00(0.00-0.01)	0.7881	0.8788
p__Deferribacteres	0.01(0.00-0.04)	0.00(0.00-0.06)	0.02(0.00-0.05)	0.8788	0.8788
p__Firmicutes|c__Clostridia	40.47(0.17-52.98)	33.80(15.31-44.77)	38.42(21.54-57.71)	<0.0001	0.0003
p__Proteobacteria|c__Betaproteobacteria	1.29(0.02-2.42)	3.40(0.53-8.28)	1.61(0.54-5.59)	0.0002	0.0015
p__Actinobacteria|c__Coriobacteriia	1.26(0.01-3.34)	0.95(0.11-2.10)	1.55(0.08-3.12)	0.0014	0.0057
p__Bacteroidetes|c__Bacteroidia	11.76(0.21-20.81)	14.54(8.43-35.87)	9.26(3.70-15.92)	0.0081	0.0244
p__Firmicutes|c__Bacilli	6.42(0.07-47.36)	19.98(2.71-49.04)	14.09(1.37-40.57)	0.0406	0.0973
p__Proteobacteria|c__Epsilonproteobacteria	0.01(0.00-0.09)	0.03(0.00-0.10)	0.01(0.00-0.09)	0.0967	0.1934
p__Proteobacteria|c__Gammaproteobacteria	0.96(0.01-5.90)	2.46(0.33-7.02)	1.02(0.36-10.01)	0.1843	0.3084
p__Fusobacteria|c__Fusobacteriia	22.69(0.22-32.78)	15.60(12.39-30.58)	21.81(11.23-38.15)	0.2056	0.3084
p__Actinobacteria|c__Actinobacteria	0.05(0.03-10.58)	0.05(0.03-6.42)	0.06(0.04-13.33)	0.4529	0.6039
p__Firmicutes|c__Erysipelotrichi	2.33(0.19-18.64)	2.53(0.53-13.05)	2.69(0.36-10.45)	0.7524	0.8598
p__Tenericutes|c__Mollicutes	0.00(0.00-0.01)	0.00(0.00-0.01)	0.00(0.00-0.01)	0.7881	0.8598
p__Deferribacteres|c__Deferribacteres	0.01(0.00-0.04)	0.00(0.00-0.06)	0.02(0.00-0.05)	0.8788	0.8788
p__Firmicutes|c__Clostridia|o__Clostridiales	40.47(0.17-52.98)	33.80(15.31-44.77)	38.42(21.54-57.71)	<0.0001	0.0005
p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales	1.29(0.02-2.42)	3.40(0.53-8.28)	1.61(0.54-5.59)	0.0002	0.0020
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales	1.26(0.01-3.34)	0.95(0.11-2.10)	1.55(0.08-3.12)	0.0014	0.0076
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales	11.76(0.21-20.81)	14.54(8.43-35.87)	9.26(3.70-15.92)	0.0081	0.0325

Table 7. Continued.

	median (minumum-maximum)				
Taxa	**Baseline**	**Cholestyramine**	**Washout**	**p-value**	**q-value**
p__Firmicutes|c__Bacilli|Other	0.00(0.00-0.00)	0.00(0.00-0.00)	0.00(0.00-0.00)	0.0128	0.0411
p__Firmicutes|c__Bacilli|o__Lactobacillales	2.29(0.06-46.94)	19.35(1.29-48.58)	9.86(0.60-35.97)	0.0267	0.0711
p__Firmicutes|c__Bacilli|o__Turicibacterales	1.53(0.02-9.62)	1.62(0.25-8.37)	3.32(0.26-7.97)	0.0385	0.0880
p__Actinobacteria|c__Actinobacteria|o__Actinomycetales	0.00(0.00-0.01)	0.00(0.00-0.01)	0.00(0.00-0.02)	0.0794	0.1547
p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacteriales	0.07(0.00-5.02)	0.46(0.05-6.74)	0.13(0.03-9.70)	0.0870	0.1547
p__Proteobacteria|c__Epsilonproteobacteria|o__Campylobacterales	0.01(0.00-0.09)	0.03(0.00-0.10)	0.01(0.00-0.09)	0.0967	0.1547
p__Fusobacteria|c__Fusobacteriia|o__Fusobacteriales	22.69(0.22-32.78)	15.60(12.39-30.58)	21.81(11.23-38.15)	0.2056	0.2991
p__Proteobacteria|c__Gammaproteobacteria|o__Aeromonadales	0.78(0.01-1.54)	0.73(0.15-4.00)	0.84(0.31-2.47)	0.4071	0.5428
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales	2.33(0.19-18.64)	2.53(0.53-13.05)	2.69(0.36-10.45)	0.7524	0.9144
p__Actinobacteria|c__Actinobacteria|o__Bifidobacteriales	0.05(0.03-10.57)	0.04(0.02-6.41)	0.05(0.03-13.32)	0.8559	0.9144
p__Deferribacteres|c__Deferribacteres|o__Deferribacterales	0.01(0.00-0.04)	0.00(0.00-0.06)	0.02(0.00-0.05)	0.8788	0.9144
p__Tenericutes|c__Mollicutes|o__Anaeroplasmatales	0.00(0.00-0.01)	0.00(0.00-0.01)	0.00(0.00-0.01)	0.9144	0.9144
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae	19.15(0.09-31.66)	14.44(6.94-29.30)	19.45(12.59-31.35)	0.0001	0.0023
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Bacteroidaceae	9.12(0.11-12.43)	13.46(6.18-25.93)	6.30(3.63-12.76)	0.0001	0.0023
p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Alcaligenaceae	1.29(0.02-2.42)	3.40(0.53-8.28)	1.61(0.54-5.59)	0.0002	0.0026
p__Firmicutes|c__Clostridia|o__Clostridiales|Other	0.02(0.00-0.04)	0.01(0.00-0.03)	0.03(0.01-0.06)	0.0003	0.0026
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Veillonellaceae	1.99(0.02-4.82)	0.97(0.28-1.57)	2.08(0.09-4.43)	0.0007	0.0043
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae	1.26(0.01-3.34)	0.95(0.11-2.10)	1.55(0.08-3.12)	0.0014	0.0079
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae	0.38(0.00-30.38)	6.09(0.46-22.38)	1.38(0.18-29.51)	0.0049	0.0221
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Porphyromonadaceae	0.04(0.00-0.31)	0.01(0.00-0.07)	0.02(0.00-0.21)	0.0054	0.0221
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae	12.44(0.04-17.62)	9.58(4.43-20.09)	12.49(5.12-21.08)	0.0060	0.0221
p__Actinobacteria|c__Actinobacteria|o__Actinomycetales|f__Actinomycetaceae	0.00(0.00-0.01)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.0083	0.0274
p__Firmicutes|c__Bacilli|Other|Other	0.00(0.00-0.00)	0.00(0.00-0.00)	0.00(0.00-0.00)	0.0128	0.0385
p__Actinobacteria|c__Actinobacteria|o__Actinomycetales|f__Microbacteriaceae	0.00(0.00-0.01)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.0383	0.0977

Table 7. Continued.

	median (minumum-maximum)				
Taxa	**Baseline**	**Cholestyramine**	**Washout**	**p-value**	**q-value**
p__Firmicutes|c__Bacilli|o__Turicibacterales|f__Turicibacteraceae	1.53(0.02-9.62)	1.62(0.25-8.37)	3.32(0.26-7.97)	0.0385	0.0977
p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacteriales|f__Enterobacteriaceae	0.07(0.00-5.02)	0.46(0.05-6.74)	0.13(0.03-9.70)	0.0870	0.1994
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptococcaceae	0.49(0.00-1.74)	0.15(0.03-1.57)	0.57(0.03-2.90)	0.0946	0.1994
p__Proteobacteria|c__Epsilonproteobacteria|o__Campylobacterales|f__Helicobacteraceae	0.01(0.00-0.09)	0.03(0.00-0.10)	0.01(0.00-0.09)	0.0967	0.1994
p__Fusobacteria|c__Fusobacteriia|o__Fusobacteriales|f__Fusobacteriaceae	22.69(0.22-32.78)	15.60(12.39-30.58)	21.81(11.23-38.15)	0.2056	0.3991
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Paraprevotellaceae]	2.66(0.04-4.96)	1.18(0.07-6.87)	1.85(0.04-3.56)	0.2445	0.4483
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Prevotellaceae	0.64(0.01-4.18)	0.02(0.01-4.85)	0.22(0.02-2.30)	0.3037	0.5274
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Odoribacteraceae]	0.00(0.00-0.04)	0.00(0.00-0.02)	0.00(0.00-0.03)	0.3764	0.6210
p__Proteobacteria|c__Gammaproteobacteria|o__Aeromonadales|f__Succinivibrionaceae	0.78(0.01-1.54)	0.73(0.15-4.00)	0.84(0.31-2.47)	0.4071	0.6398
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptostreptococcaceae	0.43(0.00-0.90)	0.41(0.11-1.68)	0.50(0.19-3.23)	0.4625	0.6938
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Lactobacillaceae	1.75(0.05-29.12)	9.37(0.32-26.20)	3.34(0.41-27.05)	0.5061	0.7262
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__	0.00(0.00-0.29)	0.00(0.00-0.30)	0.00(0.00-0.31)	0.6007	0.8259
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae	2.33(0.19-18.64)	2.53(0.53-13.05)	2.69(0.36-10.45)	0.7524	0.9501
p__Firmicutes|c__Clostridia|o__Clostridiales|f__	0.41(0.00-0.73)	0.38(0.22-0.64)	0.45(0.16-1.11)	0.7959	0.9501
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__S24-7	0.21(0.02-3.61)	0.03(0.01-2.49)	0.29(0.00-1.52)	0.8251	0.9501
p__Actinobacteria|c__Actinobacteria|o__Bifidobacteriales|f__Bifidobacteriaceae	0.05(0.03-10.57)	0.04(0.02-6.41)	0.05(0.03-13.32)	0.8559	0.9501
p__Deferribacteres|c__Deferribacteres|o__Deferribacterales|f__Deferribacteraceae	0.01(0.00-0.04)	0.00(0.00-0.06)	0.02(0.00-0.05)	0.8788	0.9501
p__Tenericutes|c__Mollicutes|o__Anaeroplasmatales|f__Anaeroplasmataceae	0.00(0.00-0.01)	0.00(0.00-0.01)	0.00(0.00-0.01)	0.9144	0.9501
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae	2.32(0.01-3.96)	2.11(1.09-5.35)	2.21(0.54-3.74)	0.9194	0.9501
p__Firmicutes|c__Clostridia|o__Clostridiales|f__[Mogibacteriaceae]	0.01(0.00-0.04)	0.01(0.00-0.07)	0.01(0.00-0.03)	0.9213	0.9501
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Eubacteriaceae	0.00(0.00-0.04)	0.00(0.00-0.04)	0.00(0.00-0.03)	0.9857	0.9857
p__Fusobacteria|c__Fusobacteriia|o__Fusobacteriales|f__Fusobacteriaceae|Other	0.56(0.00-0.85)	0.21(0.09-0.57)	0.42(0.14-0.67)	<0.0001	0.0003
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|Other	14.12(0.06-27.31)	9.88(4.01-24.37)	14.80(9.95-26.31)	<0.0001	0.0007
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__Slackia	0.05(0.00-0.11)	0.01(0.00-0.09)	0.05(0.00-0.14)	<0.0001	0.0007

Table 7. Continued.

Taxa	median (minumum-maximum)				
	Baseline	Cholestyramine	Washout	p-value	q-value
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__Ruminococcus	0.01(0.00-0.03)	0.00(0.00-0.01)	0.01(0.00-0.04)	0.0001	0.0015
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__	0.83(0.01-1.40)	0.44(0.22-0.79)	0.79(0.18-1.84)	0.0001	0.0015
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Bacteroidaceae|g__Bacteroides	9.12(0.11-12.43)	13.46(6.18-25.93)	6.30(3.63-12.76)	0.0001	0.0015
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__Catenibacterium	0.30(0.00-1.54)	0.05(0.01-1.08)	0.23(0.01-0.84)	0.0002	0.0020
p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Alcaligenaceae|g__Sutterella	1.29(0.02-2.42)	3.40(0.53-8.28)	1.61(0.54-5.59)	0.0002	0.0020
p__Firmicutes|c__Clostridia|o__Clostridiales|Other|Other	0.02(0.00-0.04)	0.01(0.00-0.03)	0.03(0.01-0.06)	0.0003	0.0022
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Blautia	7.23(0.02-11.12)	5.26(1.53-8.21)	6.67(2.46-13.29)	0.0005	0.0031
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__SMB53	0.10(0.00-0.17)	0.08(0.03-0.15)	0.12(0.06-0.19)	0.0006	0.0033
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__[Ruminococcus]	1.13(0.01-1.98)	0.74(0.30-1.85)	1.18(0.48-2.63)	0.0013	0.0062
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Veillonellaceae|g__Megamonas	0.85(0.01-3.10)	0.06(0.02-0.62)	0.80(0.04-2.26)	0.0013	0.0062
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Coprococcus	0.06(0.00-0.10)	0.04(0.01-0.11)	0.05(0.02-0.11)	0.0014	0.0062
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__Collinsella	1.20(0.01-3.27)	0.91(0.09-2.00)	1.52(0.03-3.02)	0.0029	0.0123
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Veillonellaceae|g__Phascolarctobacterium	1.06(0.01-1.99)	0.80(0.23-1.55)	1.33(0.04-3.04)	0.0044	0.0167
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__Oscillospira	0.03(0.00-0.09)	0.01(0.01-0.02)	0.02(0.01-0.08)	0.0045	0.0167
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae|g__Streptococcus	0.38(0.00-30.38)	6.09(0.46-22.38)	1.38(0.18-29.50)	0.0049	0.0168
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Dorea	1.91(0.01-2.68)	1.75(0.66-3.70)	2.33(1.04-3.32)	0.0051	0.0168
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Porphyromonadaceae|g__Parabacteroides	0.04(0.00-0.31)	0.01(0.00-0.07)	0.02(0.00-0.21)	0.0054	0.0170
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__	1.84(0.02-3.14)	1.80(0.99-3.15)	2.01(1.67-4.20)	0.0082	0.0238
p__Actinobacteria|c__Actinobacteria|o__Actinomycetales|f__Actinomycetaceae|g__Actinomyces	0.00(0.00-0.01)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.0083	0.0238
p__Firmicutes|c__Bacilli|Other|Other|Other	0.00(0.00-0.00)	0.00(0.00-0.00)	0.00(0.00-0.00)	0.0128	0.0352
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__Coprobacillus	0.04(0.00-0.16)	0.02(0.00-0.15)	0.05(0.01-0.21)	0.0168	0.0440
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__Clostridium	1.12(0.00-4.04)	1.89(0.47-10.19)	1.34(0.34-6.89)	0.0259	0.0654
p__Fusobacteria|c__Fusobacteriia|o__Fusobacteriales|f__Fusobacteriaceae|g__Cetobacterium	0.00(0.00-0.01)	0.00(0.00-0.04)	0.00(0.00-0.04)	0.0332	0.0805
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__	0.25(0.00-0.62)	0.16(0.05-1.50)	0.33(0.08-0.81)	0.0381	0.0836
p__Actinobacteria|c__Actinobacteria|o__Actinomycetales|f__Microbacteriaceae|g__Leucobacter	0.00(0.00-0.01)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.0383	0.0836

Table 7. Continued.

	median (minumum-maximum)				
Taxa	**Baseline**	**Cholestyramine**	**Washout**	**p-value**	**q-value**
p__Firmicutes|c__Bacilli|o__Turicibacterales|f__Turicibacteraceae|g__Turicibacter	1.53(0.02-9.62)	1.62(0.25-8.37)	3.32(0.26-7.97)	0.0385	0.0836
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__Faecalibacterium	1.60(0.00-2.90)	1.63(0.60-4.46)	1.20(0.32-2.40)	0.0477	0.1002
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Veillonellaceae|g__Veillonella	0.00(0.00-0.15)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.0521	0.1058
p__Proteobacteria|c__Epsilonproteobacteria|o__Campylobacterales|f__Helicobacteraceae|g__Helicobacter	0.01(0.00-0.08)	0.02(0.00-0.10)	0.01(0.00-0.08)	0.0608	0.1198
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Paraprevotellaceae]|g__[Prevotella]	1.80(0.03-3.88)	0.33(0.04-3.52)	1.50(0.03-2.99)	0.0682	0.1302
p__Proteobacteria|c__Epsilonproteobacteria|o__Campylobacterales|f__Helicobacteraceae|g__	0.00(0.00-0.00)	0.00(0.00-0.00)	0.00(0.00-0.00)	0.0746	0.1383
p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacteriales|f__Enterobacteriaceae|g__	0.07(0.00-5.02)	0.46(0.05-6.74)	0.13(0.03-9.70)	0.0870	0.1567
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__	0.70(0.00-1.11)	0.55(0.30-1.26)	0.69(0.23-1.44)	0.0916	0.1603
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptococcaceae|g__Peptococcus	0.49(0.00-1.74)	0.15(0.03-1.57)	0.57(0.03-2.90)	0.0946	0.1611
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptostreptococcaceae|Other	0.06(0.00-0.29)	0.08(0.03-1.12)	0.08(0.03-2.02)	0.1011	0.1676
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__[Eubacterium]	0.36(0.00-1.76)	0.41(0.06-3.38)	0.22(0.03-1.57)	0.1664	0.2688
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Paraprevotellaceae]|g__CF231	0.50(0.00-1.14)	0.69(0.02-3.27)	0.38(0.01-1.22)	0.1773	0.2793
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__	0.00(0.00-0.20)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.1915	0.2943
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Epulopiscium	0.00(0.00-0.01)	0.00(0.00-0.72)	0.00(0.00-0.08)	0.2245	0.3367
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|Other	0.66(0.00-2.31)	0.84(0.20-6.18)	0.55(0.15-3.00)	0.2431	0.3561
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Roseburia	0.00(0.00-0.01)	0.01(0.00-0.03)	0.00(0.00-0.01)	0.2569	0.3665
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__Allobaculum	0.72(0.18-18.34)	0.35(0.08-12.81)	1.21(0.09-10.05)	0.2658	0.3665
p__Fusobacteria|c__Fusobacteriia|o__Fusobacteriales|f__Fusobacteriaceae|g__Fusobacterium	22.11(0.21-32.09)	15.31(12.18-30.46)	21.27(10.89-37.49)	0.2676	0.3665
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Prevotellaceae|g__Prevotella	0.64(0.01-4.18)	0.02(0.01-4.85)	0.22(0.02-2.30)	0.3037	0.4064
p__Proteobacteria|c__Gammaproteobacteria|o__Aeromonadales|f__Succinivibrionaceae|g__	0.01(0.00-0.09)	0.02(0.00-0.13)	0.01(0.00-0.05)	0.3096	0.4064
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Odoribacteraceae]|g__Odoribacter	0.00(0.00-0.04)	0.00(0.00-0.02)	0.00(0.00-0.03)	0.3764	0.4839
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|Other	0.09(0.00-0.29)	0.08(0.02-0.14)	0.10(0.01-0.18)	0.3906	0.4921
p__Proteobacteria|c__Gammaproteobacteria|o__Aeromonadales|f__Succinivibrionaceae|g__Anaerobiospirillum	0.77(0.01-1.50)	0.72(0.14-3.89)	0.83(0.27-2.46)	0.4382	0.5413
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__Adlercreutzia	0.01(0.00-0.04)	0.00(0.00-0.04)	0.00(0.00-0.02)	0.5015	0.6016
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Lactobacillaceae|g__Lactobacillus	1.75(0.05-29.12)	9.37(0.32-26.20)	3.34(0.41-27.05)	0.5061	0.6016

Table 7. Continued.

Taxa	median (minumum-maximum)				
	Baseline	Cholestyramine	Washout	p-value	q-value
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__|g__	0.00(0.00-0.29)	0.00(0.00-0.30)	0.00(0.00-0.31)	0.6007	0.7008
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptostreptococcaceae|g__	0.34(0.00-0.76)	0.32(0.08-1.32)	0.40(0.16-1.22)	0.7823	0.8906
p__Firmicutes|c__Clostridia|o__Clostridiales|f__|g__	0.41(0.00-0.73)	0.38(0.22-0.64)	0.45(0.16-1.11)	0.7959	0.8906
p__Tenericutes|c__Mollicutes|o__Anaeroplasmatales|f__Anaeroplasmataceae|Other	0.00(0.00-0.01)	0.00(0.00-0.01)	0.00(0.00-0.01)	0.8058	0.8906
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__S24-7|g__	0.21(0.02-3.61)	0.03(0.01-2.49)	0.29(0.00-1.52)	0.8251	0.8963
p__Actinobacteria|c__Actinobacteria|o__Bifidobacteriales|f__Bifidobacteriaceae|g__Bifidobacterium	0.05(0.03-10.57)	0.04(0.02-6.41)	0.05(0.03-13.32)	0.8559	0.9139
p__Deferribacteres|c__Deferribacteres|o__Deferribacterales|f__Deferribacteraceae|g__Mucispirillum	0.01(0.00-0.04)	0.00(0.00-0.06)	0.02(0.00-0.05)	0.8788	0.9228
p__Firmicutes|c__Clostridia|o__Clostridiales|f__[Mogibacteriaceae]|g__	0.01(0.00-0.04)	0.01(0.00-0.07)	0.01(0.00-0.03)	0.9213	0.9515
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Eubacteriaceae|g__Pseudoramibacter_Eubacterium	0.00(0.00-0.04)	0.00(0.00-0.04)	0.00(0.00-0.03)	0.9577	0.9732
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Paraprevotellaceae]|g__	0.01(0.00-0.04)	0.01(0.01-0.08)	0.01(0.00-0.02)	0.9828	0.9828
p__Fusobacteria|c__Fusobacteriia|o__Fusobacteriales|f__Fusobacteriaceae|Other|Other	0.56(0.00-0.85)	0.21(0.09-0.57)	0.42(0.14-0.67)	<0.0001	0.0004
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|Other|Other	14.12(0.06-27.31)	9.88(4.01-24.37)	14.80(9.95-26.31)	<0.0001	0.0007
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Blautia|s__	3.51(0.01-7.13)	2.30(0.70-3.40)	3.87(0.85-8.25)	<0.0001	0.0007
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__Slackia|s__	0.05(0.00-0.11)	0.01(0.00-0.09)	0.05(0.00-0.14)	<0.0001	0.0007
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__Ruminococcus|s__	0.01(0.00-0.03)	0.00(0.00-0.01)	0.01(0.00-0.04)	0.0001	0.0016
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__|s__	0.83(0.01-1.40)	0.44(0.22-0.79)	0.79(0.18-1.84)	0.0001	0.0016
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Bacteroidaceae|g__Bacteroides|s__	9.02(0.11-12.35)	13.38(6.12-25.87)	6.16(3.63-12.70)	0.0001	0.0016
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__Catenibacterium|s__	0.30(0.00-1.54)	0.05(0.01-1.08)	0.23(0.01-0.84)	0.0002	0.0022
p__Proteobacteria|c__Betaproteobacteria|o__Burkholderiales|f__Alcaligenaceae|g__Sutterella|s__	1.29(0.02-2.42)	3.40(0.53-8.28)	1.61(0.54-5.59)	0.0002	0.0022
p__Firmicutes|c__Clostridia|o__Clostridiales|Other|Other|Other	0.02(0.00-0.04)	0.01(0.00-0.03)	0.03(0.01-0.06)	0.0003	0.0025
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__Clostridium|s__hiranonis	0.04(0.00-0.07)	0.03(0.01-0.05)	0.04(0.03-0.07)	0.0003	0.0025
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__SMB53|s__	0.10(0.00-0.17)	0.08(0.03-0.15)	0.12(0.06-0.19)	0.0006	0.0038
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__Collinsella|s__stercoris	1.11(0.01-3.12)	0.82(0.09-1.89)	1.44(0.03-2.81)	0.0011	0.0070
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Veillonellaceae|g__Megamonas|s__	0.85(0.01-3.10)	0.06(0.02-0.62)	0.80(0.04-2.26)	0.0013	0.0073

Table 7. Continued.

Taxa	median (minumum-maximum)				
	Baseline	Cholestyramine	Washout	p-value	q-value
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Coprococcus|s__	0.06(0.00-0.10)	0.04(0.01-0.11)	0.05(0.02-0.11)	0.0014	0.0073
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__[Ruminococcus]|s__gnavus	1.05(0.01-1.88)	0.67(0.25-1.77)	1.12(0.43-2.49)	0.0017	0.0083
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__[Ruminococcus]|s__	0.07(0.00-0.24)	0.05(0.03-0.17)	0.07(0.04-0.21)	0.0024	0.0112
p__Actinobacteria|c__Actinobacteria|o__Actinomycetales|f__Actinomycetaceae|g__Actinomyces|s__	0.00(0.00-0.01)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.0037	0.0163
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Veillonellaceae|g__Phascolarctobacterium|s__	1.06(0.01-1.99)	0.80(0.23-1.55)	1.33(0.04-3.04)	0.0044	0.0181
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__Oscillospira|s__	0.03(0.00-0.09)	0.01(0.01-0.02)	0.02(0.01-0.08)	0.0045	0.0181
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae|g__Streptococcus|s__	0.38(0.00-30.32)	6.07(0.45-22.33)	1.37(0.18-29.46)	0.0049	0.0181
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Dorea|s__	1.91(0.01-2.68)	1.75(0.66-3.70)	2.33(1.04-3.32)	0.0051	0.0181
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Porphyromonadaceae|g__Parabacteroides|s__	0.04(0.00-0.31)	0.01(0.00-0.07)	0.02(0.00-0.21)	0.0054	0.0181
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__Clostridium|Other	0.72(0.00-3.48)	1.47(0.27-9.57)	0.67(0.21-6.07)	0.0054	0.0181
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Blautia|Other	0.05(0.00-0.10)	0.03(0.01-0.07)	0.04(0.01-0.13)	0.0058	0.0187
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__|s__	1.84(0.02-3.14)	1.80(0.99-3.15)	2.01(1.67-4.20)	0.0082	0.0251
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Blautia|s__producta	2.97(0.01-5.86)	2.55(0.83-4.89)	2.84(1.32-6.77)	0.0098	0.0291
p__Firmicutes|c__Bacilli|Other|Other|Other|Other	0.00(0.00-0.00)	0.00(0.00-0.00)	0.00(0.00-0.00)	0.0128	0.0367
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__Coprobacillus|s__	0.04(0.00-0.16)	0.02(0.00-0.15)	0.05(0.01-0.21)	0.0168	0.0463
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__[Eubacterium]|s__	0.00(0.00-0.00)	0.00(0.00-0.01)	0.00(0.00-0.00)	0.0274	0.0692
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Lactobacillaceae|g__Lactobacillus|s__ruminis	0.01(0.00-1.34)	0.01(0.00-0.05)	0.01(0.00-0.03)	0.0276	0.0692
p__Fusobacteria|c__Fusobacteriia|o__Fusobacteriales|f__Fusobacteriaceae|g__Cetobacterium|s__somerae	0.00(0.00-0.01)	0.00(0.00-0.04)	0.00(0.00-0.04)	0.0277	0.0692
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__Collinsella|s__	0.07(0.00-0.16)	0.05(0.00-0.16)	0.09(0.00-0.21)	0.0326	0.0790
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__|s__	0.25(0.00-0.62)	0.16(0.05-1.50)	0.33(0.08-0.81)	0.0381	0.0855
p__Actinobacteria|c__Actinobacteria|o__Actinomycetales|f__Microbacteriaceae|g__Leucobacter|s__	0.00(0.00-0.01)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.0383	0.0855
p__Firmicutes|c__Bacilli|o__Turicibacterales|f__Turicibacteraceae|g__Turicibacter|s__	1.53(0.02-9.62)	1.62(0.25-8.37)	3.32(0.26-7.97)	0.0385	0.0855
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae|g__Streptococcus|Other	0.00(0.00-0.04)	0.01(0.00-0.05)	0.00(0.00-0.04)	0.0461	0.0997
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__Faecalibacterium|s__prausnitzii	1.60(0.00-2.89)	1.62(0.60-4.45)	1.19(0.32-2.40)	0.0477	0.1004
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Veillonellaceae|g__Veillonella|s__	0.00(0.00-0.15)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.0521	0.1068

Table 7. Continued.

Taxa	median (minumum-maximum)				
	Baseline	Cholestyramine	Washout	p-value	q-value
p__Proteobacteria|c__Epsilonproteobacteria|o__Campylobacterales|f__Helicobacteraceae|g__Helicobacter|s__	0.01(0.00-0.08)	0.02(0.00-0.10)	0.01(0.00-0.08)	0.0608	0.1217
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Paraprevotellaceae]|g__[Prevotella]|s__	1.80(0.03-3.88)	0.33(0.04-3.52)	1.50(0.03-2.99)	0.0682	0.1331
p__Proteobacteria|c__Gammaproteobacteria|o__Enterobacteriales|f__Enterobacteriaceae|g__|s__	0.07(0.00-5.02)	0.46(0.05-6.74)	0.13(0.03-9.70)	0.0870	0.1658
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__|s__	0.70(0.00-1.11)	0.55(0.30-1.26)	0.69(0.23-1.44)	0.0916	0.1704
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptococcaceae|g__Peptococcus|s__	0.49(0.00-1.74)	0.15(0.03-1.57)	0.57(0.03-2.90)	0.0946	0.1720
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptostreptococcaceae|Other|Other	0.06(0.00-0.29)	0.08(0.03-1.12)	0.08(0.03-2.02)	0.1011	0.1797
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__[Eubacterium]|s__biforme	0.36(0.00-1.75)	0.40(0.05-3.38)	0.21(0.02-1.56)	0.1098	0.1909
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__[Eubacterium]|s__dolichum	0.01(0.00-0.12)	0.01(0.00-0.07)	0.01(0.00-0.01)	0.1135	0.1932
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Paraprevotellaceae]|g__CF231|s__	0.50(0.00-1.14)	0.69(0.02-3.27)	0.38(0.01-1.22)	0.1773	0.2956
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|g__Faecalibacterium|Other	0.00(0.00-0.01)	0.01(0.00-0.02)	0.00(0.00-0.01)	0.1823	0.2976
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__|s__	0.00(0.00-0.20)	0.00(0.00-0.00)	0.00(0.00-0.01)	0.1915	0.3065
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Bacteroidaceae|g__Bacteroides|s__coprophilus	0.06(0.00-0.17)	0.04(0.01-0.22)	0.06(0.00-0.16)	0.2123	0.3331
p__Proteobacteria|c__Epsilonproteobacteria|o__Campylobacterales|f__Helicobacteraceae|g__|s__	0.00(0.00-0.03)	0.00(0.00-0.01)	0.00(0.00-0.02)	0.2191	0.3371
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Epulopiscium|s__	0.00(0.00-0.01)	0.00(0.00-0.72)	0.00(0.00-0.08)	0.2245	0.3388
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|Other|Other	0.66(0.00-2.31)	0.84(0.20-6.18)	0.55(0.15-3.00)	0.2431	0.3601
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Lachnospiraceae|g__Roseburia|s__	0.00(0.00-0.01)	0.01(0.00-0.03)	0.00(0.00-0.01)	0.2569	0.3736
p__Firmicutes|c__Erysipelotrichi|o__Erysipelotrichales|f__Erysipelotrichaceae|g__Allobaculum|s__	0.72(0.18-18.34)	0.35(0.08-12.81)	1.21(0.09-10.05)	0.2658	0.3756
p__Fusobacteria|c__Fusobacteriia|o__Fusobacteriales|f__Fusobacteriaceae|g__Fusobacterium|s__	22.11(0.21-32.09)	15.31(12.18-30.46)	21.27(10.89-37.49)	0.2676	0.3756
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Streptococcaceae|g__Streptococcus|s__luteciae	0.00(0.00-0.01)	0.00(0.00-0.01)	0.00(0.00-0.01)	0.2817	0.3886
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Prevotellaceae|g__Prevotella|s__copri	0.64(0.01-4.18)	0.02(0.01-4.85)	0.22(0.02-2.30)	0.3037	0.4118
p__Proteobacteria|c__Gammaproteobacteria|o__Aeromonadales|f__Succinivibrionaceae|g__|s__	0.01(0.00-0.09)	0.02(0.00-0.13)	0.01(0.00-0.05)	0.3096	0.4128
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__Clostridium|s__perfringens	0.05(0.00-2.23)	0.16(0.03-0.26)	0.07(0.01-0.68)	0.3314	0.4347
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Odoribacteraceae]|g__Odoribacter|s__	0.00(0.00-0.04)	0.00(0.00-0.02)	0.00(0.00-0.03)	0.3764	0.4857
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Ruminococcaceae|Other|Other	0.09(0.00-0.29)	0.08(0.02-0.14)	0.10(0.01-0.18)	0.3906	0.4959
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Clostridiaceae|g__Clostridium|s__	0.19(0.00-0.88)	0.29(0.03-0.53)	0.24(0.05-0.68)	0.4375	0.5393

Table 7. Continued.

	median (minumum-maximum)				
Taxa	**Baseline**	**Cholestyramine**	**Washout**	**p-value**	**q-value**
p__Proteobacteria|c__Gammaproteobacteria|o__Aeromonadales|f__Succinivibrionaceae|g__Anaerobiospirillum|s__	0.77(0.01-1.50)	0.72(0.14-3.89)	0.83(0.27-2.46)	0.4382	0.5393
p__Firmicutes|c__Bacilli|o__Lactobacillales|f__Lactobacillaceae|g__Lactobacillus|s__	1.75(0.05-29.07)	9.36(0.32-26.14)	3.34(0.41-27.01)	0.4839	0.5865
p__Actinobacteria|c__Coriobacteriia|o__Coriobacteriales|f__Coriobacteriaceae|g__Adlercreutzia|s__	0.01(0.00-0.04)	0.00(0.00-0.04)	0.00(0.00-0.02)	0.5015	0.5988
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Bacteroidaceae|g__Bacteroides|s__plebeius	0.01(0.00-0.03)	0.01(0.00-0.07)	0.01(0.00-0.02)	0.5784	0.6804
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__|g__|s__	0.00(0.00-0.29)	0.00(0.00-0.30)	0.00(0.00-0.31)	0.6007	0.6964
p__Actinobacteria|c__Actinobacteria|o__Bifidobacteriales|f__Bifidobacteriaceae|g__Bifidobacterium|s__	0.00(0.00-0.07)	0.00(0.00-0.02)	0.00(0.00-0.08)	0.6434	0.7353
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__Bacteroidaceae|g__Bacteroides|s__uniformis	0.01(0.00-0.12)	0.01(0.00-0.11)	0.01(0.00-0.16)	0.7459	0.8405
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Peptostreptococcaceae|g__|s__	0.34(0.00-0.76)	0.32(0.08-1.32)	0.40(0.16-1.22)	0.7823	0.8595
p__Firmicutes|c__Clostridia|o__Clostridiales|f__|g__|s__	0.41(0.00-0.73)	0.38(0.22-0.64)	0.45(0.16-1.11)	0.7959	0.8595
p__Actinobacteria|c__Actinobacteria|o__Bifidobacteriales|f__Bifidobacteriaceae|g__Bifidobacterium|Other	0.05(0.03-10.49)	0.04(0.02-6.40)	0.05(0.03-13.22)	0.7976	0.8595
p__Tenericutes|c__Mollicutes|o__Anaeroplasmatales|f__Anaeroplasmataceae|Other|Other	0.00(0.00-0.01)	0.00(0.00-0.01)	0.00(0.00-0.01)	0.8058	0.8595
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__S24-7|g__|s__	0.21(0.02-3.61)	0.03(0.01-2.49)	0.29(0.00-1.52)	0.8251	0.8686
p__Deferribacteres|c__Deferribacteres|o__Deferribacterales|f__Deferribacteraceae|g__Mucispirillum|s__schaedleri	0.01(0.00-0.04)	0.00(0.00-0.06)	0.02(0.00-0.05)	0.8788	0.9131
p__Firmicutes|c__Clostridia|o__Clostridiales|f__[Mogibacteriaceae]|g__|s__	0.01(0.00-0.04)	0.01(0.00-0.07)	0.01(0.00-0.03)	0.9213	0.9449
p__Firmicutes|c__Clostridia|o__Clostridiales|f__Eubacteriaceae|g__Pseudoramibacter_Eubacterium|s__	0.00(0.00-0.04)	0.00(0.00-0.04)	0.00(0.00-0.03)	0.9577	0.9698
p__Bacteroidetes|c__Bacteroidia|o__Bacteroidales|f__[Paraprevotellaceae]|g__|s__	0.01(0.00-0.04)	0.01(0.01-0.08)	0.01(0.00-0.02)	0.9828	0.9828

Discussion

In this study, 12 healthy dogs were administered cholestyramine in their food once daily for a duration of two weeks. The fecal microbiome and bile acid profile were evaluated. When dogs received cholestyramine, there were significant increases in the fecal concentration of DCA, secondary fUBA, and total fUBA. When measured as a percent of total fUBA, LCA significantly decreased while DCA significantly increased while dogs were on cholestyramine.

There were significant shifts in microbial communities based on both beta diversity metrics. No significant changes were noted for species richness between the groups. Univariate statistics identified a multitude of significant differences in bacterial abundances after adjusting for multiple comparisons.

A previous study showed that the sum of DCA, CDCA, and CA excretion increases with administration of cholestyramine in a dose dependent fashion up to 6 grams per day (i.e., 446 mg of bile acids/day) (Jansen and Zanetti, 1965). The main goal of that study was to investigate dose and resin particulate size and its ability reduce plasma cholesterol in healthy patients as it may pertain to patients suffering from coronary heart disease. That study reported that plasma cholesterol increased as resin doses increased from 1 to 3, 6, and 10 gm/dog/day. Other studies evaluating the effects of cholestyramine on dogs have used similar dosage strategies as that in the present study (i.e., 0.7 g/kg) and provides sufficient evidence that this dosing strategy is likely to be safe and efficacious in eliciting systemic changes in dogs when administering cholestyramine (Gans and Cater, 1971). In dogs, cholestyramine has also been used to treat cyanobacterial (microcystin) toxicosis

(dose: 172 mg/kg q 24 h) (Rankin et al., 2013). The findings of the current study are similar to the study described previously in dogs fed cholestyramine.

Cholestyramine as a bile acid sequestrant has been utilized and is efficacious in managing primary bile acid diarrhea which occurs in approximately 32% of patients with diarrhea predominant IBS and general chronic GI disease (Wedlake et al., 2009). Bile acid diarrhea can be diagnosed by the serum C4 test or SeHCAT retention test, and by clinical response to bile acid sequestrants (Vijayvargiya et al., 2013). Bile acid dysmetabolism is prevalent in a subset of dogs with CE. Anecdotal evidence suggest that some dogs with chronic diarrhea unresponsive to traditional therapy (e.g., antimicrobials, immunosuppressive drugs, and dietary trials) may respond to cholestyramine.

Unpublished data from our lab suggests that the fecal bile acid pool in a subset of canine patients with chronic diarrhea is comprised almost exclusively of fecal primary bile acids. In the present study, cholestyramine significantly increased the concentration of DCA in feces. Deoxycholic acid is found in the highest concentration in feces compared to the other bile acids measured. In patients with chronic diarrhea, primary bile acids are found to be in the highest concentration in feces and decreasing this concentration may be useful.

Cholestyramine has reportedly been useful in cases of *Clostridium difficile* infection (Moncino and Falletta, 1992). It is thought that the factor that allows for germination of *Clostridium difficile* spores is associated with bile salts thereby allowing cholestyramine to effectively promote inhibition thereof. Cholestyramine has also been shown to bind *C. difficile* toxins A and B in vitro (Taylor and Bartlett, 1980).

Cholestyramine is not the first method of therapeutics for this *C. difficile* infection as cholestyramine is thought to bind to Vancomycin, which is a commonly used antimicrobial for the first defense against *C. difficile* infection.

There is no available evidence in scientific literature that has yet to evaluate the effect of cholestyramine on the fecal microbiota. This study provides useful insight into understanding the effects of cholestyramine in the GIT given the importance of bile acids and microbiota in maintaining gut. In the current study, dogs fed cholestyramine had lower fecal scores (firmer stools) than controls (mean; p-value: 2.48 and 1.98, respectively; p=0.0028). Interestingly, the change in microbial communities (i.e., beta diversity) was not accompanied by a change in species richness (i.e., alpha diversity). *Clostridium hiranonis* was found to significantly decrease after cholestyramine administration. This interaction may be explained by the 7α-dehyxroxylating function that belongs to this organism which converts primary to secondary bile acids. This could suggest a lack of substrate availability for the organism and may explain the decreased proportion of *C. hiranonis* according to sequencing results (Kitahara et al., 2001).

The present study provides a foundation to understanding the effects of cholestyramine in healthy dogs which may later be useful in extrapolating these effects to dogs with chronic GI disease. This study is limited in that it did not explore varieties of dosage. This may be useful in future studies when evaluating if dogs with chronic GI disease may be able to benefit from cholestyramine administration.

CHAPTER VI

CONCLUSIONS

CE in dogs encompasses a number of diseases that are idiopathic in nature, and therefore, difficult to diagnose. Current research in dogs with CE has focused on a number of components to explain and categorize these different disease phenotypes. The microbiome is an area of focus in dogs with CE as well as in humans with IBD. A microbial dysbiosis has been identified in dogs with CE and IBD and remains present even after months of therapy. Describing the intestinal microbiota on a phylogenetic level (e.g., using sequencing of 16S rRNA genes) does not provide information pertaining to the function of bacteria within the GI tract. Researchers are now using platforms such as gas or liquid chromatography coupled with mass spectrometry to assess metabolite profiles that can better describe functional aspects of the GI tract. An untargeted approach is beneficial in that it unbiasedly identifies and then calculates the relative abundance of metabolites in a given sample. A targeted approach has the benefit of selectively identifying compounds and measuring the actual concentration based on standard curves. Recently, in an unpublished pilot study, our laboratory acquired untargeted metabolomic data from the feces of healthy dogs and dogs with CE. Fecal bile acids were significantly altered in dogs with CE. Bile acids are being widely considered as an important regulator of host health, given that bile acids mechanisms contribute to obesity, glycemic control, and the treatment of recurrent *Clostridium difficile* infection in humans (Oduyebo and Camilleri, 2017). Recent evidence in people suggests that bile acid malabsorption can account for 30% of cases of chronic diarrhea.

These findings coupled with current literature describing bile acid dysmetabolism in a number of human diseases prompted further investigation into the role bile acids have in the GI tract in healthy dogs and dogs with CE. We developed an assay for fUBA that was precise and reproducible. Benefits for developing an in-house assay included the rapid identification and quantification of fUBA. A targeted assay requires less time data mining as opposed to untargeted metabolomics. The purpose of this assay was to validate our previous untargeted metabolomic data in dogs with CE in a larger sample size of new and well-characterized patients with CE. This assay was developed using known standards to accurately identify and quantify fUBA. The developed assay may be beneficial to clinicians in that it has a fast turn-around time (i.e., approximately 2 days) and requires a single fecal sample. Naturally passed fecal samples are inherently excellent candidates for diagnostic assays. Fecal samples are non-invasive to collect and provide a snapshot of upstream metabolic activity. Fecal samples can also be collected by patient's owners in the comfort of their own home. This decreases the likelihood of the patient's surroundings affecting systemic metabolic activity (i.e., stress and fear). The entire GI tract has influence over fecal output which also contributes to the quality of the sample. Pitfalls of fecal samples are that they are heterogeneous in composition and can often require multiple steps of isolation to generate an extract with few impurities. The assay described within this body of research was time efficient and required minimal isolation of bile acids from feces.

Our study identified that dogs with CE had significantly decreased secondary fUBA. A variety of reports in humans had identified bile acid malabsorption either by

serum tests (C4) or by the radio labeled SeHCAT test (i.e., the gold standard for testing bile acid malabsorption). Recent literature by Duboc et al., demonstrated that patients with IBD have decreased proportions of secondary bile acids and increased proportions of primary bile acids. While we did not measure serum bile acids or use standardized tests for bile acid malabsoprtion, we were able to identify bile acid malabsorption in dogs with CE through measuring fecal bile acids. We measured fecal bile acids in a number of healthy dogs. Secondary fUBA are expected to be found almost completely in the colon because of bacterial deconjugation and dehydroxylation. Duboc et al., also measured conjugated BA in feces, which accounted for approximately 3% of bile acids in healthy human subjects and approximately 9% of bile acids in human patients with active IBD. Unfortunately, our assay was unable to measure conjugated bile acids. An imbalance in secondary to primary fUBA may indicate a lack of bile acid absorption as well as microbial imbalance for those microbes responsible for this conversion. Most healthy dogs had between 90-100% secondary fUBA.

It was evident that the fecal bile acid profile may be impacted long term by antibiotic administration. Many dogs that were considered clinically healthy but had received antibiotics within two weeks prior to sample collection had a fecal bile acid profile almost completely absent of secondary fUBA. It is possible that antibiotic administration or microbial dysbiosis weeks or maybe even months to years before fecal collection may cause residual decreased secondary fUBA proportions in otherwise clinically healthy dogs. A proposed reference interval for the proportion of secondary fUBA was created for healthy dogs. Twenty out of 34 dogs with CE had a proportion of

secondary fUBA outside of the reference interval. The proportion of secondary fUBA significantly increased in patients with CE that were treated with immunosuppressive therapy over time and fUBA profiles returned within the reference interval described for the proportion of secondary fUBA. In the future, a new cohort of healthy dogs should be carefully screened and the sample size should be increased to validate the reference interval for healthy dogs in this study. Decreased proportions of secondary fUBA in dogs with CE was counteracted by increased CA in the feces of dogs with CE. It is unclear if increased CA or decreased presence of secondary fUBA is a driver of clinical signs of GI disease. UDCA and LCA have been established as having anti-inflammatory and cytoprotective effects in the colon, which may suggest the latter (Ward et al., 2017).

Bile acid receptors such as the farnesoid X receptor may be an area of therapeutic manipulation since it is a key regulator of bile acid synthesis found in both the enterocytes and hepatocytes. When activated, it inhibits the transcription of genes that participate in bile acid synthesis. When activated in enterocytes, it is an agonist for fibroblast growth factor 19 which in turn navigates to the hepatocytes closing the negative feedback loop (Pavlidis et al., 2015).

Characterizing the fecal bile acid profile in dogs with CE prompts further questioning in how clinicians may implement this knowledge in practice. The microbiome and fecal bile acid profile was evaluated in healthy dogs administered cholestyramine. Cholestyramine is a bile acid sequestrant well-known for its ability to mitigate bile acid diarrhea in human patients. Secondary fUBA increased after cholestyramine administration. Dogs with CE have long been treated using a variety of different

approaches including antimicrobials, food trials, and immunosuppressive drugs. Empirical treatment of CE using these methods however, can cause antibiotic induced microbial dysbiosis and immunosuppressive therapy can weaken the patient's immune system and subsequently becoming less resistant to infection. Cholestyramine may be a candidate for therapeutic management of chronic canine GI disease. Cholestyramine could two-fold increase our level of understanding and care for patients with CE by 1) treating and properly identifying the underlying cause of GI clinical signs and disease and 2) potentially avoid side effects of other commonly used modes of therapy.

While we did not trial cholestyramine in dogs with CE we would likely expect a different outcome than what was observed in healthy dogs. In dogs that received cholestyramine the concentration of DCA was found to be increased. DCA is also the most predominant fUBA identified in the feces of healthy dogs so it makes sense that cholestyramine primarily affects this compound. In patients with CE, our aim may be to restore the fecal bile acid profile to that of healthy dogs (i.e., mostly secondary fUBA). If we were to expect cholestyramine to have the greatest effect on the most predominant fUBA in dogs with CE then we might expect CA to be significantly altered. This may not be so simple, however, since some literature suggests cholestyramine binds preferentially to DCA and CDCA. In this scenario, other bile acid sequestrants should be trialed.

An inherent difficulty in understanding the underlying etiology behind CE in dogs is that patients are lost to follow-up. Long term outcome measures can help to establish whether certain serological or fecal markers are prognostic of positive or negative outcomes after months or years of treatment. In this collection of research, an untargeted

metabolomics approach was used to characterize the feces of dogs with IBD. This analysis revealed little overlap between healthy dogs and dogs with IBD at baseline according to 95% confidence intervals. Over the course of treatment and after 1 year later, the 95% confidence interval of dogs with IBD began to share considerable more overlap with healthy dogs. Amino acids were identified as being significantly different between healthy dogs and dogs with IBD sampled at baseline, 3 weeks post therapy, 8 weeks post therapy, and more than 1 year after therapy. Almost 100 additional compounds were found to be significantly altered between the sample groups. This study suggests that there are many more changes occurring in the feces of dogs with IBD than simply changes in bile acid metabolism. Nevertheless, fecal bile acid dysmetabolism in dogs with CE is clearly evident and likely a major player in disease etiology at least in a subset of dogs. Studies using fecal microbial transplantation in humans with *Clostridium difficile* infection are now providing some of the most supportive evidence behind the importance of maintaining normal bile acid metabolism in the GI tract. Many patients with *C. diff.* infection also have bile acid dysmetabolism and clinical symptoms that can be ameliorated by fecal microbial transplantation.

In summary, a fecal bile acid dysmetabolism was reported in dogs with CE. This is characterized by a decrease in the percent of fUBA in dogs with CE. Cholestyramine, is effective in modulating the fecal bile acid composition and microbial community in healthy dogs and may be effective in treating those dogs with CE that fall outside of the reference interval described previously. These studies support the need for further investigation of bile acids along the GI tract. Our lab is currently evaluating

cholestyramine as a therapeutic approach in treating dogs with CE as well as characterizing fecal bile acid profiles in dogs that are administered fecal microbial transplantation.

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