INTESTINAL MICROBIOTA AND ITS FUNCTIONAL ASPECTS IN DOGS WITH GASTROINTESTINAL DISEASES

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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August 2015

Major Subject: Biomedical Sciences

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ABSTRACT

Accumulating evidence has shown a significant relationship between the GI microbiota and GI health of the host. An alteration in the intestinal microbial community structure, referred to as intestinal dysbiosis, can significantly affect host GI health and play a pivotal role in the pathogenesis of GI diseases. This study aimed to evaluate the relationship between an enteric pathogen and intestinal dysbiosis, and to describe the functional aspects of the intestinal microbiota in dogs with GI diseases. Firstly, this study evaluated the relationship between diarrhea, intestinal dysbiosis, and the presence of *C. perfringens by* quantitative PCR and its enterotoxin (CPE) by ELISA in feces. The presence of CPE as well as fecal dysbiosis were associated with GI disease. However, the presence of *C. perfringens* was not indicative of GI disease. Furthermore, an increased abundance of enterotoxigenic *C. perfringens* may be a feature of intestinal dysbiosis that is associated with GI disease. Secondly, this study characterized fecal concentrations of short-chain fatty acids (SCFAs) in dogs with clinical signs of chronic GI disease. Dogs with clinical signs of chronic GI disease had decreased fecal concentrations of acetate, propionate, and total short-chain fatty acids. Finally, this study characterized the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease (IBD) by using 454-pyrosequencing of 16S rRNA genes and an untargeted metabolomics approach. This study also evaluated the effects of 3 weeks of medical treatment on both the GI microbiota and serum metabolite profiles in

dogs with IBD. Significantly lower bacterial diversity and distinct microbial communities were observed in dogs with IBD compared to healthy control dogs. Based on the metabolite profiles, this study identified several potential biomarkers. Although a clinical improvement was observed after medical therapy in all dogs with IBD, this was not accompanied by significant changes in the fecal microbiota or in serum metabolite profiles, suggesting an ongoing intestinal disease process. In conclusion, the findings of these studies provided new insights into the pathogenesis of canine GI diseases and highlight the importance of balanced microbial communities for canine GI health.

DEDICATION

To Tomomi and my family

ACKNOWLEDGEMENTS

I would like to thank my committee members Dr. James Barr, Dr. Sara Lawhon and Dr. Rosemary Walzem for their guidance and support through the course of this research.

My special thanks go to Dr. Jan Suchodolski and Dr. Jörg Steiner for giving me the opportunity to pursue my research career and providing guidance and support. Finally, thanks to my friends, colleagues, and staff at the GI lab for their contributions to this research.

NOMENCLATURE

16S rRNA	16S ribosomal RNA
ANOSIM	Analysis of similarities
GC-TOF/MS	Gas chromatography coupled with time-of-flight mass spectrometry
CIBDAI	Canine IBD activity index
CPE	*Clostridium perfringens* enterotoxin
cpe gene	*C. perfringens* enterotoxin gene
ELISA	Enzyme-linked immunosorbent assay
FDR	False Discovery Rate
GI	Gastrointestinal
GPRs	G-protein coupled receptors
HDAC	Histone deacetylase
IBD	Idiopathic inflammatory bowel disease
KEGG	Kyoto Encyclopedia of Genes and Genomes
LEfSe	Linear discriminant analysis (LDA) effect size
MCT	Monocarboxylate transporter
PCA	Principal component analysis
PCoA	Principal coordinates analysis
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states

qPCR	Quantitative real-time polymerase chain reaction
ROC	Receiver operating characteristic
RPLA	reverse passive latex agglutination assay
SMCT	Sodium-coupled monocarboxylate transporter
SCFAs	Short-chain fatty acids
T_{reg}	Regulatory T cell

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I.1 Microbial communities in the canine gastrointestinal tract

The mammalian gastrointestinal (GI) tract harbors trillions of microorganisms (bacteria, archaea, fungi, protozoa, and viruses), collectively termed the GI microbiota.[1] Bacteria are the most predominant domain and may represent more than 98% of GI microbiota.[2,3] Due to physiological and environmental differences, the composition of the microbiota differs along the length of GI tract.[1,4] In general, the microbiota increases in abundance along the GI tract, from the stomach to the colon. Anaerobic bacterial groups predominate in the distal portions of the GI tract, whereas a more equal distribution of aerobic and anaerobic bacteria has been observed in the proximal portions of the GI tract.[4] This distribution of bacterial groups is similar to those observed in humans and other animal species including cats, a finding that highlights the genetic and environmental factors that play a major role in shaping the host microbiota.[5,6]

A study using bacterial cultivation techniques reported that up to 10^6 cfu /g of bacteria are present in the canine stomach.[7] Recent study using 454-pyrosequencing of 16S rRNA genes, fluorescence in situ hybridization (FISH), and quantitative real-time PCR (qPCR), evaluated the microbial composition of gastric mucosal biopsies from healthy dogs.[8] A median of 36 (range of 18–119) operational taxonomic units (OTUs) were detected. The majority of the sequences belonged to the phylum Proteobacteria (99.6%), with only a few sequences belonging to the phylum Firmicutes (0.3%). The

genus *Helicobacter* spp. was the most abundant genus (98.6 %) within the phylum Proteobacteria.[8]

In studies using cultivation techniques, the reported duodenal bacterial counts ranged from 10^2 to 10^6 cfu/g of luminal content in healthy dogs.[7,9] However, up to 10^9 cfu/g of bacteria have been reported in clinically healthy dogs.[7,8] In duodenal mucosal biopsy samples from healthy dogs, 9 bacterial phyla, with Proteobacteria (average 34.9%), Bacteroidetes (24.2%), Fusobacteria (20.7%), and Firmicutes (17.7%) being the most abundant, were identified in a study using 454-pyrosequencing of 16S rRNA genes.[12] At the genus level, *Fusobacterium* spp. (14.5%), *Bacteroides* spp. (11.2%), and *Clostridium* spp. (7.2%) were the most abundant in duodenal biopsy samples.[12]

The total bacterial count in the jejunum was similar to that in the duodenum and was reported to be approximately 10^6 cfu/g.[7] In a study using 454-pyrosequencing of 16S rRNA genes, 10 different bacterial phyla were identified in jejunal luminal contents. The major bacterial phyla were Proteobacteria (46.7%), Firmicutes (15.0%), Actinobacteria (11.2%), Spirochaetes (14.2%), Bacteroidetes (6.2%), and Fusobacteria (5.4%). The most abundant bacterial classes were Gamma-proteobacteria (~40%), followed by Spirochaetes (~13%), Actinobacteria (~12%), Clostridia (~10%), Bacteroidetes (~6%), Fusobacteria (~4%), and Alpha-proteobacteria (~3%).[13]

Along the small intestine, the ileum harbored the highest bacterial load (approximately 10^7 cfu/g) in healthy dogs.[7] In a study analyzing constructed 16s rRNA gene clone libraries, the predominant bacterial phyla in luminal contents from the ileum were Fusobacteria, Firmicutes, and Bacteroidetes. At the order level, Fusobacteriales

(32.6%), Clostridiales (24.8%), Bacteroidales (22.7%), and Enterobacteriales (18.4%) dominated in ileal contents.[4]

The large intestine harbors the highest density and diversity of microbial communities.[4] The reported cultivated bacterial counts in the colon in dogs range between 10^9 to 10^{11} cfu/g with the predominant phyla being Fusobacteria, Bacteroidetes, and Firmicutes.[4,7] Bacteroidales (30.3%) is the most predominant order in the colonic contents of healthy dogs, followed by Fusobacteriales (28.9%) and Clostridiales (26.1%).[4] The microbial communities in feces have been studied intensively due to the noninvasive nature of sample collection and accessibility. The phylum Firmicutes, Bacteroidetes and Fusobacteria are the most predominant phyla reported in most studies.[3,9,14–17] However, there are inconsistencies in regard to the reported proportions of these bacterial groups between studies. The reported proportion of Firmicutes ranged from 15% to >95% and the reported proportion of Bacteroidetes ranged from 0.1% to 38%.[3,16] These discrepancies between studies may be due to the differences in sample handling (e.g., storage condition, repeated freeze and thaw cycles of samples), DNA extraction protocols, targeted regions of the 16S rRNA gene, and the techniques utilized to characterize the microbiota.[18,19]

I.2 Alterations of the canine intestinal microbiota in gastrointestinal disease

An alteration of the intestinal microbial community structure, called intestinal dysbiosis, can significantly affect host GI health and play a pivotal role in the pathogenesis of various GI diseases.[20–22] Intestinal dysbiosis may result in a

dysregulation of adaptive immune responses, and lead to inflammation and/or reduced immunological activity against infection.[21] Furthermore, microbial disturbances may result in functional changes of the intestine, leading to altered intestinal permeability, and changes in metabolic functions (e.g., deconjugation of bile acids and reduced carbohydrate utilization) that lead to malabsorption and maldigestion.[20] As a consequence, intestinal dysbiosis does not only affect the GI tract, but also the overall host health. In humans and rodent models, also a wide variety of extra-intestinal diseases, including diabetes mellitus, stress, asthma, and atopy, have been associated with intestinal dysbiosis.[23,24]

With recent advances in high-throughput DNA sequencing technology, molecular approaches have become routine tools for ecological studies of the intestinal microbiota in the field of veterinary medicine and have revealed a highly diverse and complex intestinal ecosystem in the GI tract of dogs.[3,4] Studies have also shown the presence of intestinal dysbiosis in dogs with various GI diseases.[12,16,17,25–29]

I.2.1 Alterations of the canine intestinal microbiota in acute gastrointestinal disease

Bell et al. assessed changes in the GI microbiota associated with acute episodes of diarrhea by using T-RFLP and qPCR, and observed large-scale changes of the GI microbiota during episodes of diarrhea.[25] These included an increased abundance of Clostridiales (especially *C. perfringens*) and *Enterococcus* spp. In another recent study using 454-pyrosequencing of 16S rRNA genes, distinct fecal microbial communities were also observed between healthy control dogs and dogs with acute diarrhea.[16]

Authors also applied qPCR assays and found profound changes of specific bacterial groups in dogs with acute hemorrhagic diarrhea. While *Blautia* spp., *Faecalibacterium* spp., and *Turicibacter* spp. decreased, *Sutterella* spp. and *C. perfringens* increased significantly in dogs with acute hemorrhagic diarrhea compared to healthy dogs.[16]

I.2.2 Alterations of the canine intestinal microbiota in chronic gastrointestinal disease

Intestinal dysbiosis has also been observed in dogs with chronic GI disease. Jia et al. utilized FISH and DGGE to investigate the composition of the fecal microbiota in dogs with chronic diarrhea. FISH analysis revealed a significantly higher *Bacteroides* spp. count in dogs with chronic diarrhea compared to healthy control dogs.[17] The proportion of the *Lactobacillus–Enterococcus* and *Bifidobacterium* spp. were not significantly different between control dogs and dogs with chronic diarrhea.[17] Allenspach et al. constructed 16S rRNA gene clone libraries from duodenal brush samples from healthy Greyhounds and German shepherd dogs with chronic enteropathy.[27] At the phylum level, a higher abundance of Actinobacteria was observed in dogs with chronic enteropathy than in healthy controls. At the order level, the abundances and frequencies of Lactobacillales, Actinomycetales, and Erysipelotrichales were significantly higher than those in healthy dogs. Within the order Lactobacillales, *Streptococcus* spp. and *Abiotrophia* spp. were more frequently found in dogs with chronic enteropathy than in healthy controls.[27]

I.2.3 Alterations of the canine intestinal microbiota in idiopathic inflammatory bowel disease

Idiopathic inflammatory bowel disease (IBD) is a common chronic GI disorder in dogs.[30] Similarly to humans, an interplay between the intestinal microbiota together with an underlying genetic susceptibility of the host and dietary and environmental factors, are implicated in the development of IBD.[31] In humans with IBD, a lower abundance of Firmicutes and Bacteroidetes, and a higher abundance of Proteobacteria compared to healthy subjects have been observed.[32] Similar changes in the small intestinal microbiota have been observed in dogs with IBD. Xenoulis et al. constructed 16S rRNA gene clone libraries from duodenal brush samples from healthy dogs and dogs with IBD.[29] In this study, a significantly lower species richness and a significantly higher proportion of Enterobacteriaceae were observed in dogs with IBD.[29] Suchodolski et al. applied the same technique to duodenal mucosal biopsies from dogs with IBD. These dogs had a significantly higher abundance of Proteobacteria and a significantly lower abundance of Clostridia.[4] More recently, two studies evaluating the fecal microbiota reported a lower abundance of *Faecalibacterium* spp. in dogs with IBD.[16,33] In addition to this finding, Rossi et al. also observed a lower abundance of *Turicibacter* spp..[33]

Commonly observed alterations in the fecal microbiota in dogs with GI disease are decreases in Ruminococcaceae, *Faecalibacterium* spp., *Turicibacter* spp., and Bacteroidetes, with concurrent increases in Proteobacteria, specifically *E. coli*.[16,33] However, compared to what is known in humans, the information regarding the bacterial composition of the intestinal microbiota in dogs is still limited. Understanding changes

in the microbiota, together with the associated functional alterations may lay the foundation for future studies aiming to develop interventions (e.g., antibiotics, prebiotics or probiotics) to manipulate GI microbial communities in dogs with GI diseases.

I.3 Functional aspects of the gastrointestinal microbiota

Animals have co-evolved to exist with their GI microbiota in a mutualistic relationship where animals provide a uniquely suited environment for the microbiota in return for physiologic benefits provided to the host.[1,21,34] The resident microbiota provides many health benefits to the host. For example, resident microbes are able to protect the host from invading pathogens.[35] They aid in digestive processes and harvest energy from the diet that can be utilized by the host, thereby providing nutritional support for enterocytes.[36] Furthermore, the presence of the enteral microbiota is an important trigger for the development and constant stimulation of the immune system.[37] In fact, an underdevelopment of lymphoid organs (e.g., Peyer's patches, spleen) and immune cells (e.g., T-cells, dendritic cells, macrophages) was observed in germfree experimental animals.[34,38,39]

The metabolic functions of micro-organisms are assumed to be one of the main evolutionary driving forces behind the coevolution of the GI microbiota with their host.[40] Molecular tools allow the identification of previously uncharacterized intestinal microbes and these techniques are also able to provide information about the functionality of the microbiome by means of metagenomics.[31] High-throughput sequencing platforms enable a metagenomics approach (i.e., shotgun sequencing of

genomic DNA).[41] This approach yields identification of host and microbial genes present in a sample, and offers an opportunity to assess the functional aspects of the microbiota.[41] However, a genomic approach only provides a prediction of downstream biological events, because there are several downstream regulatory mechanisms including transcriptional/translational regulations and pathway interactions.[42] Therefore, metabolomics, the study of downstream biochemical end products (metabolites), is deemed to have the potential to provide new insights into functional aspects of the GI microbiota (details follow below).[43,44]

I.4 Enteric pathogens in dogs

Recent data suggest that the composition of the intestinal microbiota is an important factor for activation of virulence genes of some enteropathogens.[45] For example, a study using a mouse model showed that specific patterns in intestinal microbial communities had a direct effect on the pathogenicity of *Salmonella* spp.[46] The exact mechanisms behind this interplay between commensal bacteria and the virulence factors of enteropathogens have not been well elucidated. Recent reports have associated intestinal dysbiosis and changes in bacterial metabolism (e.g., altered short-chain fatty acid [SCFA] concentrations and bile acid profiles) with the activation of toxin production in patients with *Clostridium difficile* infection.[47] There is evidence that alterations in intestinal bile acid composition enhance germination of *C. difficile*, increasing the susceptibility to infection.[48,49] This suggests that there may be an important cross-talk between enteropathogens and commensals. In addition,

understanding the response of enteric pathogens to the changing intestinal environment may be the key to develop new ways to prevent and treat enteric infections.

The invasion and/or colonization of the GI tract with specific pathogens may profoundly disturb the integrity of the intestinal epithelial barrier.[50] Several potential GI pathogens have been recognized in dogs, including *Clostridium difficile*, *Clostridium perfringens*, *Campylobacter jejuni*, *Salmonella* spp., and *E. coli*.[51] However, several of these potential enteropathogens are commensals in the GI tract and have been isolated at similar frequencies from diarrheic and non-diarrheic animals (e.g., *C. perfringens* and *C. difficile*).[52] This complicates the clinical interpretation when presumptive enteropathogens are merely identified in the feces, as the isolation of those organisms from dogs does not always indicate the cause for the GI disease.[51] Therefore, the cause-effect relationship between those organisms and GI disease need to be interpreted with caution.

One of the potential GI pathogens, *Clostridium perfringens* is a Gram-positive, spore-forming, anaerobic bacillus.[53] This organism is a common inhabitant of the canine GI tract[51] and responsible for a spectrum of diseases. *C. perfringens* enterotoxin (CPE) is thought to be an important virulence factor in dogs with *C. perfringens*-associated diarrhea.[54] CPE is a single polypeptide, heat-labile protein with 35 kDa molecular weight. This enterotoxin is encoded by the *C. perfringens* enterotoxin gene (*cpe* gene) and synthesized during sporulation. After release from a sporulating cell, CPE induces toxicity by interacting with intestinal tight junctions, forming transmembrane pores on the cytoplasmic membrane and leading to altered epithelial permeability.[53] In humans,

CPE is responsible for several GI diseases, including *C. perfringens* type A food poisoning,[55] antibiotic-associated diarrhea,[56] and nosocomial diarrheal disease.[57] Detection of CPE in feces from human patients with diarrhea is a criterion for the diagnosis of CPE-associated diarrhea.[58] Several studies have evaluated the role of *C. perfringens* in dogs, and have reported *C. perfringens* as a potential cause of nosocomial diarrhea[59] and acute hemorrhagic diarrhea.[60,61] Clinical signs are usually mild and self-limiting.[51] Therefore, further diagnostic modalities, such as an endoscopy and abdominal exploration, are rarely conducted. Recently, evaluation of histopathological changes and the presence of bacteria in duodenal mucosal biopsies from dogs with acute hemorrhagic diarrhea revealed an increased abundance of mucosa-adherent *C. perfringens* in 6/9 of dogs, and demonstrated an association between the presence of this organism and the occurrence of acute hemorrhagic diarrhea.[61] The author hypothesized *C. perfringens* may have a primary pathogenic role in dogs with acute hemorrhagic diarrhea.[61]

Despite these studies, the exact role of *C. perfringens* in canine GI disease remains unknown, because this organism has been detected at similar isolation rates in healthy and diarrheic dogs.[51,52,54,62] Moreover, limited information is available regarding the relationship between diarrhea, intestinal dysbiosis, and/or the presence of *C. perfringens* and its virulence factors CPE. The lack of understanding of these relationships may have led to inappropriate therapy (e.g., indiscriminate use of antibiotics) which may result in an undesired clinical outcome (i.e., further disturbance of the intestinal microbiota). To prevent this, studies evaluating the relationship between commensal bacteria and enteric pathogens in dogs are warranted.

I.5 Metabolomics

Despite the well documented evidence that the intestinal microbiota play a role in the pathogenesis of various GI diseases, the actual mechanisms of the host-microbe interactions remain elusive, but are believed to be mediated in part by microbial products (metabolites) that are derived from the intestinal microbiota and are locally and/or systemically absorbed by the host.[34] Metabolomics is the comprehensive study of small molecules to evaluate cellular processes.[63] Recent studies utilizing this approach have suggested that there may be various bacterial metabolites that play a significant role in GI health.[64–67]

There are two main approaches to metabolomics. The first approach, targeted metabolomics, utilizes a variety of analytical methods and focuses on one or few specific metabolite(s).[43] One example of targeted metabolomics is the measurement of serum concentrations of cobalamin (vitamin B12) and folate (vitamin B9). The measurement of these vitamins in serum has been utilized to diagnose GI disease in dogs for several years.[68] The second approach, untargeted metabolomics, utilizes mass spectrometry platforms, including nuclear magnetic resonance (NMR) spectroscopy, gas chromatography/mass spectrometry (GC/MS), and liquid chromatography/mass spectrometry (LC/MS), and ion cyclotron resonance-fourier transform mass spectrometry (ICR-FT/MS) to identify hundreds of metabolites in biological samples simultaneously and thus provides global metabolite profiles, although the identity of all metabolites may not be known.[43]

I.5.1 Short-chain fatty acids

Short-chain fatty acids (SCFAs) are primary end products of bacterial fermentation of non-digestible dietary fiber and have been the focus of many studies due to their immunomodulatory effects and their importance as an energy source for intestinal epithelial cells.[69] SCFAs, primarily acetate, propionate, and butyrate, are produced through multiple pathways by the mutual relationship of different microbial groups.[70] For example, the primary fermenters Bacteroidetes are able to transform simple sugars derived from the breakdown of complex carbohydrates to acetate. Then, butyrate producing bacteria (e.g., *Clostridium* spp.) further utilize acetate to generate butyrate. Propionate can be produced by Bacteroidetes and some Firmicutes (e.g., Lachnospiraceae) from lactate or succinate through the acetylate or succinate pathway, respectively.[71,72]

Approximately 95% of SCFAs produced in the intestine are rapidly absorbed from the gut lumen either by directly crossing the epithelial barrier or by uptake through specialized transporters.[73,74] Undissociated (i.e., unionized) SCFAs (< 5% of total SCFAs) can be transported from the apical surface into colonocytes by passive diffusion. On the other hand, the transport of dissociated (i.e., ionized) SCFAs (> 95% of total SCFAs) requires an active transport by several different transporters.[69] The monocarboxylate transporter (MCT) 1 and the sodium-coupled monocarboxylate transporter (SMCT) 1 are highly expressed on the apical membrane of epithelia cells along the intestinal tract including the small intestine, the cecum, and the large intestine.[73] SCFAs are cotransported with cations such as H^+ and Na^+.[69] Therefore,

SCFAs absorption also stimulates water absorption and this mechanism provides an anti-diarrheic effect.[75] Although the identity of the actual transporter remains unidentified, a considerable amount of SCFAs seems to be transported via SCFA-HCO_3- exchange.[76] SCFAs that are not utilized by epithelial cells are suspected to be transported across the basolateral membrane by other families of MCT, such as MCT4 and MCT5, or SCFA-HCO_3- exchange, and then reach the portal circulation.[69] However, the exact mechanism for the basolateral transport remains elusive.

SCFAs are also sensed by SCFAs receptors such as G-protein coupled receptors (GPRs), including GPR41 (also known as FFAR3), GPR43 (also known FFAR2), and GPR109A (also known as NIACR1).[77,78] These receptors are expressed not only on intestinal epithelial cells but also on immune cells, adipocytes, endocrine L-cells and smooth muscle cells.[79–82] The affinities of each receptor for SCFAs differ. The affinity of GPR 41 for SCFA in decreasing order is propionate, butyrate, and acetate; that of GPR 43 is acetate and propionate followed by butyrate. GPR109A only has affinity for butyrate.[78]

The degree of absorption and utilization differs for each SCFA. Butyrate is preferentially utilized as an energy source by intestinal epithelial cells, and only small amounts (<10%) of butyrate reach the portal circulation.[83] In contrast, utilization of acetate and propionate by intestinal epithelial cells is limited. Therefore, most of acetate and propionate (up to 70%) enter the portal circulation and are metabolized in the liver and utilized as a source for lipid metabolism (acetate) and gluconeogenesis (propionate).[36,83] Only acetate reaches the systemic circulation at relatively high

concentration and is utilized by muscle, heart, adipose tissue, and kidney.[84] A small proportion of unabsorbed SCFAs are excreted in feces.[84–87]

While the main role of SCFAs is to function as an energy source for intestinal epithelial cells and for further metabolism, several other physiological roles have been identified. While the exact mechanisms remain to be fully elucidated, two major mechanisms by which SCFAs exert physiological effects on the host have been proposed. The first mechanism is inhibition of histone deacetylase (HDAC).[88] This mechanism can be achieved by direct action of intracellular SCFAs and/or indirect activation through GPRs signaling. The inhibition of HDAC promotes hyperacetylation of histones and can modify gene expression and cell differentiation.[88] The second mechanism is signaling through GPRs. As mentioned previously, these receptors are located on a variety of cells/tissues.[34] Therefore, the systemic effects of SCFAs, including chemotaxis, stimulation of satiation through leptin production, regulation of GI motility, and immune cell development/differentiation, have been reported in studies in animal model and in vitro studies.[77,81,89,90] Similarly, studies in cats have revealed the importance of SCFAs for proper intestinal function (e.g., maintenance of mucosal health and stimulation of longitudinal colonic smooth muscle), demonstrating their potential to increase intestinal motility and to provide an energy source in cats with colonic motility disorders .[89,91]

Clinical studies in humans have revealed associations between fecal SCFAs concentrations and GI disorders such as IBD[92] and colorectal cancer,[93] and SCFAs are thought to confer a protective role against further disease progression.[94] Regarding the

association between GI disease and SCFAs, the anti-inflammatory effect of SCFAs is of interest. Through these signaling transductions, SCFAs decrease the production of pro-inflammatory cytokines such as IL-6, IL-8, and TNFα.[82,95] Moreover, recent evidence shows the regulatory effect of propionate and butyrate on colonic regulatory T cells (T_{regs}).[90] T_{regs} from GF mice treated with SCFAs showed an increased expression of anti-inflammatory cytokine IL-10 and Foxp3, which are crucial for suppressing immune response toward intestinal inflammation and the immune tolerance to commensal bacteria.[96] Moreover, SCFAs treatment augmented the T_{regs} population and function through the GPR43 signaling mentioned above. Authors also demonstrated that these effects ameliorate the severity of induced colitis in mice.[90]

SCFAs not only affect host physiology. The production of SCFAs provides an acidic luminal environment, which prevents overgrowth of pH-sensitive pathogenic bacteria, such as Enterobacteriaceae and Clostridia.[70] Data from *in vitro* studies and experimental animal models suggest that the composition of intestinal microbiota and SCFAs are important factors for activation of virulence genes of some enteropathogens.[35,46,97,98] Lawhon et al. reported on the effects of SCFAs on virulence genes of *Salmonella typhimurium*.[98] These effects were shown to depend on the dose and composition of SCFAs, and were different between different SCFAs. At low total SCFAs concentration with a predominance of acetate, the expression of the invasion gene of *Salmonella* pathogenicity island (SPI-1) was induced.[98] However, at high total SCFAs concentrations with a higher proportion of propionate and butyrate, the expression of the SPI-1 gene was suppressed.[98] Similar findings, where propionate and

butyrate suppressed the expression of the SPI-1 gene have been reported in *in-vitro* studies.[97,99]

In veterinary medicine, studies in dogs have mainly evaluated the effects of nutritional intervention on fecal SCFAs concentrations in healthy research dogs.[100–102] Limited information is available with regards to the status of fecal SCFAs in dogs with GI disease. Therefore, their role in the pathogenesis of canine GI disease is still unknown.

I.5.2 Untargeted metabolomics

Owing to the development of bioinformatics and analytical separation techniques, untargeted metabolomics, has been increasingly used in clinical studies in humans.[103] Using mass spectrometry platforms, this approach can identify hundreds of metabolites in biological samples simultaneously, and therefore provides a comprehensive functional overview of biochemical pathways that are being up- or down-regulated during different physiologic or pathophysiologic states.[43] Consequently, untargeted metabolomics is an emerging method for better understanding of disease pathophysiology and host-microbe interactions, with the potential to develop novel diagnostic markers and/or treatment approaches.[92,104] While metabolomics studies have been applied in human patients with GI disease and have provided new insights into disease pathogenesis,[42,103] only few studies have utilized this approach in veterinary clinical patients,[64,65] and no studies using this approach have been reported in dogs with GI disease. One of the first metabolomics studies in human IBD patients, evaluated fecal extracts, and differentiated IBD patients from healthy controls.[92] Decreased levels of

SCFAs and some ammonia derivatives were observed. This finding revealed a connection between IBD and dysbiosis.[92] Further studies characterizing serum metabolite profiles in human patients with IBD have reported impaired amino acid and lipid metabolism.[66,67,105]

As mentioned above, information about the intestinal microbiota and especially its functional aspects in dogs with GI disease is limited. Therefore, studies aiming to characterize the microbial composition and the metabolite profiles in healthy dogs and dogs with GI disease are warranted. These studies will improve our knowledge of the pathogenesis of various GI diseases in dogs and have the potential to aid in the development of new biomarkers and new treatments and/or drug targets, which ultimately have the potential to improve the quality of life in dogs with GI disease.

I.6 Hypotheses and specific objectives

The hypothesis of this study is that dogs with GI disease have an altered microbial composition, an altered function of the intestinal microbiota, and altered metabolite profiles compared to healthy control dogs.

The objectives of the proposed research project are 1) to characterize the microbial composition within the intestinal tract of healthy control dogs and dogs with various GI diseases, 2) to evaluate the relationship between enteric pathogens and commensal bacteria, and 3) to describe the functional aspects of the intestinal microbiota by characterizing bacterial metabolite concentrations in healthy control dogs and dogs with GI diseases

CHAPTER II

PREVALENCE OF *CLOSTRIDIUM PERFRINGENS*, *CLOSTRIDIUM PERFRINGENS* ENTEROTOXIN AND DYSBIOSIS IN FECAL SAMPLES OF DOGS WITH DIARRHEA*

II.1 Overview

Clostridium perfringens has been suspected as an enteropathogen in dogs. However, its exact role in gastrointestinal (GI) disorders in dogs remains unknown. Recent studies suggest the importance of an altered intestinal microbiota in the activation of virulence factors of enteropathogens. The aim of this study was to evaluate the relationship between diarrhea, dysbiosis, and the presence of *C. perfringens* and its enterotoxin (CPE). Fecal samples were collected prospectively from 95 healthy control dogs and 104 dogs with GI disease and assessed for bacterial abundances and the presence of CPE using quantitative PCR and ELISA, respectively. *C. perfringens* was detected in all dogs. Potentially enterotoxigenic *C. perfringens* were detected in 33.7 % (32/95) of healthy control dogs and 48.1% (50/104) diseased dogs, respectively. CPE was detected by ELISA in 1.0% (1/95) of control dogs and 16.3% (17/104) of diseased dogs. Abundances of Fusobacteria, Ruminococcaceae, *Blautia*, and *Faecalibacterium* were significantly decreased in diseased dogs, while abundances of *Bifidobacterium*, *Lactobacillus,* and *E. coli* were significantly increased compared to control dogs. The

*Reprinted with permission from Prevalence of *Clostridium perfringens*, *Clostridium perfringens* enterotoxin and dysbiosis in fecal samples of dogs with diarrhea. By Minamoto Y, Dhanani N, Markel M.E., Steiner JM, Suchodolski JS., 2014. Veterinary Microbiology, 174(3-4):463-473, Copyright 2014 by Elsevier B.V

microbial dysbiosis was independent of the presence of the enterotoxigenic *C. perfringens* or CPE. In conclusion, the presence of CPE as well as fecal dysbiosis was associated with GI disease. However, the presence of *C. perfringens* was not indicative of GI disease in all cases of diarrhea, and the observed increased abundance of enterotoxigenic *C. perfringens* may be part of intestinal dysbiosis occurring in GI disease. The significance of an intestinal dysbiosis in dogs with GI disease deserves further attention.

II.2 Introduction

Clostridium perfringens is a commensal in the canine gastrointestinal (GI) tract[51] and responsible for a spectrum of diseases. *C. perfringens* enterotoxin (CPE) is thought to be an important virulence factor in dogs with *C. perfringens*–associated diarrhea.[54] CPE is encoded by the enterotoxin gene (*cpe* gene) and synthesized during sporulation. After release from sporulating cell, CPE induces toxicity by interacting with intestinal tight junctions, forming transmembrane pores on the cytoplasmic membrane and leading to altered epithelial permeability.[53] In humans, CPE is responsible for several GI diseases including *C. perfringens* type A food poisoning,[55] antibiotic-associated diarrhea,[56] and nosocomial diarrheal disease.[57] Detection of CPE in feces from human patients with diarrhea is a criterion for diagnosis of CPE-associated diarrhea.[58] Several studies have evaluated the role of *C. perfringens* in dogs, and have reported *C. perfringens* as a potential cause of nosocomial diarrhea[59] and acute hemorrhagic diarrhea.[60,61] Clinical signs are usually mild and self-limiting. Therefore, further

diagnostic modalities, such as an endoscopy and abdominal exploration, are rarely conducted. Recently, evaluation of histopathological changes and the presence of bacteria in duodenal biopsies from dogs with acute hemorrhagic diarrhea revealed an increased abundance of mucosa-adherent *C. perfringens* in 6/9 of dogs.[61]

However, despite these studies, the exact role of *C. perfringens* in canine GI disease remains unknown because this organism is detected at similar isolation rates in healthy and diarrheic dogs. Furthermore, these studies were conducted in a different country (i.e., Canada),[54,106] used samples from shelter animals or referral hospitals,[60,62,106,107] used a different ELISA assay,[59] or used different detection methods (reverse passive latex agglutination assay [RPLAA]).[62] There is currently also no gold standard for the diagnosis of *C. perfringens*-associated diarrhea in dogs. Therefore, confirmatory interpretation of data from different diagnostic assays (i.e., detection of the *cpe* gene by PCR and CPE by ELISA in fecal samples from dogs) is recommended.[51]

Recently, molecular studies have evaluated the diverse bacterial communities in the canine GI tract, and have shown the presence of intestinal dysbiosis, defined as altered GI microbial communities, in dogs with GI diseases. Commonly observed alterations are decreases in Ruminococcaceae, *Faecalibacterium*, *Turicibacter*, and Bacteroidetes, with concurrent increases in Proteobacteria, especially *E. coli*.[4,12,16,29,51,108] Of particular interest is that, while it has been shown that GI inflammation induces GI dysbiosis,[109] it is also thought that prolonged dysbiosis may aggravate intestinal

inflammation.[110] Therefore, dysbiosis plays a pivotal role in the pathogenesis of GI disease.[20–22]

Limited information is available regarding the relationship between diarrhea, dysbiosis, and/or the presence of an enteric pathogen and its virulence factors. This is of importance, as recent studies suggest that activation of virulence factors of *C. difficile* and *Salmonella* is associated with dysbiosis and concurrent changes in metabolite profiles such as altered bile acid and short-chain fatty acid (SCFA) concentrations.[46,49] Therefore, this study first aimed to investigate the prevalence of *C. perfringens* and CPE in healthy dogs and dogs with clinical signs of GI disease. The second aim was to quantify the abundance of *C. perfringens* and enterotoxigenic *C. perfringens* by detecting *C. perfringens* 16S ribosomal RNA (16S rRNA) gene and *C. perfringens* enterotoxin gene (*cpe* gene), respectively. The third aim was to evaluate the relationships between diarrhea, dysbiosis, and the presence of *C. perfringens* and its enterotoxin (CPE).

II.3 Materials and methods

II.3.1 Fecal samples

Fecal samples from healthy control dogs and dogs with clinical signs of GI disease were collected prospectively from April 2010 to June 2012. The protocol for sample collection was approved by the Texas A&M University Institutional Animal Care and Use Committee (#2012-83).

Healthy control dogs: A total of 95 privately owned dogs without clinical signs of GI disease (i.e., vomiting, diarrhea, anorexia, weight loss, etc.) within the past three months of sample collection were enrolled. Dogs that received antibiotics within the past three months were excluded. Fresh fecal samples were collected at home or at public dog parks, and transported on ice to the Gastrointestinal Laboratory.

Diseased dogs: A total of 104 left-over fecal samples from submissions to the Gastrointestinal Laboratory at Texas A&M University were utilized for this study. These submissions were from dogs with clinical signs of GI disease (i.e., vomiting, diarrhea, anorexia, weight loss, etc.) based on the clinical history and were submitted for enteric pathogen testing and/or fecal biomarker testing. Only the first submission sample was utilized for this study when multiple samples from same dog were submitted. The time of fecal sample collection after onset of diarrhea varied between samples depending on the time the dog was presented to the veterinarian.

II.3.2 Clinical history of dogs

Questionnaires were sent to veterinarians who submitted fecal samples and the owners of healthy control dogs. The questionnaire was composed of three major parts: signalment of dogs (breed, age, sex, body weight, and body condition score), health status of dogs at time of fecal sample collection (presence of GI signs and its characteristics, duration of GI signs), and medical history of dogs (medication [use of antibiotics, probiotics, etc.], concurrent diseases). Dogs with clinical signs of GI disease were classified based on the type of diarrhea (acute, chronic, or non-diarrhea). Diarrhea

was characterized as acute in nature if present for < 3 weeks and chronic if present for ≥ 3 weeks.

II.3.3 Evaluation of the stability of CPE in fecal samples

To test the stability of the CPE in fecal samples during shipping to the laboratory, the stability at different storage conditions was evaluated. Thirteen leftover fecal samples from dogs with clinical signs of GI disease were screened for CPE. Of these samples, 8 fecal samples were initially positive for CPE and 5 fecal samples were initially negative for CPE. These samples were subdivided into 8 aliquots, then evaluated at 4 time points (day 0, 2, 5, and 10) after having been stored at 3 storage conditions (room temperature, 4°C, and -20°C). Each aliquot was stored unprocessed in microcentrifuge tubes at each storage condition until processing for the ELISA.

II.3.4 DNA extraction

DNA was extracted from an aliquot of 100 mg (wet weight) of each fecal sample using a commercial DNA extraction kit (ZR Fecal DNA Kit™; Zymo Research Corporation, Irvine, CA) following the manufacturer's instructions. The bead-beating step was performed on a homogenizer (FastPrep-24; MP Biomedicals, Santa Ana, CA) for 60 s at a speed of 4 m/s. Fecal DNA was stored at -80°C until analysis.

II.3.5 Quantitative PCR (qPCR) assay

The abundances of *C. perfringens* 16S rRNA gene and *C. perfringens* enterotoxin gene (*cpe* gene) in feces were evaluated by qPCR assays using published oligonucleotides (Table 1). Quantitative PCR for detection of *C. perfringens* 16S rRNA gene was conducted using a total volume of 10 µL, with the mastermix containing 5 µL

of SsoFast™ Probes supermix (Biorad Laboratories, Hercules, CA), 2.2 µL of water, 0.3 µL of each forward and reverse primer (300 nM final concentration), 0.2 µL of the probe (200 nM final concentration), and 2 µL of DNA. The qPCR cycling conditions were: an initial incubation at 94°C for 10 min, 45 cycles of denaturation at 94°C for 10 s, annealing at 58 °C for 20 s, and extension at 70 °C for 10 s. The qPCR for the detection of the *cpe* gene in feces was conducted using a total volume of 10 µL, with the mastermix containing 5 µL of SsoFast™ Probes supermix, 2.35 µL of water, 0.25 µL of each primer (final concentration: 250 nM), 0.15 µL of the probe (150 nM final concentration), and 2 µL of DNA. The qPCR cycling conditions were: an initial incubation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 5 s, and annealing for 10 s at 55°C.

To assess the abundances of bacterial groups, which previously have been shown to be altered in canine GI diseases (Rossi et al., 2014; Suchodolski et al., 2012a; Suchodolski et al., 2008; Suchodolski et al., 2012b; Suchodolski et al., 2012c; Xenoulis et al., 2008), qPCR assays were performed for total bacteria, Fusobacteria, Ruminococcaceae, *Bifidobacterium* spp., *Blautia* spp., *Faecalibacterium* spp., *Lactobacillus* spp., and *E. coli*. The assay conditions, the oligonucleotide sequences of primers and probes, and respective annealing temperatures were described previously (Suchodolski et al., 2012c). A commercial real-time PCR thermal cycler (CFX384 Touch™ Real-Time PCR Detection System; Biorad Laboratories, Hercules, CA) was used for all qPCR assays and all samples were run in duplicate.

Table 1. Oligonucleotides used for the detection of the *C. perfringens* 16S rRNA gene and the *C. perfringens* enterotoxin gene.

Target	qPCR primers/probes	Sequence (5'- 3')	Annealing temperature (°C)	Ref
C. perfringens 16S rRNA gene	CPerf165F	CGCATAACGTTGAAAGATGG	58	
	CPerf269R	CCTTGGTAGGCCGTTACCC		111
	CPerf187F (probe)	**FAM-**TCATCATTCAACCAAAGGAGCAATCC-**TAMURA**		
C. perfringens enterotoxin gene	cpe F	AACTATAGGAGAACAAAATACAATAG	55	
	cpe R	TGCATAAACCTTATAATATACATATTC		112
	cpe Pr (probe)	**FAM-**TCTGTATCTACAACTGCTGGTCCA-**TAMURA**		

II.3.6 ELISA for CPE

C. perfringens enterotoxin (CPE) was detected using a commercially available ELISA kit (*C. perfringens* Enterotoxin Test; TechLab, Blacksburg, VA). The test was performed according to the manufacturer's instructions. Briefly, fecal samples (an amount equal to 3 mm of formed feces or 50 µl of liquid feces) were emulsified in 200 µl of diluent and vortexed for 10 seconds. One hundred µl of the diluted sample was then transferred to the microassay well containing the detecting polyclonal antibody against the toxin. The ELISA reaction was evaluated spectrophotometrically using a commercial multi-mode microplate reader (Synergy 2 Multi-Mode Microplate Reader; BioTek, Winooski, VT) at 450 nm wavelength. Samples with optical density $(OD)_{450} \geq 0.120$ were considered positive, and samples with $OD_{450} < 0.120$ were considered negative.

II.3.7 Statistical analysis

Datasets for healthy control dogs and dogs with clinical signs of GI disease were tested for normality using a Shapiro-Wilk test, and then compared using a Wilcoxon rank-sum test (for 2 groups) or Kruskal-Wallis tests with Dunn's post-tests (> 2 groups) where appropriate. A $p < 0.05$ was considered significant. For the evaluation of GI microbiota, all data were adjusted for multiple comparisons using a Bonferroni correction and an adjusted $p < 0.05$ was considered significant. A Spearman's rank correlation coefficient was used to evaluate the correlation between the abundance of *C. perfringens* 16s rRNA gene and the *cpe* gene. All statistical analyses were conducted using a statistical software package (JMP® Pro version 10, SAS Institute Inc, Cary, NC).

II.4 Results

II.4.1 Clinical history of dogs

A total of 95 fecal samples from healthy control dogs and 104 fecal samples from dogs with clinical signs of GI disease were utilized for this study. The median age of healthy dogs and dogs with clinical signs of GI disease was 3 years (range: 0.6 to 12 y) and 5 years (range: 0.4 to 15 y), respectively ($p = 0.006$; Figure 1). Of the healthy control dogs, 38 were male (3 intact, 35 castrated) and 57 female (4 intact, 53 spayed). Of the dogs with clinical signs of GI disease, 54 were male (11 intact, 43 castrated) and 50 female (13 intact, 37 spayed). There was no significant difference between the gender between the two groups ($p = 0.117$). The median body weight of healthy control dogs

and dogs with clinical signs of GI disease was 23.9 kg (range: 3.0 to 83.2 kg) and 23.2 kg (range: 1.5 to 106.0 kg), respectively ($p = 0.856$). A large variety of breeds was represented in this study population. The healthy control group consisted of dogs of 39 breeds and the 3 most common breeds were mixed breed (19/95 [20.0%]), Labrador Retriever [18/95 (18.9%)], and Australian Shepherd (6/95 [6.3%]). The diseased group consisted of dogs of 51 breeds and the 3 most common breeds were Labrador Retriever (17/104 [16.3%]), mixed breed (15/104 [14.4%]), and German Shepherd dogs (7/104 [6.7%]). None of the healthy control dogs received antibiotics for at least 3 months before sample collection. Of the dogs with clinical signs of GI disease, 38 dogs received antibiotics at the time of sample collection, while 58 dogs did not receive antibiotics at the time of sample collection, and 8 dogs had an unknown history of antibiotic administration.

II.4.2 Evaluation of the stability of CPE in fecal samples

A total of 104 fecal aliquots made from 8 CPE positive and 5 CPE negative fecal samples were analyzed to evaluate the stability of CPE over 10 days for the 3 storage conditions. All fecal aliquots were consistent with the initial result, regardless of storage condition or time (Table 2).

Table 2. Results of CPE stability study. Each value represents OD_{450} value. Sample with OD_{450} ≥0.120 was considered positive, and sample with OD450 <0.120 was considered negative.

		Time point and storage condition							
		Day 0	**Day 2**		**Day 5**		**Day 10**		
Sample	**Initial result**		**RT**	**4°C**	**RT**	**4°C**	**RT**	**4°C**	**-20°C**
1	positive	0.765	0.631	0.770	0.477	0.388	1.241	0.574	0.589
2	positive	0.181	0.495	0.338	0.408	0.215	0.269	0.393	0.274
3	positive	0.258	0.602	0.371	0.413	0.297	0.466	0.525	0.464
4	positive	0.609	0.646	0.820	0.802	0.248	0.531	0.882	0.563
5	positive	0.556	0.756	0.574	0.621	0.519	0.492	0.611	0.694
6	positive	1.956	1.371	1.501	1.968	1.934	1.077	1.722	1.840
7	positive	0.843	0.947	0.864	0.549	0.510	0.492	1.097	0.749
8	positive	0.220	0.275	0.426	0.457	0.263	0.530	0.224	0.394
9	negative	0.045	0.048	0.042	0.041	0.059	0.069	0.051	0.048
10	negative	0.048	0.099	0.046	0.045	0.047	0.061	0.055	0.060
11	negative	0.045	0.049	0.069	0.045	0.051	0.046	0.048	0.081
12	negative	0.046	0.041	0.049	0.063	0.053	0.044	0.065	0.050
13	negative	0.050	0.053	0.082	0.041	0.063	0.049	0.057	0.048

Each value represents OD450 value. Sample with OD450 ≥ 0.120 was considered positive, and sample with OD450 < 0.120 was considered negative. ELISA, enzyme-linked immunosorbent assay; RT, room temperature

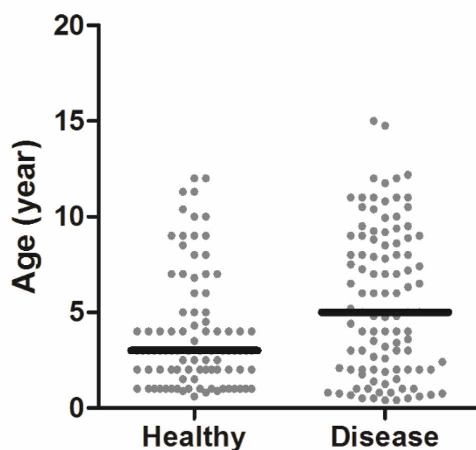

Figure 1. Distribution of ages in healthy control dogs and dogs with signs of GI disease. The lines represent the medians of both groups.

II.4.3 Prevalence of **C. perfringens** *(***C. perfringens** *16S rRNA gene)*

The *C. perfringens* 16S rRNA gene was detected in all samples from either healthy control dogs or dogs with clinical signs of GI disease. The abundance (i.e., amount of DNA) of the *C. perfringens* 16S rRNA gene was significantly higher in dogs with clinical signs of GI disease ($p < 0.001$) than in healthy control dogs. A subset analysis, in which dogs with clinical signs of GI disease were divided into 3 groups based on the type of diarrhea, revealed that a significantly higher abundance of the *C. perfringens* 16S rRNA gene was observed in dogs with acute and chronic diarrhea compared to the healthy control dogs ($p = 0.003$ and 0.010, respectively; Figure 2A). No significant difference was observed between control dogs and those dogs with clinical signs of GI disease but without diarrhea, and between dogs with acute and chronic diarrhea (Figure 2A).

II.4.4 Prevalence of potentially enterotoxigenic **C. perfringens** *(***C. perfringens** *enterotoxin gene; cpe gene)*

The prevalence of the *cpe* gene was 32/95 (33.7%) in healthy dogs and 50/104 (48.1%) in dogs with clinical signs of GI disease, and was significantly different between the groups ($p = 0.044$; Table 3). The abundance (i.e., amount of DNA) of the *cpe* gene was significantly higher in dogs with clinical signs of GI disease than in healthy dogs ($p = 0.021$). A subset analysis, in which dogs with clinical signs of GI disease were divided into 3 groups based on the type of diarrhea, revealed that a significantly higher abundance of *cpe* gene was observed in dogs with acute diarrhea compared to healthy control dogs ($p = 0.002$), but no significant differences were

observed among other groups (i.e., between healthy control, chronic diarrhea, and non-diarrheic groups; Figure 2B). Of the samples positive for the *cpe* gene, 15/82 (18.3%; healthy control dog, n = 1; dogs with acute diarrhea, n = 4; chronic diarrhea, n = 7; non diarrhea, n = 4) were positive for the enterotoxin (CPE), and 67/82 (81.7%) samples were negative for CPE. A significant positive correlation was observed between the abundances of the *C. perfringens* 16S rRNA gene and the *cpe* gene ($r = 0.428$, $p < 0.001$; Figure 2C).

II.4.5 Prevalence of **C. perfringens** *enterotoxin (CPE)*

The prevalence of CPE was significantly different ($p < 0.001$) between healthy control dogs and dogs with clinical signs of GI disease (1/95 [1.0 %] and 17/104 [16.3%], respectively; Table 3). Fecal samples from dogs that were positive for CPE had significantly higher abundances of *C. perfringens* 16S rRNA gene and *cpe* gene compared to those dogs that were CPE negative (both $p < 0.001$; Figure3).

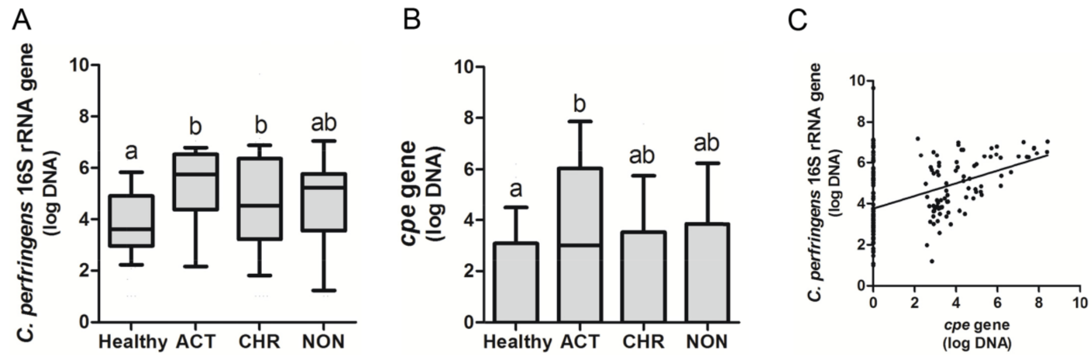

Figure 2. Abundances of the *C. perfringens* 16S rRNA gene (A) and the *cpe* gene (B) in healthy control dogs and dogs with clinical signs of GI disease, and correlation of the *C. perfringens* 16S rRNA gene and the *cpe* gene (C). The bottom and top of the box represent the 25th and 75th percentiles, and the line of the box represents the medians. Whiskers represent the 10th and the 90th percentile. Columns not sharing a common superscript are significantly different ($p < 0.05$). ACT, dogs with acute diarrhea; CHR, dogs with chronic diarrhea; NON, dogs with clinical signs of GI disease but without diarrhea

Table 3**.** The prevalence of enterotoxigenic *C. perfringens* or *C. perfringens* enterotoxin in feces.

	ACT (n = 22)	**CHR (n = 58)**	**NON (n = 24)**	**Total GI disease (n =104)**	**Healthy (n = 95)**	**p-value (Healthy vs. GI disease)**
***cpe*-gene**						
positive	16	24	10	50 (48.1%)	32 (33.7%)	p = 0.044
negative	6	34	14	54 (51.9%)	63 (66.3%)	
CPE						
positive	4	8	5	17 (16.3%)	1 (1.0%)	p < 0.001
negative	18	50	19	87 (83.7%)	94 (99.0%)	

cpe-gene, *C. perfringens* enterotoxin gene; CPE, *C. perfringens* enterotoxin*;* ACT, dogs with acute diarrhea; CHR, dogs with chronic diarrhea; NON, dogs with clinical signs of GI disease but without diarrhea

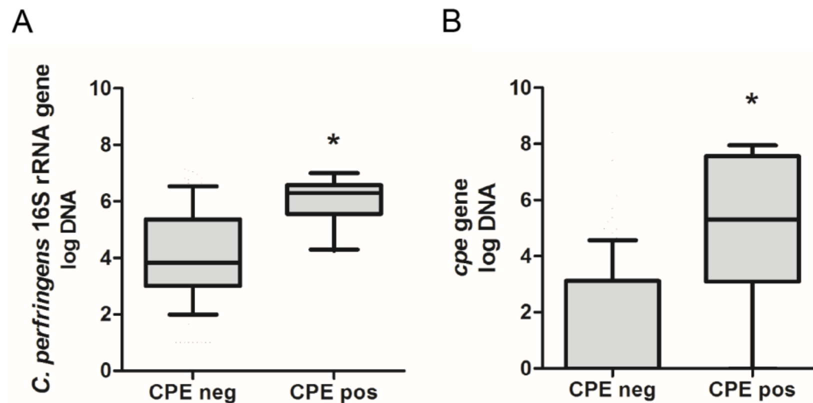

Figure 3. Abundance of the *C. perfringens* 16S rRNA gene (A) and the *cpe* gene (B) in samples either positive or negative for CPE. The bottom and top of the box represent the 25th and 75th percentiles, and the line of the box represents the median. Whiskers represent the 10th and the 90th percentile. *significantly different ($p < 0.05$) compared to the CPE negative samples. CPE neg, *C. perfringens* enterotoxin assay negative; CPE pos, *C. perfringens* enterotoxin assay positive

II.4.6 Evaluation of the fecal microbiota

Comparison between healthy control dogs and dogs with clinical signs of GI disease: The abundances of Fusobacteria ($p < 0.001$), Ruminococcaceae ($p < 0.001$), *Blautia* spp. ($p < 0.001$), and *Faecalibacterium* spp. ($p < 0.001$) were significantly decreased in dogs with clinical signs of GI disease, while the abundances of *Bifidobacterium* spp. ($p < 0.001$), *Lactobacillus* spp. ($p < 0.001$), and *E. coli* ($p < 0.001$) were significantly increased compared to healthy control dogs (Figure 4). No significant difference was observed in the abundance of total bacteria between healthy control dogs and dogs with clinical signs of GI disease ($p = 0.888$; Figure 4). A subset analysis, in

which dogs with clinical signs of GI disease were divided into 3 groups based on the type of diarrhea (i.e., either acute, chronic, or non-diarrhea), revealed that dogs with chronic diarrhea had significantly decreased abundances of Fusobacteria ($p = 0.002$), Ruminococcaceae ($p < 0.001$), *Blautia* spp. ($p < 0.001$), and *Faecalibacterium* spp. ($p < 0.001$) and significantly increased abundances of *Bifidobacterium* spp. ($p = 0.001$), *Lactobacillus* spp. ($p < 0.001$), and *E. coli* ($p < 0.001$) compared to healthy control dogs (Figure 5). Dogs with acute diarrhea and dogs with clinical signs of GI disease but without diarrhea had significantly decreased abundances of Ruminococcaceae (both $p < 0.001$), *Blautia* spp. (both $p < 0.001$), and *Faecalibacterium* spp. (both $p < 0.001$) and significantly increased abundances of *E. coli* ($p < 0.001$, $p = 0.027$, respectively) compared to healthy control dogs (Figure 5).

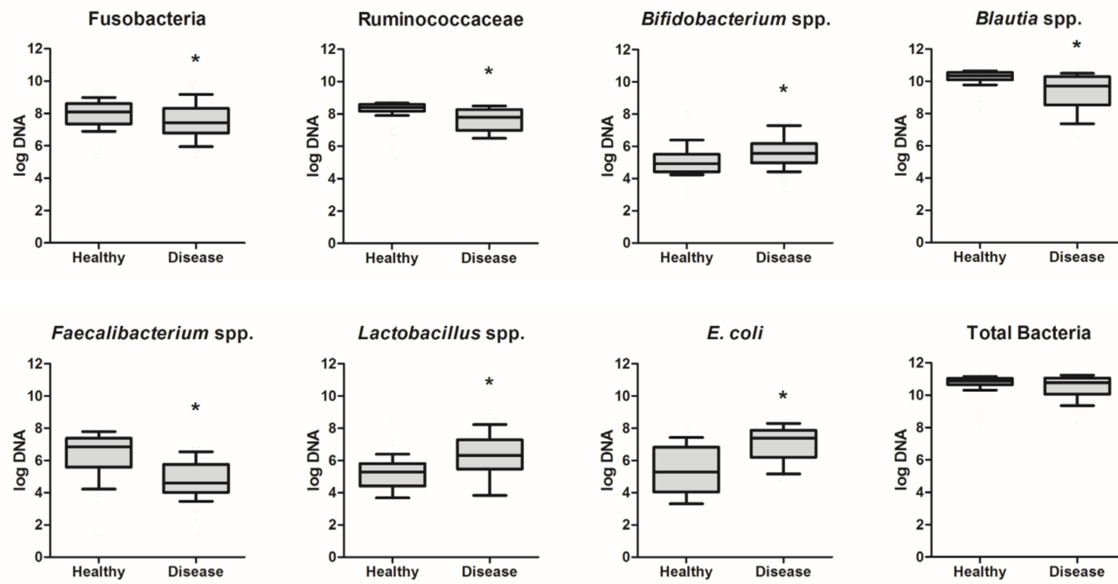

Figure 4. Abundances of bacterial groups in healthy control dogs and dogs with clinical signs of GI disease. The bottom and top of the box represent the 25th and the 75th percentiles, and the line of the box represents the median. Whiskers represent the 10th and the 90th percentile. *significantly different (adjusted $p < 0.05$) compared to healthy control dogs.

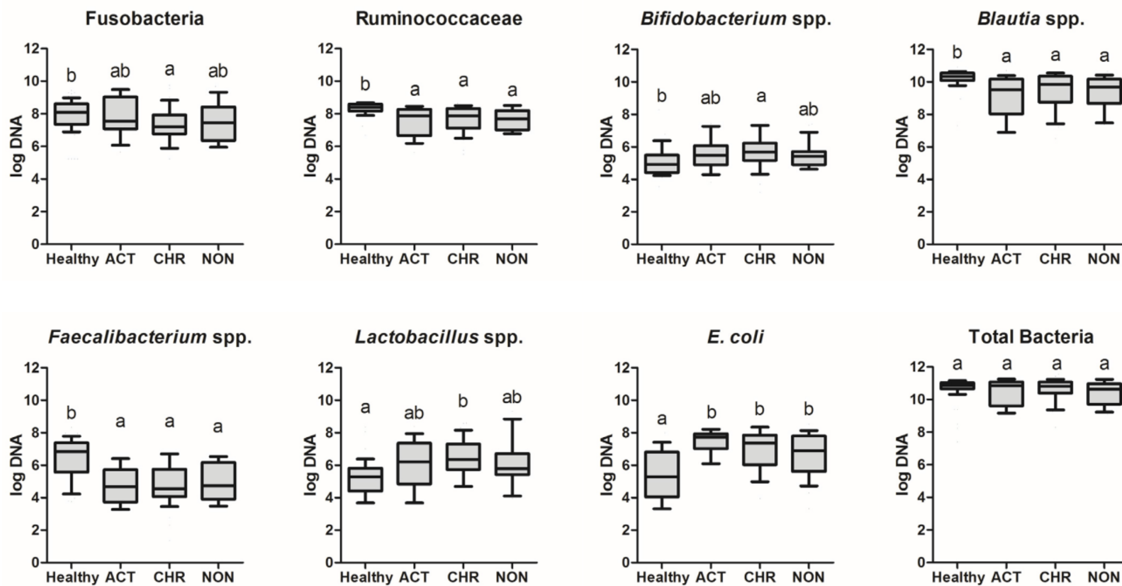

Figure 5. Abundances of bacterial groups in healthy control dogs and dogs with clinical signs of GI disease. The bottom and top of the box represent the 25th and the 75th percentiles, and the line of the box represents the median. Whiskers represent the 10th and the 90th percentile. Columns not sharing a common superscript are significantly different (adjusted $p < 0.05$). ACT, dogs with acute diarrhea; CHR, dogs with chronic diarrhea; NON, dogs with clinical signs of GI disease but without diarrhea

Relationship between intestinal dysbiosis and the presence of the *cpe* gene: Based on the qPCR results, both healthy control dogs and dogs with clinical signs of GI disease were classified into 2 groups (*cpe* gene positive or negative; total 4 groups; Figure 6). There were no significant differences in the abundance of any bacterial group between dogs that were *cpe* gene positive and *cpe* gene negative within either the healthy control group or the disease group (Figure 6). The results indicated that the differences in

bacterial groups between diseased and healthy control dogs were generally independent of the presence of the *cpe* gene.

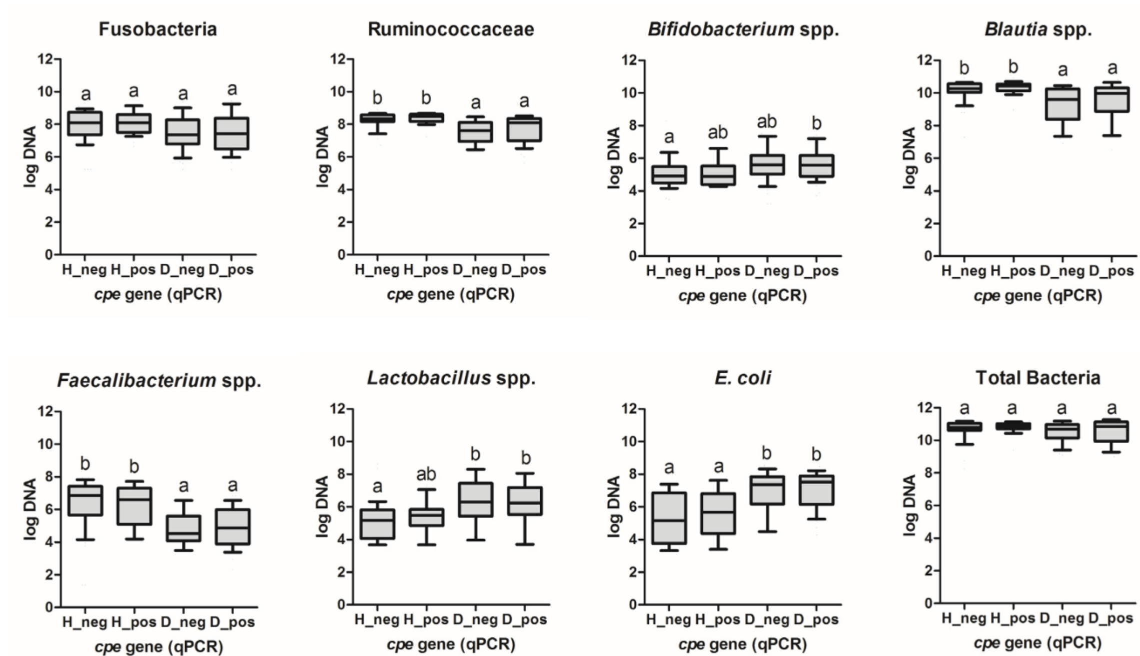

Figure 6. Relationship between the presence of the *cpe* gene and the abundance of bacterial groups. The bottom and top of the box represent the 25th and the 75th percentiles, and the line of the box represents the median. Whiskers represent the 10th and the 90th percentile. Columns not sharing a common superscript are significantly different (adjusted $p < 0.05$). H_neg, healthy control dogs that were *cpe* gene negative; H_pos, healthy control dogs that were *cpe* gene positive; D_neg, dogs with clinical signs of GI disease that were *cpe* gene negative; D_pos, dogs with clinical signs of GI disease that were *cpe* gene positive

Relation between dysbiosis and the presence of CPE: According to the ELISA results, healthy control dogs and dogs with clinical signs of GI disease were classified into 2 groups (CPE positive or negative: total 3 groups). The healthy control dog that was positive for CPE was excluded from this analysis because only one sample was present in this group. There were no significant differences in the abundance of any bacterial groups between dogs with clinical signs of GI disease that were CPE positive and CPE negative, indicating that differences in bacterial groups between diseased and healthy control dogs were generally independent of the presence of CPE (Figure 7).

II.4.7 Effects of antibiotics

To evaluate if the administration of antibiotics had a confounding effect on the prevalence of enterotoxigenic *C. perfringens*, the prevalence of CPE, and the abundances of bacterial groups, samples from dogs with signs of GI disease were analyzed based on the history of antibiotic administration. No significant differences were observed in the prevalence of enterotoxigenic *C. perfringens* and CPE between

dogs with signs of GI disease that receiving antibiotics and those not receiving antibiotics ($p = 0.946$ and 0.419, respectively). No significant differences were also observed when dogs were divided into 3 groups based on the type of diarrhea, and compared based on the history of antibiotics within each group (all $p > 0.100$). With regards to the effects on the abundance of bacterial groups, no significant differences were observed in the abundances of *C. perfringens* 16S rRNA gene, *cpe* gene, and any other bacterial group analyzed between dogs with signs of GI disease that receiving antibiotics and those not receiving antibiotics (Table 4).

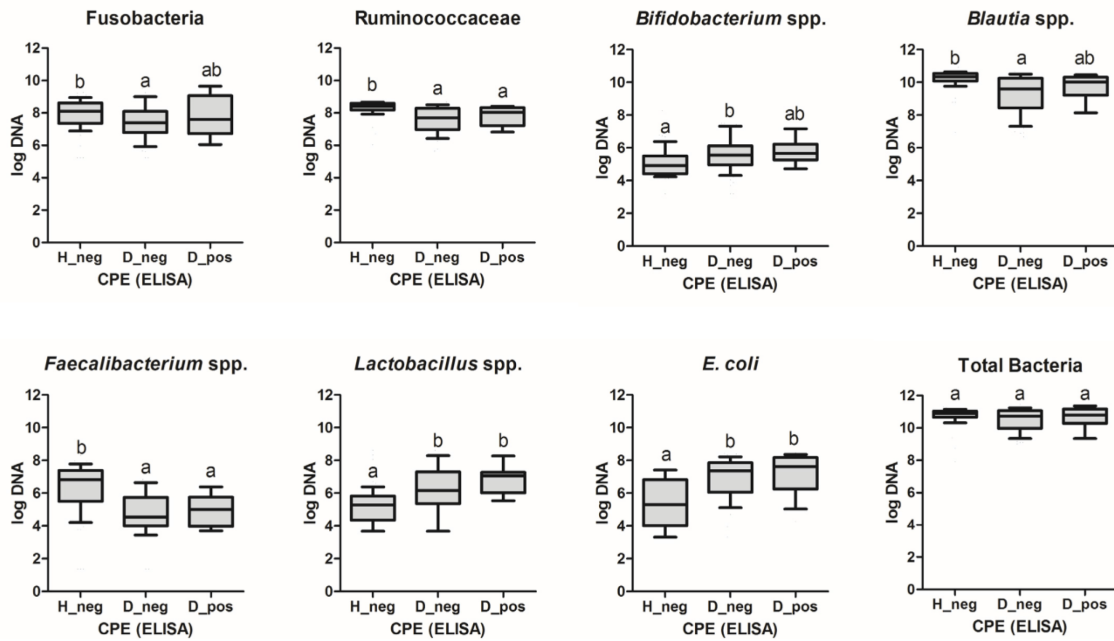

Figure 7. Relationship between the presence of CPE and the abundance of bacterial groups. The bottom and top of the box represent the 25th and the 75th percentiles, and the line of the box represents the median. Whiskers represent the 10th and the 90th percentile. Columns not sharing a common superscript are significantly different (adjusted $p < 0.05$). H_neg, healthy control dogs that were CPE negative; D_neg, dogs with signs of GI disease that were CPE negative; D_pos, dogs with signs of GI disease that were CPE positive

Table 4. Abundance of bacterial groups in dogs with signs of GI disease that did or did not receive antibiotics at the time of sample collection.

Target organism	Dogs WITHOUT antibiotics (log DNA)		Dogs WITH antibiotics (log DNA)		Adjusted p-value
	Median	**Range**	**Median**	**Range**	
C. perfringens	5.3	(1.2-7.2)	5.0	(1.0-9.6)	1.0
Enterotoxigenic C. *perfringens*	2.9	(0.0-8.4)	0.0	(0.0-7.8)	1.0
Fusobacteria	7.5	(5.2-9.7)	7.3	(5.7-9.5)	1.0
Ruminococcaceae	8.0	(5.2-8.6)	7.7	(6.0-9.0)	1.0
Bifidobacterium spp.	5.6	(3.7-7.5)	5.6	(3.2-8.1)	1.0
Blautia spp.	9.9	(6.5-10.7)	9.5	(6.9-10.8)	1.0
Faecalibacterium spp.	4.5	(2.3-6.8)	4.8	(1.4-7.8)	1.0
Lactobacillus spp.	6.3	(3.7-9.3)	6.4	(3.7-8.9)	1.0
E. coli	7.3	(3.3-8.4)	7.3	(3.3-8.5)	1.0
Total bacteria	10.7	(8.3-11.6)	10.9	(9.2-11.3)	1.0

II.5 Discussion

A previous study reported the effects of storage on the detection of CPE by RPLA in canine fecal samples stored for 24 hours at 25°C and 4°C. Although there were some samples with a discordant RPLA result, overall no significant differences among storage conditions were reported.[113] To exclude the possibility that lack of stability of CPE during long-term storage confounded our results, the stability of CPE as assessed by ELISA was evaluated. The results indicate that CPE is stable in feces under the tested conditions. However, we have not tested and, therefore, cannot exclude the possibility that the ongoing production of enterotoxin due to environmental changes and storage may yield a false positive result as assessed by ELISA.

In this study, *C. perfringens* was detected in all dogs based on the 16S rRNA gene. Potentially, enterotoxigenic *C. perfringens* (i.e., harboring the *cpe* gene) were detected in 33.7 % of control dogs and 48.1% of dogs with clinical signs of GI disease. These results are consistent with previous cultivation and/or PCR-based studies.[52,54,60,106] In previous reports, prevalence of CPE ranged between 5%[54] and 16%[60] in non-diarrheic dogs, and between 14%[60] and 41% in diarrheic dogs.[59] In our study, the prevalence of CPE in dogs with clinical signs of GI disease was similar with 16.3%. In contrast, the prevalence of CPE in control dogs (1.0%) was slightly lower than previously reported. The results of our stability study make it unlikely that this lower prevalence was due to sample degradation. Another possible explanation for lower prevalence may be due to geographical differences because all samples from control dogs were collected only in one state (i.e., Texas). In humans, the important role of CPE has been highlighted in acute diarrhea, but the role of CPE in chronic diarrhea has been rarely reported. In our current study, the prevalence of CPE was not significantly different between acute diarrhea and chronic diarrhea. Therefore,the role of CPE in chronic diarrhea warrants further evaluation. To conclude the first part of this study, dogs with clinical signs of GI disease had a significantly higher prevalence of enterotoxigenic *C. perfringens* compared to control dogs. However, its virulence factor CPE was detected in only 18.3% of dogs that were positive for enterotoxigenic strains. This indicates that the detection of *C. perfringens* by PCR for 16S rRNA gene and enterotoxigenic *C. perfringens* by PCR for the *cpe* gene is not always indicative of the presence of CPE in dogs with diarrhea.

The quantitative analysis revealed significantly higher abundances of both genes (16S rRNA gene for *C. perfringens* and *cpe gene*) in dogs with acute diarrhea compared to the control group. A recent study using sequencing of 16S rRNA gene showed changes in the fecal microbiome in dogs with acute diarrhea, and significant increases of *C. perfringens* in dogs with hemorrhagic diarrhea.[16] In the current study, we evaluated the correlation between these genes, and found that the abundance of the *cpe* gene was positively correlated with the abundance of the *C. perfringens* 16S rRNA gene. These findings may suggest that increases in populations of enterotoxigenic *C. perfringens* are associated with acute diarrhea, but also that such an increase may be simply due to the increased overall population of *C. perfringens* with concurrent reduction of commensal microbiota (i.e., intestinal dysbiosis) as indicated by results of qPCR assays. Our results are also consistent with previous studies showing significant changes in abundances of several bacterial groups in dogs with GI diseases.[4,12,16,29,108] Evaluating changes in the intestinal microbiota in GI disease are important for better understanding of disease pathogenesis. It has been well documented that the intestinal microbiota plays a crucial role in intestinal health, and that reduction of normal protective microbiota may confer susceptibility to intestinal inflammation. Prolonged imbalances of the GI microbiota may result in a dysregulation of immune responses and reduced activity against infection.[21] Furthermore, recent data suggest that the composition of intestinal microbiota and its associated metabolite profile is an important factor for activation of virulence genes of some enteropathogens. For example, a study using a mouse model showed that specific patterns in intestinal microbial communities had a direct effect on the pathogenicity of

Salmonella spp..[46] The exact mechanisms behind this interplay between commensal bacteria and virulence factors of enteropathogens have not been well elucidated. Recent reports have associated intestinal dysbiosis and changes in bacterial metabolisms (e.g., altered SCFA and bile acid profiles) with the activation of toxin production in patients with *C. difficile* infection.[47] There is evidence that alterations in intestinal bile acid composition enhance germination of *C. difficile*, increasing susceptibility to infection .[48,49] This suggests an important cross-talk between enteropathogens and commensals.

We observed an association between the abundance of enterotoxigenic *C. perfringens* and fecal dysbiosis, as the abundances of *C. perfringens* 16S rRNA gene and *cpe* gene were higher in dogs with diarrhea and these dogs had a dysbiosis manifested as reduction of several commensal bacterial groups. Furthermore, there was a strong association between the presence of CPE and GI disease. This may suggest that the increased abundance of *C. perfringens* and enterotoxigenic *C. perfringens* may be part of dysbiosis and may not necessarily play a primary pathological role in diarrhea. However, it may also be possible that initial dysbiosis due to various causes and subsequent changes in bacterial metabolite profiles within the intestinal lumen may trigger the production of CPE from enterotoxigenic *C. perfringens*, as has been demonstrated with C. *difficile*. Further studies evaluating the activity of transcription of CPE are warranted. Clearly, the relationships between enteropathogens, fecal altered microbial communities, and altered bacterial metabolite profiles deserve further studies in dogs with GI disease.

There are several observations in this study that deserve discussion. There were 6 samples from non-diarrheic dogs that were positive for CPE (5 dogs with clinical signs of GI disease but without diarrhea and 1 healthy control dog). Possible explanations for this are that there may be a threshold amount of enterotoxin required to cause diarrhea in dogs. For example, in food poisoning in humans due to enterotoxin produced by *C. perfringens* type A, a certain bacterial load is needed to induce disease.[114] Other virulence factors of *C. perfringens* may play a role in canine diarrhea. Finally, it is important to note that the assay used here has limitations. The ELISA has not been properly validated for canine fecal samples. Therefore, potentially false positive results due to nonspecific binding, and potentially false negative results due to low binding affinity of the antibodies utilized towards canine isolates of *C. perfringens* should be considered. Of all samples that were positive for the *cpe* gene, 67/82 (81.7%) samples were negative for CPE. This may suggest that abundance of CPE was below the detection limit of the ELISA assay or enterotoxigenic *C. perfringens* presented in feces, but enterotoxin was not produced due to the lack of spore formation. The latter explanation could be validated by quantifying spores. However, previous studies showed no statistical correlation between the presence of CPE and spore counts in feces.[54,62] Therefore, further studies investigating the CPE expression by evaluating RNA levels may provide the activity of CPE production. Finally, of samples negative for the *cpe* gene, 3/117 (2.5%) were positive for CPE. This suggests unspecific cross-reactivity in the ELISA assay. Alternatively, this finding may have been due to a low sensitivity of the qPCR assay.

There are some limitations to this study. There was a significant age difference between control dogs and dogs with clinical signs of GI disease. However, as Figure 1 shows, the age range of control group did overlap with that of the disease group, thus, the statistically significant difference in age may not have been a major bias in this study. As mentioned previously, the CPE ELISA assay has not been validated for use in dogs, and its sensitivity and specificity are unknown. In the current study, we evaluated only one of the *C. perfringens* virulence factors and its encoding gene. Other virulence factors such as *C. perfringens* β2 toxin gene have previously been detected in diarrheic dogs,[106,115] and a dog with fatal acute hemorrhagic gastroenteritis.[116] Recent studies have identified three types of *cpe* loci organizations in *C. perfringens* type A isolate, and have suggest that there were differences in the pathogenesis between these types.[58] Therefore, further evaluation of these genes may provide more information about the clinical significance of the isolated strains. The time of fecal sample collection after onset of diarrhea varied with sample in our study. This might confound the prevalence of CPE because detection of CPE is much more reliable when feces are collected early after the onset of diarrhea in humans. Lastly, we did not evaluate the presence of other potential enteric pathogens such as *Campylobacter* and *Salmonella*. Therefore, it is unknown that the cause of clinical signs was purely due to pathological effect of CPE.

In conclusion, increased abundances of *C. perfringens* and enterotoxigenic *C. perfringens* were observed in dogs with clinical signs of GI disease, and these were most apparent in dogs with acute diarrhea. Although, the presence of CPE was associated with GI disease, the presence of this organism, either non- or enterotoxigenic strain, was not

indicative of GI disease in all cases of diarrhea. On the other hand, dysbiosis was significantly associated with GI disease. Therefore, the increased abundance of *C. perfringens* and enterotoxigenic *C. perfringens* may be part of intestinal dysbiosis. It remains unknown whether dysbiosis is a cause or result of GI disease, and how dysbiosis affects enterotoxin production of *C. perfringens*. However, regardless of the initial cause of dysbiosis, an abnormal microbiota may exacerbate GI disease or may lead to metabolic changes in the intestinal lumen that may favor the activation of bacterial virulence genes in dogs with GI disease. These findings enlighten the importance of balanced microbial communities for GI health, and further evaluation of intestinal dysbiosis in dogs with GI disease is warranted.

CHAPTER III

FECAL SHORT-CHAIN FATTY ACIDS CONCENTRATIONS IN DOGS WITH CHRONIC ENTEROPATHY

III.1 Overview

Accumulating evidence has suggested a significant relationship between the GI microbiota and host GI health. Microbial metabolites are believed to play a critical role in host-microbial interactions. Short-chain fatty acids (SCFAs) are major end-products of bacterial carbohydrate fermentation in the intestinal tract. They constitute an important energy source for intestinal epithelial cells and help maintain intestinal mucosal integrity. Decreased concentrations of SCFAs have been observed in humans with GI disease. However, large scale clinical data are lacking in dogs. Therefore, the aim of this study was to evaluate fecal concentrations of SCFAs in healthy pet dogs and dogs with clinical signs of chronic GI disease.

One fresh fecal sample was collected from each of 50 healthy control dogs and 81 dogs with chronic enteropathy. Fecal concentrations of SCFAs (i.e., acetate, propionate, butyrate, and total short-chain fatty acids) were measured using gas chromatography/mass spectrometry. Fecal concentrations of SCFAs were normalized by dry matter and compared between both groups using a Wilcoxon rank-sum test. A $p < 0.05$ was considered to be statistically significant.

Fecal concentrations of acetate were significantly decreased ($p = 0.049$) in dogs with clinical signs of chronic GI disease (median: 193.4 [min-max: 20.1-1042.1] μmol/g

of fecal DM) compared to those in healthy control dogs (median: 218.7 [min-max: 87.7-672.8] µmol/g of fecal DM). Also, fecal concentrations of propionate were significantly decreased ($p < 0.001$) in dogs with clinical signs of chronic GI disease (median: 50.2 [min-max: 0-227.9] µmol/g of fecal DM) compared to those in healthy control dogs (median: 104.6 [min-max: 1.6-266.8] µmol/g of fecal DM). Moreover, fecal concentrations of total short-chain fatty acids were significantly decreased ($p = 0.012$) in dogs with clinical signs of chronic GI disease (median: 272.3 [min-max: 21.7-1378.2] µmol/g of fecal DM) compared to those in healthy control dogs (median: 383.6 [min-max: 126.6-927.0] µmol/g of fecal DM). However, there was no significant difference ($p = 0.233$) in fecal concentrations of butyrate between healthy control dogs (median: 29.1 [min-max: 8.1-148.1] µmol/g of fecal DM) and dogs with clinical signs of chronic GI disease (median: 23.8 [min-max: 0-137.6] µmol/g of fecal DM).

In this study, dogs with clinical signs of chronic GI disease had decreased fecal concentrations of acetate, propionate, and total short-chain fatty acids. The relationship between fecal short-chain fatty acid concentrations and microbial composition is warranted.

III.2 Introduction

Despite the well documented evidence that the intestinal microbiota plays a role in the pathogenesis of GI diseases, the actual mechanisms of the host-microbe interactions remain elusive, but are believed to be mediated in part by microbial products

(metabolites) that are derived from the intestinal microbiota and are locally and/or systemically absorbed by the host.[34]

SCFAs are primary end products of bacterial fermentation of non-digestible dietary fibers. Because of their beneficial effects on host health, including immunomodulatory effects, anti-diarrheic effects and regulatory effect on GI motility, SCFAs have been the focus of many studies.[75,87,89,117] SCFAs are also an important energy source for intestinal epithelial cells.[87]

SCFAs, primarily acetate, propionate and butyrate, are produced through multiple pathways by the mutual relationship of different microbial groups.[70] For example, the primary fermenters Bacteroidetes are able to transform simple sugars derived from the breakdown of complex carbohydrates to acetate and propionate. Then, secondly fermenters Firmicutes further utilize acetate to generate butyrate.[71,72]

Absorption and utilization differs between different SCFAs. Butyrate is preferentially utilized as an energy source by intestinal epithelial cells, and only small amounts (<10%) of butyrate reach the portal circulation. In contrast, most of acetate and propionate (up to 70%) enter the portal circulation and are metabolized in the liver and utilized as a source for lipid metabolism (acetate) and gluconeogenesis (propionate). Only acetate reaches the systemic circulation in relatively high concentrations and is utilized by muscle, heart, adipose tissue, and kidney. A small proportion of unabsorbed SCFAs, are also excreted in feces.[84–87]

Clinical studies in humans have revealed decreased fecal concentrations of SCFAs in patients with GI disorders such as IBD[92] and colorectal cancer.[93] Regarding

the association of GI diseases and SCFAs, the anti-inflammatory effect of SCFAs is of interest. As mentioned previously, SCFAs can regulate inflammation by increasing the production of pro-inflammatory cytokines (e.g., IL-10 and TGFβ), decreasing pro-inflammatory cytokines (e.g., IL-6, IL-8, and TNFα), and activating transcription factor Foxp3.[77,90] A study evaluating the effects of SCFAs in *vitro* and in *vivo* reported that treatment of SCFAs increased T_{reg} population and enhanced its function, and ameliorated the severity of induced colitis in mice.[90] SCFAs can not only affect the host physiology but also affect the GI microbiota. The production of SCFAs provides an acidic luminal environment that prevents overgrowth of pH-sensitive pathogenic bacteria, such as Enterobacteriaceae and Clostridia.[70,118] Data from *in vitro* studies and experimental animal models suggest that composition of intestinal microbiota and SCFAs are important factors for activation of virulence genes of some enteropathogens.[35,46,97,98]

In veterinary medicine, however, limited information is available with regards to fecal SCFAs in dogs with various GI diseases. Most of the studies mainly evaluated the effects of nutritional intervention on fecal concentrations of SCFAs in healthy research dogs.[100–102] Therefore, their role in the pathogenesis of canine GI disease is still unknown. Therefore, we aimed to characterize fecal concentrations of SCFAs in healthy dogs and dogs with chronic GI disease.

III.3 Materials and methods

III.3.1 Fecal samples

Fecal samples from healthy control dogs and dogs with clinical signs of GI

disease were collected prospectively from April 2011 to June 2013. The protocol for sample collection was approved by the Texas A&M University Institutional Animal Care and Use Committee (#2012-83). Fresh fecal samples were collected at home by the owners and immediately frozen, then transported on ice to the Gastrointestinal Laboratory.

Healthy control dogs: A total of 50 privately owned dogs without clinical signs of GI disease (e.g., vomiting, diarrhea, anorexia, weight loss, or others) within the past three months of sample collection were enrolled. All dogs lived in diverse home environments and were fed a variety of commercial diets. Dogs that received antibiotics within the past three months were excluded

Diseased dogs: A total of 81 left-over fecal samples from submissions to the Gastrointestinal Laboratory at Texas A&M University were utilized for this study. These submissions were from dogs with clinical signs of chronic GI disease (i.e., clinical signs including vomiting, diarrhea, anorexia, weight loss, others for a period of at least 3 weeks) based on the clinical history and were submitted for fecal biomarker testing. The time of fecal sample collection after onset of diarrhea varied between samples depending on the time the dog was presented to the veterinarian. The clinical status of each dog was evaluated using a published canine chronic enteropathy activity index (CCEAI).

III.3.2 Clinical history of dogs

Questionnaires were sent to veterinarians and owners who submitted fecal

samples. The questionnaire was composed of three major parts: signalment of the dogs (i.e., breed, age, sex, body weight, and body condition score), health status of the dogs at time of fecal sample collection (i.e., presence of GI signs and its characteristics, duration of GI signs), medical history of dogs (i.e., medication [use of antibiotics, probiotics, etc.], concurrent diseases), and dietary information.

III.3.3 Analysis of fecal SCFAs

Concentrations of short-chain fatty acids (SCFA; acetate, propionate, and butyrate) in feces were measured using a stable isotope dilution gas chromatography-mass spectrometry (GC-MS) assay as previously described[119] with some modifications. Briefly, the fecal samples were weighed and diluted 1:5 in extraction solution (2N hydrochloric acid). After homogenization by a multi-tube vortexer for 30 minutes at room temperature, fecal suspensions were centrifuged for 20 minutes at 2,100 x g at 4°C. Supernatants were then collected using serum filters (Fisherbrand serum filter system, Fisher Scientific Inc, Pittsburgh, Pa). Of each sample, 500 µl of supernatant were mixed with 10 µl of internal standard (200mM heptadeuterated butyric acid) and extracted using a C18 solid phase extraction column (Sep-Pak C18 1 cc Vac Cartridge, Waters Corporation, Milford, MA). Samples were derivatized using *N*-tert-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) at room temperature for 60 minutes. A gas chromatograph (Agilent 6890N, Agilent Technologies Inc, Santa Clara, CA) coupled with a mass spectrometer (Agilent 5975C, Agilent Technologies Inc, Santa Clara, CA) was used for chromatographic separation and quantification of the

derivatized samples. Separation was achieved using a DB-1ms capillary column (Agilent Technologies Inc, Santa Clara, CA). The GC temperature program was as follows: 40°C held for 0.1 min, increased to 70°C at 5°C/ min , 70°C held for 3.5 min, increased to 160°C at 20°C /min, and finally increased to 280°C at 35°C/ min, then held for 3 min. The total run time was 20.53 min. The mass spectrometer was operated in electron impact positive-ion mode with selective ion monitoring at mass-to-charge ratios (*M/Z*) of 117 (acetate), 131 (propionate), 145 (butyrate), and 152 (deuterated butyrate; internal standard). Quantification was based on the ratio of the area under the curve of the internal standard and each of the fatty acids. Final concentrations of fecal SCFAs were adjusted by fecal dry matter (DM) and expressed as μmol/g of fecal DM.

III.3.4 Statistical analysis

Datasets for healthy control dogs and dogs with clinical signs of chronic GI disease were tested for normality using a Shapiro-Wilk test, and then compared using a Wilcoxon rank-sum test. A $p < 0.05$ was considered significant. All statistical analyses were conducted using a statistical software package (JMP® Pro version 10, SAS Institute Inc, Cary, NC).

III.4 Results

III.4.1 Signalment of dogs

A total of 50 fecal samples from healthy control dogs and 81 fecal samples from dogs with clinical signs of chronic GI disease were utilized for this study. The median age of healthy dogs and dogs with clinical signs of chronic GI disease was 4.5 years

(min to max: 0.8 to 11.7 y) and 6.5 years (min to max: 0.5 to 12 y), respectively (p = 0.041). Of the healthy control dogs, 26 were male (2 intact, 24 castrated) and 24 female (all spayed). Of the dogs with clinical signs of GI disease, 39 were male (6 intact, 33 castrated) and 34 female (3 intact, 31 spayed). There was no significant difference between the gender between the two groups (p = 0.236). The median body weight of healthy control dogs and dogs with clinical signs of chronic GI disease was 17.9 kg (min to max: 1.5 to 42.2 kg) and 20.9 kg (min to max: 1.7 to 72.6 kg), respectively (p = 0.256). A large variety of breeds was represented in this study population. None of the healthy control dogs received antibiotics for at least 3 months before sample collection.

III.4.2 Evaluation of fecal SCFAs

Fecal concentrations (median [min-max]) of acetate were significantly decreased (p=0.049; Figure 8 and Table 5) in dogs with clinical signs of chronic GI disease (193.4 [20.1-1042.1] µmol/g of fecal DM) compared to those in healthy control dogs (218.7 [87.7-672.8] µmol/g of DM). Also, fecal concentrations of propionate were significantly decreased (p<0.001; Figure 8 and Table 5) in dogs with clinical signs of chronic GI

disease (50.2 [0-227.9] μmol/g of fecal DM) compared to those in healthy control dogs (104.6 [1.6-266.8] μmol/g of DM). Moreover, fecal concentrations of total short-chain fatty acids were significantly decreased ($p=0.012$; Figure 8 and Table 5) in dogs with clinical signs of chronic GI disease (272.3 [21.7-1378.2] μmol/g of fecal DM) compared to those in healthy control dogs (383.6 [126.6-927.0] μmol/g of fecal DM). However, there was no significant difference ($p=0.233$; Figure 8 and Table 5) in fecal concentrations of butyrate between healthy control dogs (29.1 [8.1-148.1] μmol/g of fecal DM) and dogs with clinical signs of chronic GI disease (23.8 [0-137.6] μmol/g of fecal DM). Without adjusting for dry matter, fecal concentrations of all SCFAs were significantly decreased in dogs with clinical signs of chronic GI disease (Table 5).

While the proportion of acetate increased in dogs with clinical signs of chronic GI disease ($p = 0.015$), the proportion of propionate decreased in dogs with clinical signs of chronic GI disease compared to healthy control dogs ($p < 0.001$; Figure 9). The proportion of butyrate did not differ between the 2 groups.

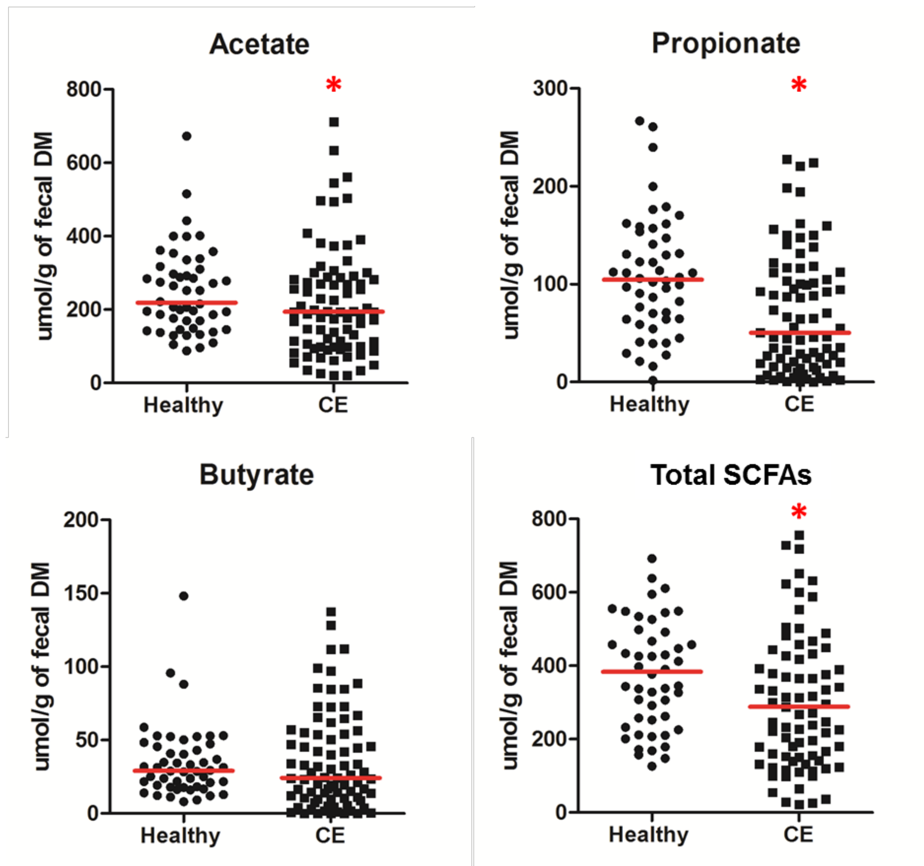

Figure 8. Fecal SCFAs concentrations in healthy control dogs and dogs with clinical signs of chronic GI disease. CE, dogs with clinical signs of chronic GI disease $*p < 0.05$

Table 5. Fecal SCFAs concentrations in healthy control dogs with dogs with clinical signs of chronic GI disease.

	WITH DM adjustment (µmol/g of fecal DM)					WITHOUT DM adjustment (µmol/g of wet feces)				
	HC		CE			HC		CE		
SCFA	**Median**	**Range**	**Median**	**Range**	**p-value**	**Median**	**Range**	**Median**	**Range**	**p-value**
Acetate	218.7	87.7-672.8	193.4	20.1-1042.1	**0.049**	81.2	25.3-224	53.3	3.8-151.3	**<0.001**
Propionate	104.6	1.6-266.8	50.2	0-227.9	**<0.001**	36.6	0.2-81.6	14.4	0-62.2	**<0.001**
Butyrate	29.1	8.1-148.1	23.8	0-137.6	0.233	10.2	1.5-30.3	7.3	0-31.2	**0.0071**
Total SCFAs	383.6	126.6-927.0	272.3	21.7-1378.2	**0.010**	129.8	27.2-292.9	72.2	4-229.3	**<0.001**

CE, dogs with clinical signs of chronic GI disease; HC, healthy control dogs; SCFA(s), short-chain fatty acid(s); DM, dry matter

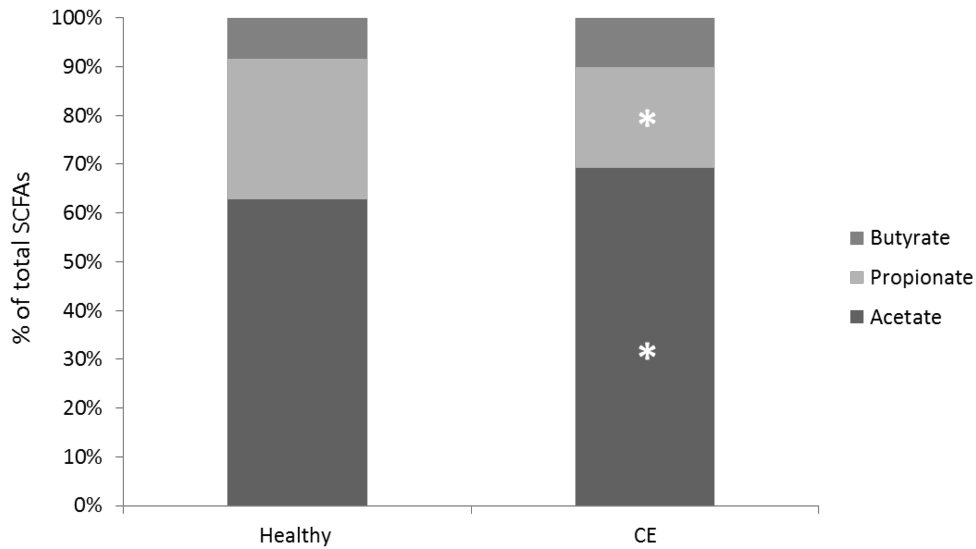

Figure 9. Composition of total fecal SCFAs in healthy control dogs and dogs with clinical signs of chronic GI disease. CE, dogs with clinical signs of chronic GI disease; * $p < 0.05$

III.5 Discussion

In this study, dogs with clinical signs of chronic GI disease had decreased fecal concentrations of total SCFAs, specifically decreased concentrations of acetate and propionate. Without dry matter adjustment, fecal concentrations of all SCFAs were significantly decreased in dogs with clinical signs of chronic GI disease compared to healthy control dogs.

While the fecal concentration of acetate was slightly decreased in dogs with clinical signs of chronic GI disease, its proportion (i.e., acetate/total SCFAs) was significantly increased in dogs with clinical signs of chronic GI disease. On the other

hand, the proportion of propionate was significantly decreased. These findings are of importance, as recent studies suggest that activation of virulence factors of *C. difficile* and *Salmonella* is associated with changes in SCFAs concentrations.[46,49] Lawhon et al. reported on the effects of SCFAs on virulence genes of *Salmonella typhimurium*. At low total SCFA concentrations with a predominant of acetate, the expression of the invasion gene, SPI-1 gene, was induced. However, at high total SCFA concentrations with a higher proportion of propionate, the expression of the SPI-1 gene was suppressed.[98] Similar findings where propionate and butyrate suppressed the expression of the SPI-1 gene have been reported in in-vitro studies.[97,99]

In the current study, the degree of change in concentration of propionate was prominent among all SCFAs. As mentioned above, propionate is metabolized in the liver and utilized as a source for gluconeogenesis. Therefore, the decreased concentration of propionate may lead to an alteration of energy metabolism in dogs with GI disease. In fact, the presence of altered energy metabolism was suspected based on the serum metabolite profiles in the other part of this study (chapter 5). Significant activation of the pentose phosphate pathway (an alternative pathway of glycolysis) and significant increased 3-hydroxybutyrate (ketone body), hexuronic acid (vitamin C), ribose, and gluconic acid lactone were observed in dogs with IBD (chapter 5). Propionate is also important because of its anti-inflammatory effect on intestinal inflammation. It has been shown that propionate decreases the production of pro-inflammatory cytokines such as IL-6, IL-8, and TNFα.[120,121] Moreover, recent evidence shows the regulatory effect of propionate on colonic regulatory T cells (T_{regs}).[90] In this study the treatment of

propionate stimulated the expression of IL-10 and one of the transcription factors Foxp3. Activation of Foxp3 is crucial for suppression /regulation of intestinal inflammation. Moreover, treatment of propionate increased T_{regs} population and improved function through GPR43 signaling.[90] Authors also demonstrated that these effects ameliorate the severity of experimental colitis in mice.[90] Therefore, a decrease in concentration of propionate in dogs with clinical signs of chronic GI disease may affect immune system responses and resistance mechanisms against enteropathogens.

Similar to our findings, studies in humans and rodent models have shown that decreased concentrations of SCFAs in the intestinal tract are associated with GI disease such as IBD and colorectal cancer.[92,93] In these studies, the protective and therapeutic effects of SCFAs on GI disease have also been reported. Therefore, therapeutic interventions by manipulating SCFAs concentrations in the GI tract may be a promising treatment for suppressing intestinal inflammation and restore proper immune responses. Further research is warranted to determine the efficacy of SCFAs manipulation in dogs with GI disease.

There are some limitations to this study. There was a significant age difference between the dogs in the control group and dogs with clinical signs of chronic GI disease. No studies have evaluated the effect of age on fecal concentrations of SCFAs. Therefore, the influence of age differences on this study is unknown. However, the age range of dogs in the control group did overlap with that of the disease group, thus, the statistically significant difference in age may not have created a major bias in this study. In our dataset, there were several outliers, and those samples tended to have a low fecal DM

value. This raised the question regarding normalization for fecal concentrations of SCFAs. While most of the studies evaluating fecal concentrations of SCFAs in dogs reported the concentrations adjusted by fecal DM (i.e., µmol/g of fecal DM), most of the clinical studies in humans reported the concentrations without adjustment to fecal DM (i.e., µmol/g of fecal content or wet feces). No consensus has been reached at this point whether normalization is necessary or not.

In conclusion, this study demonstrated decreased concentrations and altered composition of SCFAs in dogs with clinical signs of chronic GI disease. Therefore, interventions manipulating SCFAs concentrations in the GI tract may prove to be a useful treatment for suppression of intestinal inflammation and to restore proper immune responses. Further research is warranted to determine the efficacy of SCFAs manipulation in dogs with GI diseases.

CHAPTER IV

ALTERATION OF THE FECAL MICROBIOTA AND SERUM METABOLITE PROFILES IN DOGS WITH IDIOPATHIC INFLAMMATORY BOWEL DISEASE*

IV.1 Overview

Idiopathic inflammatory bowel disease (IBD) is a common cause of chronic gastrointestinal (GI) disease in dogs. The combination of an underlying host genetic susceptibility, an intestinal dysbiosis, and dietary/environmental factors are suspected as main contributing factors in the pathogenesis of canine IBD. However, actual mechanisms of the host-microbe interactions remain elusive. The aim of this study was to compare the fecal microbiota and serum metabolite profiles between healthy dogs (n=10) and dogs with IBD before and after 3 weeks of medical therapy (n=12). Fecal microbiota and metabolite profiles were characterized by 454-pyrosequencing of 16S rRNA genes and by an untargeted metabolomics approach, respectively. Significantly lower bacterial diversity and distinct microbial communities were observed in dogs with IBD compared to the healthy control dogs. While Gammaproteobacteria were overrepresented, Erysipelotrichia, Clostridia, and Bacteroidia were underrepresented in dogs with IBD. The functional gene content was predicted from the 16S rRNA gene data using PICRUSt, and revealed overrepresented bacterial secretion system and

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transcription factors, and underrepresented amino acid metabolism in dogs with IBD. The serum metabolites 3-hydroxybutyrate, hexuronic acid, ribose, and gluconic acid lactone were significantly more abundant in dogs with IBD. Although a clinical improvement was observed after medical therapy in all dogs with IBD, this was not accompanied by significant changes in the fecal microbiota or in serum metabolite profiles. These results suggest the presence of oxidative stress and a functional alteration of the GI microbiota in dogs with IBD, which persisted even in the face of a clinical response to medical therapy.

IV.2 Introduction

The close relationship between the gastrointestinal (GI) microbiota and the host has a crucial impact on the health status of an animal.[122] Intestinal dysbiosis, which describes an alteration of the GI microbiota, has been observed in human patients with chronic GI inflammation[22] but also in dogs.[12,16,28,29] Idiopathic inflammatory bowel disease (IBD) is a common cause of chronic GI disease in dogs. Similarly to human IBD, the combination of an underlying host genetic susceptibility, an intestinal dysbiosis, and dietary and/or environmental factors are suspected as main contributing factors in the pathogenesis of canine IBD.[123–125] Furthermore, similarly to humans, intestinal inflammation develops spontaneously in dogs living in natural home environments, and the most frequently used treatment modalities for these disorders include antibiotic therapy and immunosuppression.[126]

Despite well documented evidence that the intestinal microbiota plays a role in the pathogenesis of canine IBD, the actual mechanisms of the host-microbe interactions remain elusive, but are believed to be mediated in part by microbial products (metabolites) derived from the GI microbiota and locally and/or systemically absorbed by the host.[34] Therefore, studies that assess functional aspects of the GI microbiota are needed. Metabolomics, the comprehensive study of small molecules present in biological samples, is an emerging method for better understanding of disease pathophysiology and host-microbe interactions, with the potential to develop novel diagnostic and/or treatment approaches.[92,104] Metabolomics studies have been reported in human patients with IBD as well as animal models of IBD, and provided new insights into disease pathogenesis.[42,103] Using mass spectrometry platforms, an untargeted metabolomics approach can identify hundreds of metabolites in biological samples simultaneously, and therefore provides a comprehensive functional overview of biochemical pathways that are being up- or down-regulated during different physiologic or pathophysiologic state.[43] In veterinary medicine, only few studies have utilized this approach in clinical patients,[64,65] and to our knowledge, no studies using an untargeted metabolomics approach have been reported in dogs with IBD. Therefore, we aimed to characterize the fecal microbiota and serum metabolite profiles in healthy dogs and compare those to dogs with IBD. We also investigated the effects of 3 weeks of medical intervention on these profiles. To investigate the global differences in GI microbial communities and metabolic profiles, we utilized 454-pyrosequencing of 16S ribosomal RNA (16S rRNA)

genes and an untargeted metabolomics approach using gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF/MS).

IV.3 Materials and methods

IV.3.1 Animals and sample collection

The collection and analysis of serum and fecal samples from healthy control dogs and dogs with IBD were reviewed and approved by the Texas A&M University Institutional Animal Care and Use Committee (#2012-83). Written informed consent was obtained from all owners of enrolled dogs. A blood sample was obtained during initial physical examination and serum was frozen within a few hours of collection and stored at -80°C until analysis. Left-over naturally-passed feces collected for routine fecal examination were frozen within a few hours of collection at -80°C, and were stored frozen until processing of samples for DNA extraction.

Healthy control dogs: Ten healthy dogs were enrolled. All dogs were privately owned, lived in diverse home environments, and were fed a variety of commercial diets (Supplementary Table 4). All dogs were judged to be healthy on the basis of normal clinical examination findings and routine laboratory testing. None of the dogs had exhibited clinical signs of GI disease for at least 6 weeks prior to clinical evaluation. None of the dogs had received antibiotics or other drug therapy for at least 2 months

prior to clinical examination. All dogs were eating a commercial canine maintenance diet (Supplementary Table 4).

Dogs with IBD: Dogs with clinical signs of chronic GI disease (i.e., vomiting, diarrhea, anorexia, weight loss, etc.) were diagnosed with idiopathic inflammatory bowel disease (IBD) by a board certified veterinary internist (AEJ) based on the World Small Animal Veterinary Association (WSAVA) criteria: (i) chronic (i.e., > 3 weeks) GI signs; (ii) histopathologic evidence of mucosal infiltration with inflammatory cell; (iii) inability to document other causes of GI inflammation; (iv) inadequate response to dietary, antibiotic, and anthelmintic therapies, and (v) clinical response to anti-inflammatory or immunosuppressive agents. Histological samples were obtained endoscopically. The clinical status of each dog was evaluated using a published clinical canine IBD activity index (CIBDAI).[125] Twelve dogs that showed clinical improvement after therapy were enrolled this study. All medications had been discontinued at least 2 weeks prior to the first sample collection time point (IBD-PRE). After the first sample collection, dogs were then treated with immunosuppressive drug therapy (i.e., prednisone [1-2 mg/kg, q24h, PO], prednisolone [1-2 mg/kg, q24h, PO], budesonide [1-3 mg/kg, q24h, PO], cyclosporine [3-5 mg/kg, q12h or q24h, PO], or a combination of these), and 5 of 12 dogs also received the antibiotic metronidazole (10 mg/kg, q12h, PO). A second set of serum and fecal samples was obtained from these dogs after 21 days of treatment (IBD-POST). In addition to medical therapy, all IBD dogs were fed various commercial diets (Supplementary Table 5).

IV.3.2 DNA extraction

An aliquot of 100 mg (wet weight) of each fecal sample was extracted by a bead-beating method using the ZR Fecal DNA Kit™ (Zymo Research Corporation, Irvine, CA) following the manufacturer's instructions. The bead-beating step was performed on a homogenizer (FastPrep-24, MP Biomedicals, Santa Ana, CA) for 60 s at a speed of 4 m/s. Fecal DNA was stored at -80°C until analysis.

IV.3.3 454-pyrosequencing

Bacterial tag-encoded FLX-titanium amplicon pyrosequencing (bTEFAP) based the V4-V6 region (*E. coli* position 530 – 1100) of the 16S rRNA gene was performed to evaluate the relative abundances of bacterial taxa with primers forward 530F: GTGCCAGCMGCNGCGG and reverse 1100R: GGGTTNCGNTCGTTR. Raw sequence data were screened, trimmed, denoised, chimera depleted, and filtered using the QIIME pipeline version 1.8.0 (http://qiime.sourceforge.net)[127] with the following settings: minimum read length of 300 bp; no ambiguous base calls; no homopolymeric runs longer than 8 bp; average quality value.q25 within a sliding window of 50 bp. Operational taxonomic units (OTUs) were defined as sequences with at least 97% similarity using QIIME. For classification of sequences on a genus level the naıve Bayesian classifier within the Ribosomal Database Project (RDP, v10.28) was used. The confidence threshold in RDP was set to 80%.

IV.3.4. Quantitative PCR (qPCR)

The qPCR assays for selected bacterial groups were performed to validate pyrosequencing result and/or to evaluate bacterial groups that are typically present at very low abundance or underrepresented in 16S rRNA gene based sequencing (total bacteria; Phylum level – Fusobacteria, Bacteroidetes; Family level – Ruminococcaceae; Genus level –*Bifidobacterium* spp., *Blautia* spp., *Faecalibacterium* spp., *Turicibacter* spp., and *Lactobacillus* spp.; and species level – *Escherichia coli* (*E. coli*). The qPCR cycling, the oligonucleotide sequences of primers and probe, and respective annealing temperatures for selected bacterial groups were described previously.[8,16] A commercial real-time PCR thermal cycler (CFX 96 Touch™ Real-Time PCR Detection System; Biorad Laboratories, Hercules, CA) was used for all qPCR assays and all samples were run in duplicate fashion.

IV.3.5 Analysis of serum metabolites

Untargeted metabolomics analysis was performed by the West Coast Metabolomics Center at the University of California (Davis, CA). Serum aliquots were extracted by degassed acetonitrile. Internal standards C08-C30 FAMEs were added and the samples were derivatized by methoxyamine hydrochloride in pyridine and subsequently by N-methyl-N-trimethylsilyltrifluoroacetamide for trimethylsilylation of acidic protons. Analytes were separated using an Agilent 6890 gas chromatograph (Santa Clara, CA) and mass spectrometry was performed on a Leco Pegasus IV time of flight mass spectrometer (St. Joseph, MI) following the published protocol.[47]

IV.3.6 Statistical analysis

Sequence data and qPCR data: Differences in bacterial abundances between the HC and IBD-PRE groups were evaluated using a Mann-Whitney test. Samples collected before and after treatment from each IBD dog were compared using a Wilcoxon signed rank test. The Benjamini & Hochberg's False Discovery Rate was used to correct for multiple comparisons and an adjusted $p < 0.05$ (i.e., $q < 0.05$) was considered to be statistically significant. To evaluate differences in overall microbiota composition (i.e., beta-diversity) between the groups, the analysis of similarities (ANOSIM) was performed on the unweighted UniFrac distance matrixes. The PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States: http://picrust.github.io/picrust/)[128] was used to predict the functional capabilities of bacteria based on the 16S rRNA gene dataset. Linear discriminant analysis (LDA) effect size (LEfSe: http://huttenhower.sph.harvard.edu/galaxy/)[129] was utilized to evaluate differentially abundant bacterial taxa and predicted function between the animal groups. To identify associations of microbial abundances with the use of antibiotic and other clinical metadata (age, weight, gender), which may be confounding factors, MaAsLin (Multivariate Analysis by Linear Models: http://huttenhower.sph.harvard.edu/maaslin)[128] was utilized

Untargeted metabolomics data: Univariate analysis; Differences in the abundance of serum metabolites between the HC and IBD-PRE groups were evaluated using a Mann-Whitney test. Serum samples collected before and after treatment from each affected dog

(IBD-PRE vs. IBD-POST) were compared using a Wilcoxon signed rank test. Univariate analysis was performed using Prism version 5 (Graph Pad Software, La Jolla, CA). Multivariate analysis; Data were normalized to the sum of the total spectral integral, log transformed, mean centered, and divided by the standard deviation of each variable prior to multivariate analysis. PCA was performed and a heatmap was generated using MetaboAnalyst.[130] Correlation and biochemical relationship networks; Correlation analyses were performed in R using the "cor" function. Relationships with a Pearson's correlation coefficient greater than 0.9, or smaller than -0.9, were used to build correlation networks. For biochemical relationship analysis, named metabolites were mapped onto a modified MetaMapp network.[24] The network originally published by Barupal et al.[131] was expanded by the inclusion of additional nodes and edges as described by Steelman et al.[64] Correlation and biochemical relationship networks were visualized in Cytoscape[132] by mapping metabolite data onto the expanded MetaMapp network. Data included alphanumeric name, level of significance ($q >$ or < 0.25), and PubChem ID as the unique identifier. A custom visual style was created using the following color scheme: white nodes represent metabolites that were not detected in the present study, light blue nodes represent metabolites that were detected but did not differ significantly among groups, and dark blue nodes represent metabolites that did differ significantly among groups. Evaluation of the performance of potential biomarkers; ROC analysis was performed using the Classical Analysis module in ROC Curve Explorer & Tester (ROCCET).[133]

IV.4 Results

The characteristics of the healthy control dogs (HC) and dogs with IBD (pre-treatment, IBD-PRE; post-treatment, IBD-POST) are summarized in Table 1. Significant differences in age and body weight were observed between HC and dogs with IBD. The canine IBD activity index (CIBDAI) was significantly decreased after 3 weeks of medical therapy (Table 6).

Table 6. Dog characteristics

	HC	IBD-PRE	IBD-POST	p-value (HC vs. IBD-PRE)
Number	10	12	12	n/a
Age (years; mean ± SD)	4.2 ± 2.9	7.6 ± 3.6	7.6 ± 3.6	0.025
Gender (male/female)	5/5	5/7	5/7	1.0
Weight (kg; median [range])	26.5 [7.0 - 42.0]	5.5 [3.0 - 23.0]	6.5 [3.0 - 23.0]	0.0005
CIBDAI (median [range])	n/a	6.0 [1.0 - 13.0]	1.0 [0-5.0]	0.0024*

HC, healthy control dogs; IBD-PRE, dogs with IBD pre-treatment; IBD-POST, dogs with IBD post-treatment; CIBDAI, canine IBD activity index * p-value from IBD-PRE vs. IBD-POST

IV.4.1 Sequence analysis

The 454-pyrosequencing pipeline yielded 297,619 quality sequences with an average of 8,753 sequences per sample. To account for unequal sequencing depth across samples, the subsequent analysis was performed on a randomly selected subset of 2,900 sequences per sample. The pre-treatment sample from one dog with IBD was excluded due to insufficient sequencing depth. A total of 11 phyla and138 genera were identified. The sequencing data have been deposited into the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under accession number SRP040310.

Fecal microbial communities in healthy control dogs and dogs with IBD: To characterize the global differences in fecal microbial communities between groups, a principal coordinates analysis (PCoA) was performed on unweighted UniFrac distances. The PCoA plots showed significant separation between the fecal samples from the HC and IBD-PRE groups (analysis of similarity [ANOSIM]; $p = 0.01$) (Figure 10A). The Shannon index and the Chao 1 metric were significantly decreased in the IBD-PRE group compared to the HC group ($p = 0.014$, 0.0146, respectively; Figure 10C). The number of observed species was also decreased in the IBD-PRE group but did not reach statistical significance ($p = 0.0656$; Figures 10B and 10C). Linear discriminant analysis (LDA) effect size (LEfSe) was utilized to determine differentially abundant bacterial taxa between the animal groups. A total of 22 bacterial groups were differentially expressed ($\alpha = 0.01$, LDA score > 3.0) between the HC and IBD-PRE groups. Gammaproteobacteria were overrepresented, while Erysipelotrichia, Clostridia, and

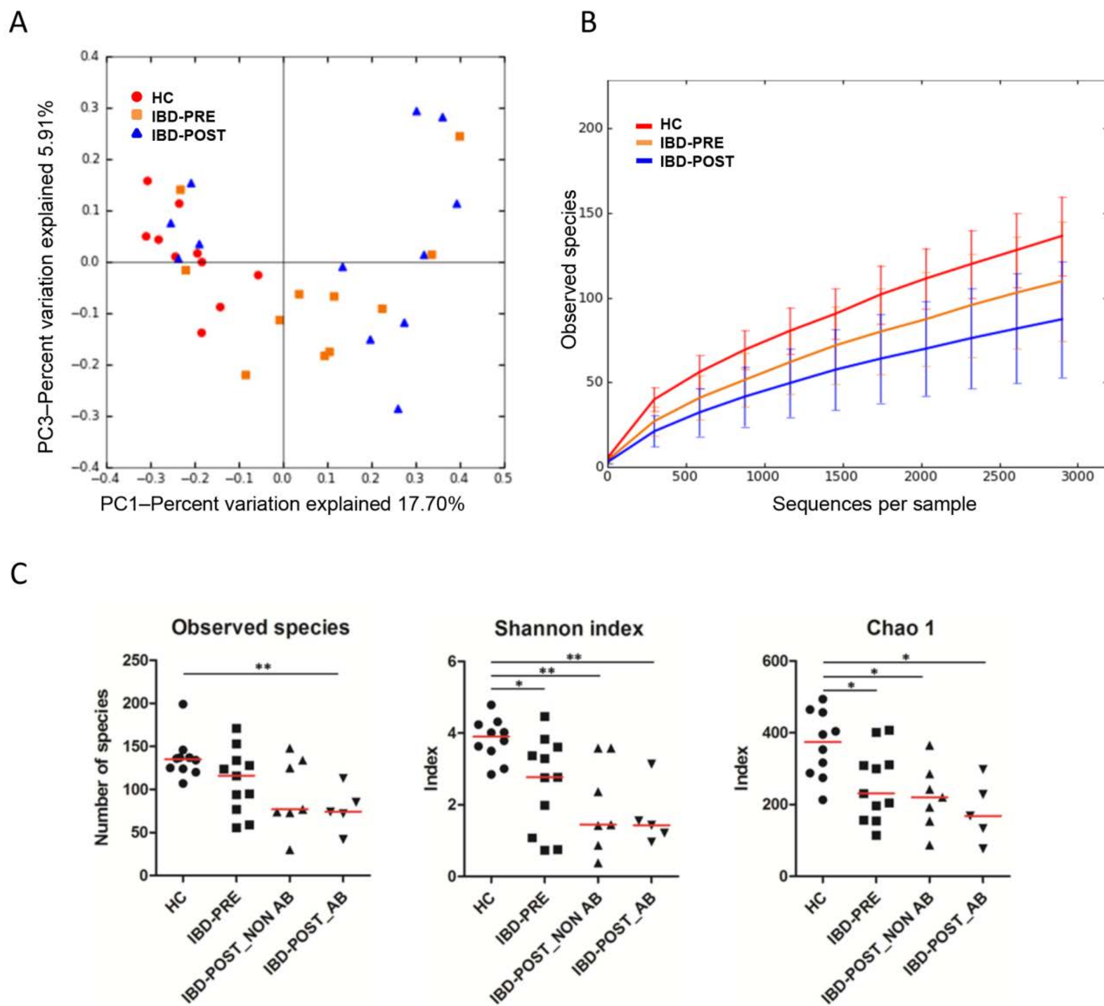

Figure 10: Bacterial diversity measures. β diversity: (A) principal coordinates analysis (PCoA) of unweighted UniFrac distances of 16S rRNA genes. PCoA plots along with Principal Coordinates (PC) 1 and PC 3. Analysis of similarity (ANOSIM) revealed clustering between healthy control dogs and dogs with IBD ($p = 0.01$), but not between IBD pre-treatment and IBD post-treatment groups ($p = 0.34$). Alpha diversity measures: (B) rarefaction analysis (number of observed species) of 16S rRNA gene sequences. Lines represent the mean of each group, while the error bars represent the standard deviations. (C) Comparisons of alpha diversity. Samples from dogs with IBD post-treatment were divided into two groups based on antibiotic administration status during 3 weeks of medical intervention. Red lines represent the median for each measure. HC, healthy control dogs; IBD-PRE, dogs with IBD pre-treatment; IBD-POST_NON AB, dogs with IBD post-treatment that did not receive antibiotic; IBD-POST_AB, dogs with IBD post-treatment that received antibiotic *$p < 0.05$; and **$p < 0.01$

Bacteroidia were underrepresented in dogs with IBD-PRE (Figure 11). Multivariate Analysis by Linear Models (MaAsLin) was utilized to identify associations of microbial abundances with clinical metadata (age, weight, gender), which may be confounding factors. None of these variables were significantly associated with microbial abundance.

qPCR analysis of fecal microbial communities: qPCR assays for selected bacterial groups were performed to confirm pyrosequencing results and/or to quantitate bacterial groups that are typically present at very low abundance or underrepresented in 16S rRNA gene sequencing data based on the authors' experience from previous studies (i.e., *Bifidobacterium* spp., *Faecalibacterium* spp.).[14,134,135] The abundances of *Blautia* spp., *Faecalibacterium* spp., and *Turicibacter* spp. were significantly decreased in the IBD-PRE group (q = 0.0277, 0.0480, 0.0380, respectively) compared to those in the HC group. *E. coli* appeared to be increased in the IBD-PRE group, but this increase did not reach statistical significance when the p-value was adjusted by False Discovery Rate (FDR) of 5% (q = 0.0560; Figure 12).

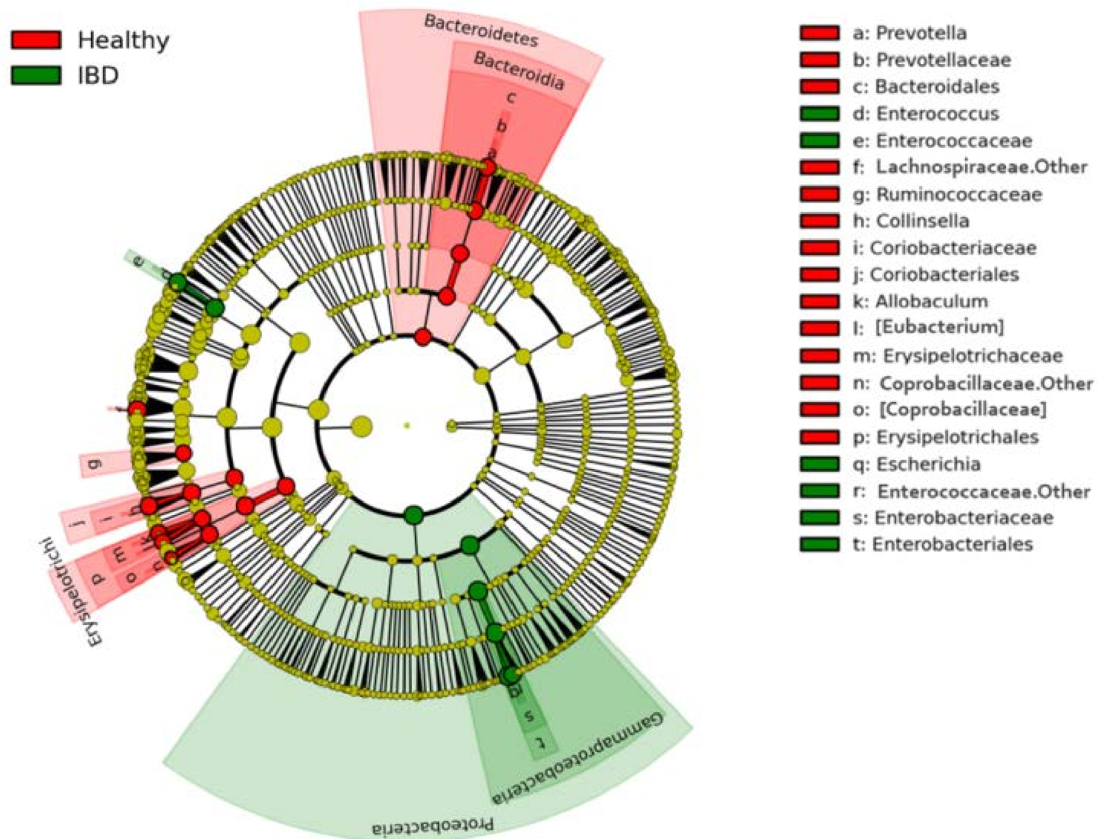

Figure 11: Linear discriminant analysis (LDA) effect size (LEfSe) of 454-pyrosequencing data sets based on 16S rRNA gene sequences.Taxonomic distribution of bacterial groups significant for IBD. A total of 22 differentially abundant bacterial taxa were detected ($\alpha = 0.01$, LDA score > 3.0). Of those, 7 bacterial taxa were significantly overrepresented in pretreatment samples from dogs with IBD (green) and 15 bacterial taxa were overrepresented in samples from healthy control dogs (red).

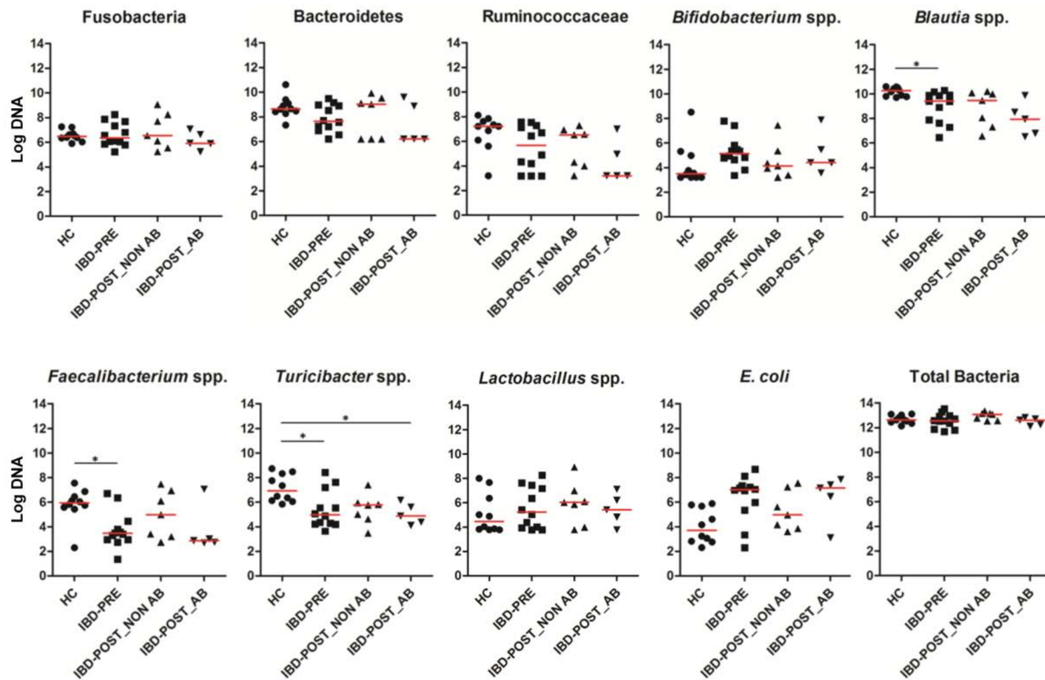

Figure 12.The abundances of selected bacterial groups in healthy dogs and dogs with IBD (pre- and post-treatment) based on qPCR. Samples from dogs with IBD post-treatment were divided into two groups based on antibiotic administration status during the 3 weeks of medical intervention. Red lines represent the median of log DNA. HC, healthy control dogs; IBD-PRE, dogs with IBD pre-treatment; IBD-POST_NON AB, dogs with IBD post-treatment that did not receive antibiotic; IBD-POST_AB, dogs with IBD post-treatment that received antibiotic *$q < 0.05$

Predicted functional composition of fecal microbial communities: To investigate alterations in GI microbial function, an analysis for Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was applied to the 16S rRNA gene sequencing data. The PICRUSt results (Figure 13A) were then analyzed using LEfSe to identify microbial functions that were significantly different in their abundance between groups. A total of 4 differentially abundant bacterial functions were observed between the HC and IBD-PRE groups ($\alpha = 0.01$, LDA score > 3.0). Of those, the functions of secretion system and transcription factors were overrepresented in the IBD-PRE group. In contrast, amino acid metabolism was underrepresented in the IBD-PRE group (Figure 13B). To investigate the alteration of some of the bacterial functions of major metabolic systems, a univariate analysis was applied on the PICRUSt data. The relative abundances of lipid, carbohydrate, and energy metabolisms were not significantly different between the HC and IBD-PRE groups (Figure 13C).

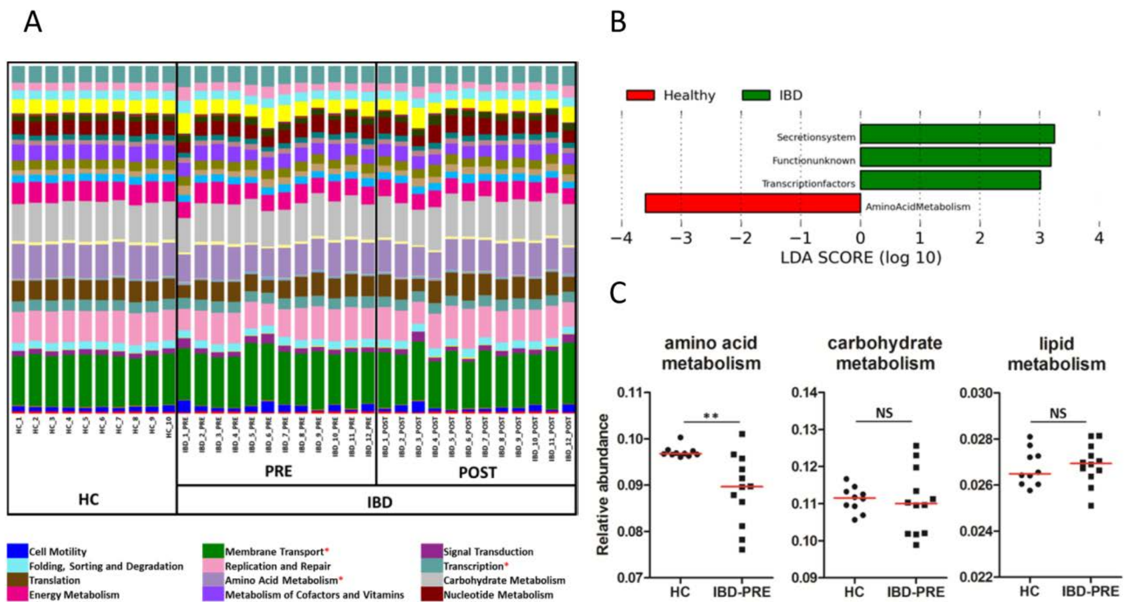

Figure 13: Predicted functional composition of metagenomes based on 16S rRNA gene sequencing data. (A) Relative abundances of predicted functions (second level of the Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog (KO) hierarchy) based on phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) data set. Each stacked bar represents relative abundances of the predicted functions of each dog. (B) LEfSe based on the PICRUSt data set (third level of the KO hierarchy) revealed a total of 4 differentially enriched bacterial functions (enriched in healthy controls: amino acid metabolism, enriched in IBD dogs: secretion system, transcription factors, and a pathway with unknown function) between healthy control dogs and dogs with IBD (pre-treatment; $\alpha = 0.01$, LDA score > 3.0). None of the bacterial functions were differentially expressed between IBD pre-treatment and IBD post-treatment. (C) Univariate analysis of major metabolisms. Red lines represent the median of relative abundance. HC, healthy control dogs; IBD-PRE, dogs with IBD pre-treatment; IBD-POST, dogs with IBD post-treatment; NS, no significance *metabolic functions that differed significantly between healthy control dogs and dogs with IBD **$q < 0.01$.

Effects of medical treatment on fecal microbial communities: We further evaluated the effects of 3 weeks of medical intervention on fecal microbial communities in dogs with IBD. All dogs received a standard therapy, which consisted of administration of an immunosuppressive drug (i.e., prednisone [1-2 mg/kg, q24h, PO], prednisolone [1-2 mg/kg, q24h, PO], budesonide [1-3 mg/kg, q24h, PO], cyclosporine [3-5 mg/kg, q12h or q24h, PO], or a combination of these) and feeding of an elimination diet (i.e., antigen-restricted or protein hydrolysate diets).

Five out of twelve IBD dogs received the antibiotic metronidazole (10 mg/kg, q12h, PO) in addition to immunosuppressive drug therapy. All dogs responded to their therapies (i.e., GI signs resolved, CIBDAI improved) and none had diarrhea after medical intervention. Although all dogs showed clinical improvement after 3 weeks of therapy, no major differences in microbial communities were observed between the IBD-PRE and IBD-POST groups (ANOSIM, $p = 0.34$; Figure 10A). In the IBD-POST group, the diversity indices further decreased compared to the IBD-PRE, but this decrease did not reach statistical significance (Figures 10B and 10C). The LEfSe analysis revealed that none of the bacterial taxa were differentially expressed between IBD-PRE and IBD-POST groups. These observations were confirmed by the qPCR assays, as none of the abundances of the evaluated bacterial groups were significantly different between IBD-PRE and IBD-POST (Figure 12). Consequently, none of the predicted bacterial functions were differently expressed between IBD-PRE and IBD-POST.

To address if the administration of metronidazole had a confounding effect on the GI microbiota in this study, the sequencing data within the IBD-POST group was compared between dogs that received antibiotic versus dogs that did not receive antibiotic. The PCoA plots showed no significant separation between the samples from dogs that received antibiotic and those that did not (ANOSIM, $p = 0.51$; Figure 14A). Also, there were no significant differences in unweighted UniFrac distances between the pre- and post-treatment samples from dogs that received antibiotic and those that did not ($p = 0.5273$; Figure 14B). The results of the qPCR assays were also statistically analyzed in the IBD-POST group based on antibiotic administration. Noteworthy trends were observed, suggesting that dogs that did not receive antibiotic in general had median abundances of Fusobacteria, Bacteroidetes, Ruminococcaceae, *Bifidobacterium* spp., *Blautia* spp., *Faecalibacterium* spp., and *E. coli* that were more similar to median abundances observed in the HC group (Figure 12). This was most notable for *Turicibacter* spp., where dogs that had received antibiotic had significantly decreased abundances of this bacterial group compared to dogs of the HC group. MaAsLin was utilized to identify associations of microbial abundance with antibiotic treatment. No significant association between antibiotic treatment and microbial abundance was identified.

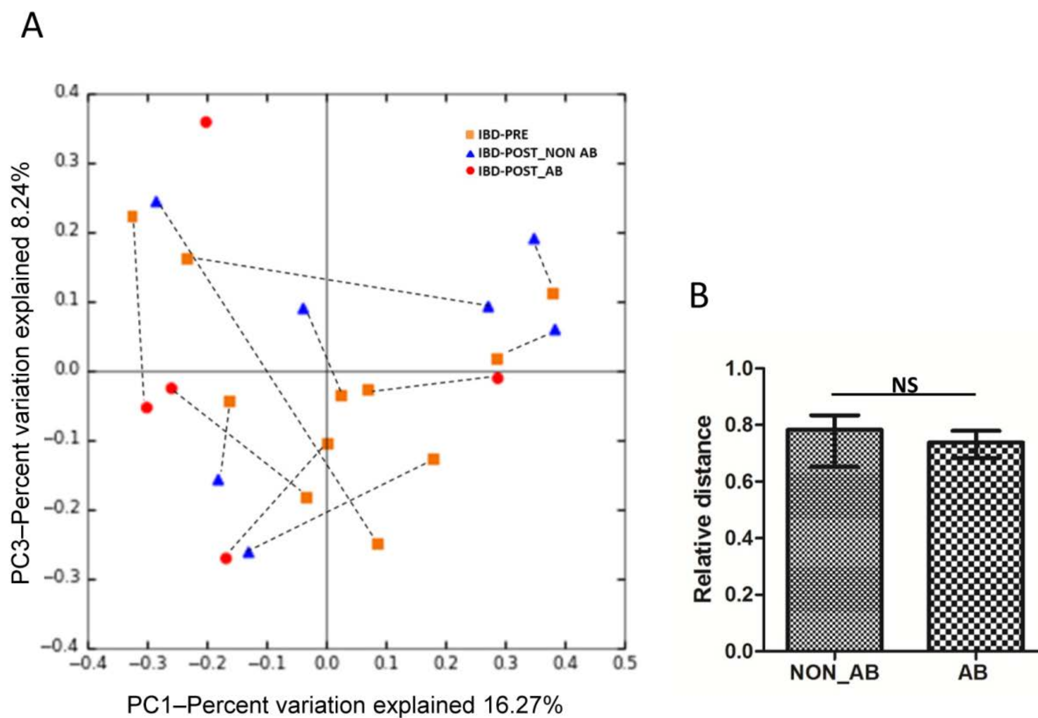

Figure 14. Effect of antibiotic administration on the fecal microbiota. (A) PCoA of unweighted UniFrac distances of 16S rRNA genes from dogs with IBD pre-treatment and post-treatment. Dogs in the IBD post-treatment group were divided into two groups based on the antibiotic administration status during 3 weeks of medical intervention. ANOSIM revealed no clustering either between IBD-PRE and IBD-POST_AB, or IBD-PRE and IBD POST-NON_AB (ANOSIM $p = 0.26$, $p = 0.51$, respectively). Dashed lines connect the pre-treatment and the post-treatment samples from each dog. The pre-treatment sample from one dog with IBD was excluded due to insufficient sequencing depth. (B) Median unweighted UniFrac distance between pre- and post-treatment samples from dogs with and without antibiotic treatment. Whiskers represent interquartile ranges. IBD-PRE, dogs with IBD pre-treatment; IBD-POST_NON AB, dogs with IBD post-treatment that did not receive antibiotic; IBD-POST_AB, dogs with IBD post-treatment that received antibiotic; NS, no significance

IV.4.2 Untargeted metabolomics analysis

A total of 359 metabolites were detected. Of those, 157 were identified metabolites, while 202 lacked full structural identification (unknown metabolites). These unknown metabolites are listed by their Binbase identification numbers (http://fiehnlab.ucdavis.edu/projects/binbase_setupx).[136]

Serum metabolite profiles in healthy control dogs and dogs with IBD: To characterize the global metabolic differences between groups, a principal component analysis (PCA) was applied. Serum metabolite profiles associated with the HC group were tightly clustered, whereas those in the IBD-PRE group were much more scattered (Figure 15A). There was considerable overlap in metabolite profiles observed between the two IBD groups. Similarly to the results of the PCA analysis, no distinct pattern was observed when the data were plotted by heatmap analysis (Figure 15B). However, two major groups of metabolites clustered in the dendrogram, with cluster 1 being more abundant in healthy animals and cluster 2 appearing to be more abundant in the IBD group. Although many unknown metabolites are included on this heatmap, cluster 1 contains a number of amino acids, whereas cluster 2 is composed of a wide variety of metabolites. Nonparametric univariate analysis using FDR of 5% revealed a total of 9 metabolites (4 identified and 5 unknown) that differed significantly between the HC and IBD-PRE groups. Identified metabolites are shown at the top of Table 7 and in Figure 15C. Complete statistical results for all metabolites with FDR less than 25% are shown in supplementary Table 1. The 4 identified metabolites include gluconic acid lactone (also known as gluconolactone), hexuronic acid (also known as ascorbic acid or vitamin

C), 3-hydroxybutanoic acid (ketone body) and ribose. All 4 metabolites were significantly increased in the IBD-PRE group (Figure 15C).

Effects of medical treatment on serum metabolite profiles: Three metabolites differed significantly between the IBD-PRE and IBD-POST groups. Of these, only one, gluconic acid lactone, was an identified metabolite (Figure 15C and supplementary Table 1). As performed in the analysis of fecal microbial communities, serum metabolite profiles were also evaluated based on antibiotic administration. The PCA plot showed no clear separation between samples from dogs that received antibiotic and those that did not (Figure 16). The univariate analysis of the selected metabolites based on antibiotic administration status also revealed no significant differences in any of the significantly different metabolites between samples from dogs that received antibiotic and those that did not (Figure 15C).

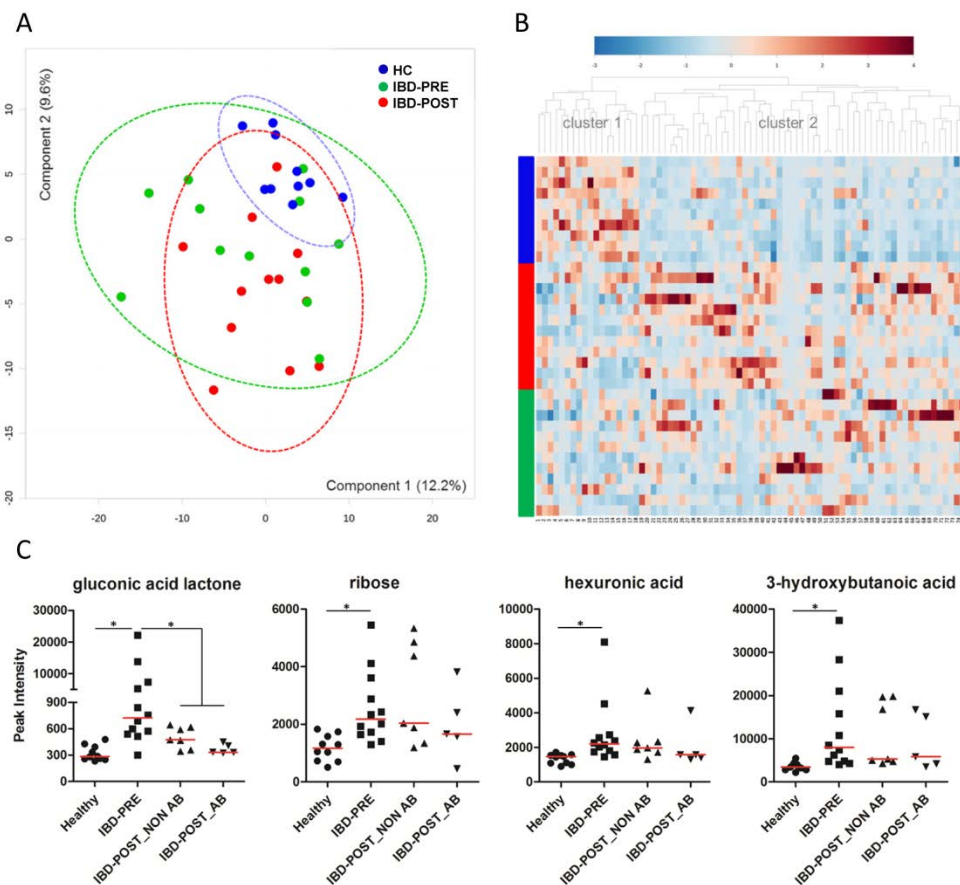

Figure 15. Serum metabolite profiles. (A) PCA score plots of metabolites in serum from healthy control dogs, dogs with IBD pre-treatment, and dogs with IBD post-treatment. Ellipses represent the 95% confidence interval of metabolite profiles for each group. No clear separations between groups were identified. (B) Hierarchical clustering and heatmap of the top 75 metabolites that were different in their peak intensity between groups. The 75 most abundant metabolites are provided on the x-axis (see detail in supplementary table 3). Each row represents the serum metabolite profile of each dog and sorted by group (colored bars on the x-axis represent groups (blue, healthy control dogs; red, dogs with IBD post-treatment; green, dogs with IBD pre-treatment) (C) Comparisons of the peak intensity for differentially expressed serum metabolites. Samples from dogs with IBD post-treatment were divided into two groups based on antibiotic administration status during 3 weeks of medical intervention. Gluconic acid lactone was the only identified metabolite that differed significantly between dogs with IBD pre-treatment and post-treatment. Red lines represent the median of peak intensity. HC, healthy control dogs; IBD-PRE, dogs with IBD pre-treatment; IBD-POST_NON AB, dogs with IBD post-treatment that did not receive antibiotic; IBD-POST_AB, dogs with IBD post-treatment that received antibiotic *$q < 0.05$

Table 7. Identified metabolites with altered peak intensity (q < 0.25) between healthy control dogs and dogs with IBD

Metabolite	HC		IBD-PRE		Fold change (IBD-PRE/HC)	p value	q value
	Median	Range	Median	Range			
gluconic acid lactone	285	(232 - 479)	723	(303 - 22,194)	14.7	3.71E-05	0.0060
hexuronic acid	1,454	(898 - 1,715)	2,206	(1,456 - 8,107)	2.1	5.88E-05	0.0063
3-hydroxybutanoic acid	3,437	(2,169 - 5,488)	7,970	(4,058 - 37,369)	3.6	9.28E-05	0.0075
ribose	1,174	(507 - 1,831)	2,180	(1,299 - 5,449)	2.2	0.0004	0.0198
aminomalonic acid	6,922	(5,936 - 9,857)	4,739	(1,756 - 9,807)	0.7	0.0034	0.0740
2-hydroxybutanoic acid	14,612	(9,599 – 44,161)	35,109	(16,902 - 64,067)	2.0	0.0044	0.0894
tryptophan	160,468	(144,526 - 308,348)	113,443	(26,157 - 209,607)	0.7	0.0056	0.0912
fucose+ rhamnose	1,133	(953 - 1,423)	1,753	(747 - 2,737)	1.5	0.0056	0.0912
tyrosine	101,389	(74,276 - 179,264)	64,785	(29,729 - 110,404)	0.6	0.0071	0.0923
cellobiotol	197	(102 - 420)	305	(140 - 1,505)	2.4	0.0071	0.0923
5-methoxytryptamine	1,600	(694 - 3,513)	3,959	(1,132 - 9,964)	2.3	0.0071	0.0923
cysteine	1,069	(570 - 1,769)	2,125	(545 - 7,414)	2.6	0.0090	0.1072
2-deoxyerythritol NIST	3,310	(2,386 - 4,314)	4,986	(2,584 - 15,227)	2.0	0.0090	0.1072
xylitol	638	(385 - 958)	1,161	(540 - 7,440)	2.7	0.0112	0.1287
xylose	1,801	(213 - 2,488)	2,475	(1,587 - 4,188)	1.6	0.0138	0.1435
nicotinic acid	191	(143 - 311)	343	(126 - 755)	2.0	0.0169	0.1654
erythritol	1,018	(778 - 1,470)	1,305	(881 - 4,254)	1.7	0.0206	0.1847
uracil	1,532	(1,022 - 2,733)	2,301	(1,170 - 3,724)	1.4	0.0249	0.2012
p-hydroquinone	3,718	(180 - 12,373)	578	(317 - 25,634)	0.6	0.0300	0.2151
homovanillic + 4-hydroxymandelic acid	2,855	(1,485 - 4,014)	1,878	(1,291 - 3,198)	0.7	0.0300	0.2151
trans-4-hydroxyproline	7,975	(2,673 - 14,850)	2,753	(713 - 11,264)	0.6	0.0358	0.2411
citric acid	85,176	(54,525 - 107,022)	124,882	(29,957 - 363,887)	1.8	0.0358	0.2411
xanthine	954	(504 - 1,665)	1,130	(155 - 2,321)	1.3	0.0426	0.2412
threonic acid	2,973	(1,750 - 4,658)	4,880	(1,479 - 15,737)	2.1	0.0426	0.2412

HC, healthy control dogs; IBD-PRE, dogs with IBD pre-treatment

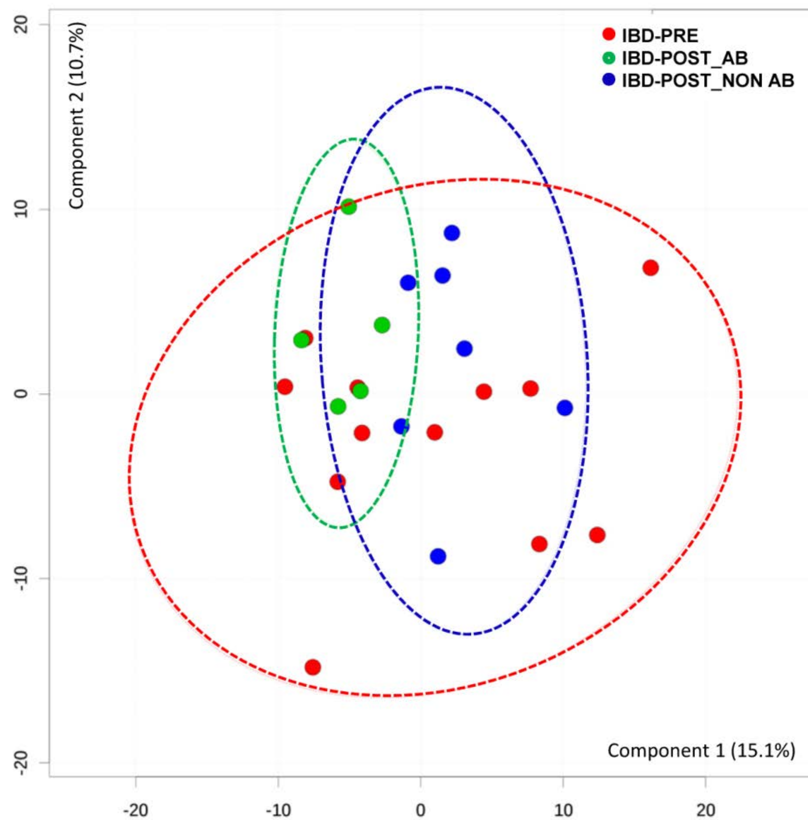

Figure 16. Principal component analysis (PCA) score plots of serum metabolomics data from dogs with IBD pre-treatment, dogs with IBD post-treatment that received antibiotic, and dogs with IBD post-treatment that did not received antibiotic. Ellipses represent the 95% confidence interval of the metabolite profile of each group. No clear separations between groups were identified. IBD-PRE, dogs with IBD pre-treatment; IBD-POST_NON AB, dogs with IBD post-treatment that did not receive antibiotic; IBD-POST_AB, dogs with IBD post-treatment that received antibiotic

Evaluation of the performance of potential biomarkers: To evaluate the utility of the differentially abundant metabolites as potential biomarkers, we conducted a receiver operating characteristic (ROC) analysis. All metabolites, both identified and unknown, as well as metabolite ratios, were screened for their ability to discriminate between the HC and IBD-PRE groups using ROCCET (ROC Curve Explorer & Tester, http://www.roccet.ca).[133] Complete results for all metabolites with area under the curve (AUC) greater than 0.75 are shown in Supplementary Table 2. Gluconic acid lactone had the highest discrimination ability among 157 identified metabolites. With an optimal cut-off peak intensity value of 497, gluconic acid lactone could be used to differentiate between the HC and IBD-PRE groups with 91.67% sensitivity and 100% specificity (Figure 17A). It could also be used discriminate between the IBD-PRE and IBD-POST groups with 75.0% sensitivity and 91.67% specificity at a cutoff value of 495 (Figure 17B).

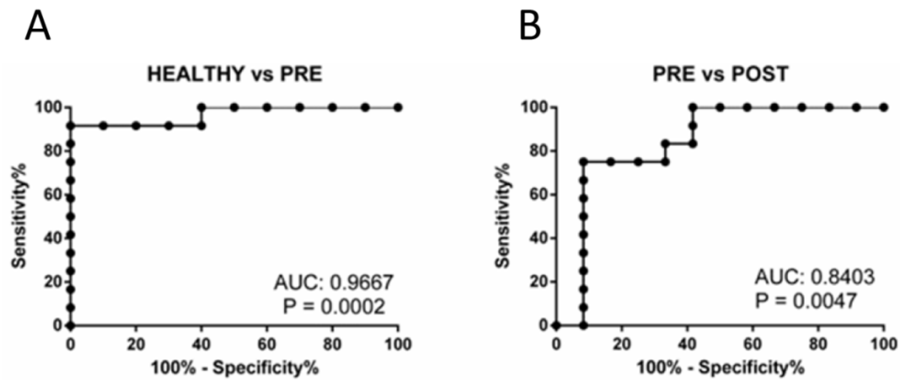

Figure 17. Receiver operating characteristic (ROC) analysis for gluconic acid lactone. (A) Discrimination ability between healthy control dogs and dogs with IBD. (B) Discrimination ability between dogs with IBD pre- and post-treatment. AUC, area under the curve

Metabolite set enrichment analysis: The pentose phosphate pathway was significantly enriched (q = 0.0012) in the IBD-PRE group compared to the HC group. Catecholamine biosynthesis (enriched in HC) and nicotinate and nicotinamide metabolism (enriched in IBD-PRE) were also differentially expressed, but did not differ statistically when p-values were adjusted by FDR of 5% (q = 0.1288, 0.1602, respectively). No significantly enriched pathways were observed between the IBD-PRE and IBD-POST groups.

Network analysis: Due to the low number of identified metabolites that were significantly different between groups using an FDR of 5%, we used an FDR of 25% (roughly corresponding to $p < 0.05$) in order to evaluate the biochemical relationships among the metabolites. This resulted in a set of 33 identified metabolites that were differentially abundant between groups.   A total of 120 metabolites, 20 of which differed significantly between groups, were mapped to a modified MetaMapp network,[64,131] which included 1,070 relationships among 293 metabolites (Figure 18A). In this network, nodes represent metabolites and edges denote relationships within a biochemical pathway. Differentially abundant metabolites were distributed throughout the network and none were directly connected to each other, except for trans-4-hydroxyproline and proline. Both of these metabolites were reduced in IBD animals, which could be reflective of the increased turnover of collagen often seen in IBD and a depletion of the substrates required for its synthesis. Creation of a sub-network using only metabolites identified in the present study as well as first neighbors showed a group of amino acids that clustered around glutamic acid (Figure 18B), suggesting a central role for glutamic acid or its associated pathways in IBD.

Correlation analysis: We next explored the metabolic networks associated with each group using a correlation analysis. Metabolites in equilibrium are positively correlated; the loss or gain of correlations thus suggests an alteration in the flow of substrates through a given pathway. The IBD groups had two clusters of correlated metabolites. One of the clusters, highlighted in green in Figure 19, was centered around citric acid and its metabolites (Figures 19B and 19C). The second cluster, highlighted in blue in

Figure 10, was composed of saturated, monounsaturated, and polyunsaturated fatty acids (Figure 10). No relationships between biochemical pathways were identified for either cluster using network analysis. In the healthy group, there was only one minor cluster which was composed of fatty acids (Figure 19A).

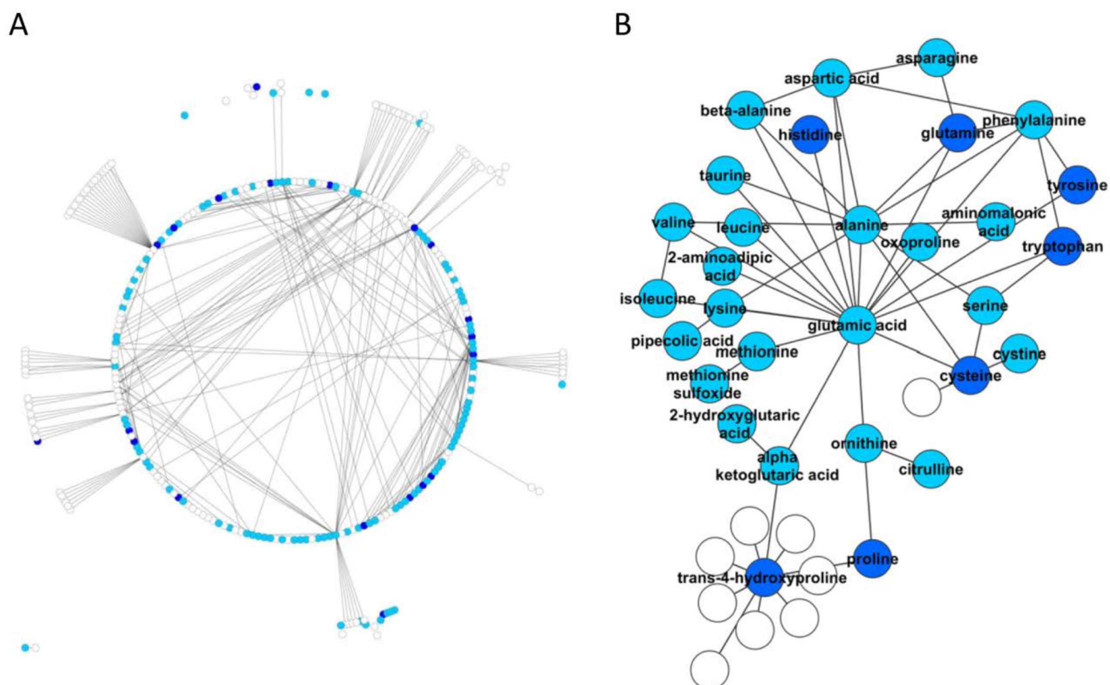

Figure 18. Network analysis of serum metabolites. (A) Overview of the network of relationships among metabolites. (B) Clustered network detected by network analysis. White nodes indicate metabolites that were not detected in this study, light blue nodes indicate metabolites that were detected but were not different ($q > 0.25$) between groups, dark blue nodes indicate metabolites that were detected and were different among groups. The edges indicate biochemical relationship among the metabolites.

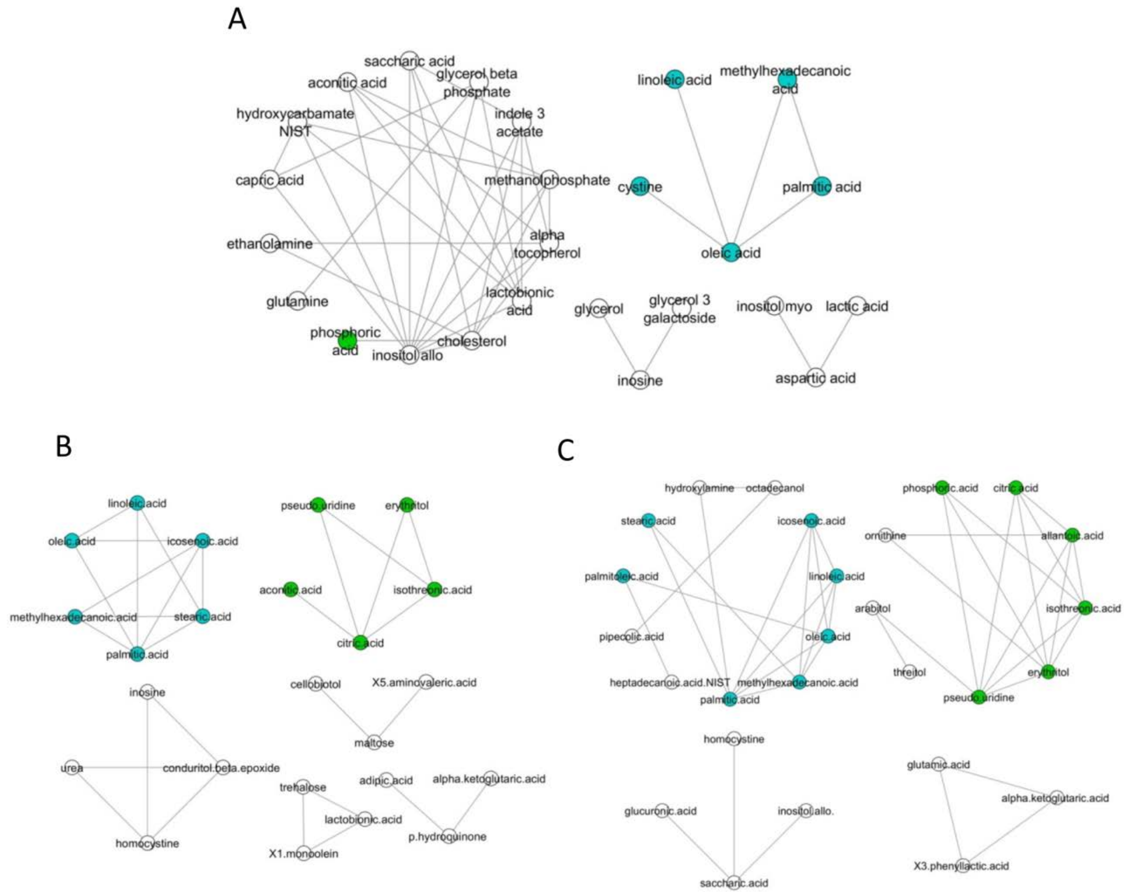

Figure 19. Correlation analysis of serum metabolites. Metabolic networks associated with (A) healthy control dogs, (B) dogs with IBD pre-treatment, and (C) dogs with IBD post-treatment. The IBD groups had two clusters of correlated metabolites. One of the clusters, highlighted in green, was centered around citric acid and its metabolites. The second cluster, highlighted in blue, was composed of saturated, monounsaturated, and polyunsaturated fatty acids. The healthy group had only one minor cluster which was composed of fatty acids.

IV.5 Discussion

In this study, we applied a dual-omics approach to improve our current understanding of the pathogenesis of canine IBD, the host-microbial interactions, and the functional aspects of the canine GI microbiota. Microbiota analysis revealed significant differences in fecal microbial communities between dogs with IBD and healthy dogs, and also significantly decreased alpha-diversity indices in dogs with IBD. On higher phylogenetic levels, the major differences were decreases in Bacteroidetes and various taxa within Firmicutes and concurrent increases in Proteobacteria in dogs with IBD. Similar compositional shifts have also been observed in intestinal biopsies and/or fecal samples from human IBD patients. [137–140] In this study, the increased abundance of Gammaproteobacteria was mainly due to increases in Enterobacteriaceae. The qPCR results suggested that this increase was due to *E. coli*, and this group is of particular interest, as previous studies have reported an increased virulent potential of *E. coli*, such as adhesive capacity, invasive capacity, toxin production, and inflammatory cytokine stimulation, in human patients with IBD.[141,142] Adherent and invasive *E. coli* strains have been reported specifically in Boxer dogs with granulomatous colitis.[143] Whether the virulent potential of *E. coli* plays a similar role in other forms of canine inflammatory bowel disease remains to be determined. Various members of the Firmicutes (*Blautia* spp., *Faecalibacterium* spp., and *Turicibacter* spp.) were decreased in IBD. Most of these bacterial groups belong to Clostridium clusters IV and XIVa and are believed to be major producers of short chain fatty acids (SCFA) and various other metabolites. Therefore, a decrease in these groups may have a significant effect on the host health.

These differences are similar to those of previous studies evaluating the GI microbiota in duodenal mucosal/luminal content and feces in dogs with IBD.[4,12,16,28,29,33]

Based on PICRUSt analysis, the relative abundances of genes associated with a given pathway may indicate an increased metabolic capacity of the GI microbiota with regard to that pathway. Inferences of the functional gene content were grouped by function according to the three-level Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog (KO) hierarchy and compared using LEfSe with the significance threshold set at α of 0.01 and a LDA score of 3.0. In dogs with IBD, the pathways for secretion system and transcription factors were overrepresented. This may reflect the activation of bacterial defense mechanisms responding to the change in intestinal environmental conditions such as availability of nutrients, which may lead to a competitive advantage/disadvantage for microbial communities. Pathways representing amino acid metabolism were, on the other hand, significantly underrepresented in IBD. It has been suggested that commensal microbiota play an important role in the extraction, regulation of absorption, or synthesis of some amino acids.[144] Recently, an association between intestinal dysbiosis and protein energy malnutrition (e.g., kwashiorkor disease) has been reported.[145] In this study, the metabolomics data also suggested alterations in amino acid metabolism and this might be, in part, affected by metabolic dysfunction of bacteria. No other pathways were significantly differently expressed between healthy control dogs and dogs with IBD. This was surprising for carbohydrate metabolism, as many of the decreased bacterial taxa in dogs with IBD are associated with the production of SCFA from carbohydrates. *Faecalibacterium* spp., one

of the main butyrate producing bacteria,[146] are prominent members of the canine GI microbiome.[15] Therefore, it was surprising that the carbohydrate metabolism did not differ between groups. A limitation of this study was that we used a prediction model based on 16S rRNA genes rather than a true metagenomic approach with shot gun sequencing of fecal DNA. Future studies are warranted to evaluate functional properties of the canine microbiota in more detail in canine IBD.

The untargeted metabolomic approach brought new insights in regards to metabolic differences in canine IBD. Gluconic acid lactone is an oxidized derivative of glucose and is capable of scavenging free radicals. Hexuronic acid is a biologically active form of vitamin C and considered an antioxidant due to its ability to donate electrons. 3-hydroxybutanoic acid is a ketone body, and increased synthesis of this metabolite reflects an energy insufficiency. Ribose is vital for biological systems and an important source for further metabolism. These findings are somewhat different than those in human IBD studies which have shown altered, for the most part, lipid and amino acid metabolism.[42] One of the potential explanations is that most of the studies in human IBD applied nuclear magnetic resonance spectroscopy. We, on the other hand, applied GC-TOF/MS which may not be sensitive enough for lipid molecules. Therefore, the difference in the analytical method can contribute to the observed differences. Metabolomics is a relatively new field and still at a descriptive stage even in the study of human IBD. Therefore, large scale multi-center studies in both human and canine IBD are needed to address this species differences. Although gluconic acid lactone showed high discrimination ability for disease status, this may simply reflect inflammation status

and may not be specific for IBD. Therefore, further studies that evaluate specific metabolites in different disease cohorts will be useful to see if those can be useful non-invasive maker for IBD. However, gluconic acid lactone has a possible utility as a biomarker for treatment efficacy. The enrichment analysis indicated that the pentose phosphate pathway was more activate in IBD, mainly due to increased abundance of gluconic acid lactone and ribose. The pentose phosphate pathway is an alternative to glycolysis and a critical pathway in cell redox balance and proliferation. This pathway produces NADPH, ribose, and energy sources for further metabolism and is required for glutathione reduction. Therefore, this pathway has an important role in the protection from oxidative stress and DNA/RNA synthesis.[147] These metabolic alterations may suggest the presence of oxidative stress. This is further supported by the decreased abundance of aminomalonic acid, and the increased abundances of 2-hydroxybutanoic acid and cysteine in dogs with IBD, although the differences of these metabolites did not reach statistical significance set at FDR of 5%. Taken all together, these results suggest that dogs with IBD are affected by oxidative stress due to their state of inflammation, and additional therapeutic interventions aimed towards mitigating oxidative stress could be beneficial. The association between the alteration of GI microbial communities and the presence of oxidative stress in GI tract has also been reported in human IBD patients, and *E. coli* seems to gain competitive advantage in an oxidative stress environment.[128,148] To validate this hypothesis, large scale studies with different treatment regimens (e.g., traditional standard therapy vs. additional antioxidant drug intervention) evaluating the

difference in the abundance of antioxidants and byproducts of oxidative stress related metabolism (e.g., gluconic acid lactone) are needed.

We also evaluated the effects of 3 weeks of medical treatment on both the GI microbial and serum metabolite profiles. Although all dogs with IBD in this study showed clinical improvement, this did not correlate with recovery from dysbiosis. This suggests an ongoing pathological process. Therefore, these dogs may need more time to recover and/or additional therapy such as antioxidant supplementation, and these dogs may have a potential risk of relapse of clinical signs. However, administration of metronidazole may also contribute to this inconsistency in some extent. Antibiotics have been shown to be a strong driver to shift GI microbial composition.[128] In this study, the qPCR assays suggested that antibiotic administration might delay a recovery from the dysbiosis, as those dogs that did not receive antibiotic had median abundances of bacterial groups more similar to those of healthy dogs. A recent large multicenter study also observed similar effects of antibiotics on human Crohn's disease patients, and the authors hypothesized that the use of antibiotics has the potential to impact the overall GI microbial community and increase the potential for exposure to dysbiosis.[149] Studies also reported different effects on the composition of the microbiome with different antibiotics. A study reported increased abundances of Actinobacteria and Bacteroidetes after metronidazole treatment in an model of experimental colitis.[150] While this was not the case in our study, we observed similar effects as observed in a study reporting the relative resistance of *Bifidobacterium* spp. to metronidazole.[151] Limited information is available with regards to the effect of antibiotics on dogs with IBD, although one study

evaluating the effects of single and combination drug regimens showed that oral prednisone monotherapy is as effective as combination therapy with prednisone and metronidazole for treatment of dogs with IBD.[126] An increased susceptibility to pathogens and bacterial community shifts (abundance change and functional change) were observed by administration of antibiotics in a rodent model of intestinal inflammation.[48] These results may suggest potential negative effects of indiscriminate administration of antibiotics on the intestinal microbiota. The effect of antibiotics on the clinical outcome itself requires further studies, as in one study, there was no clear benefit of adding metronidazole to treatment regimens, but there was also no disadvantage.[126] In our study, all 5 dogs that received metronidazole showed clinical improvement. Metronidazole treatment was initiated at the time of diagnosis in 4 of 5 dogs due to clinician's preference. Therefore, it remains unknown whether this improvement was achieved by the effect of the drug combination, or achieved solely by the effect of the immunosuppressive drug. In one dog, metronidazole was added later to the treatment regimen due to an incomplete response to immunosuppressive mono-therapy. Although, this dog showed improvement, we cannot verify whether the improvement was achieved by antimicrobial activity of metronidazole independent of their immunomodulatory effects. Consequently, further studies are needed to correlate changes in microbial communities and clinical outcome of patients, especially when antibiotics are administered.

Similarly to the effects on the GI microbial communities, antibiotic intervention did not significantly affect the serum metabolite profiles. However, in contrast to their

effects on the GI microbiota, dogs that received antibiotic had median abundances of differentially expressed metabolites that were more similar to median abundances observed in healthy dogs. This may potentially be due to an immunomodulatory effect of metronidazole. These data suggest that more extensive studies are needed to study the impact of antibiotics in canine IBD.

There are limitations to this study that need to be addressed. Firstly, the small sample size of animals used in this study limited the statistical power. Therefore, we may have missed some differences in bacterial and metabolite profiles between animal groups, especially when evaluating the effects of differences in treatment (i.e., use of antibiotic). Secondly, this study was conducted at only one institution and no standardization of diet and environment was conducted. However this study cohort represents the patients commonly seen in clinical veterinary practice. Moreover, the PCA and PCoA plots showed that healthy control dogs were tightly clustered, even though their diets varied more than the diets fed to dogs with IBD. This suggests that the dietary effects were not a confounding factor in this study. Similar findings were also observed in a previous study.[12] Finally, we did not have matching control dogs. In this study cohort, there were significant differences in body weight and age between the healthy control group and dogs with IBD. The evaluation of GI microbial communities using the UniFrac distance matric did not indicate any clustering based on body weight and age in this and previous studies.[12,16] Furthermore, the multivariate statistical analysis also did not identify any associations of microbial abundances and these variables.

Therefore, these significant differences in age and body weight may not be a major bias in this study.

In conclusion, this study demonstrates intestinal dysbiosis and altered serum metabolite profiles in dogs with IBD. These alterations may suggest the presence of oxidative stress and functional alteration of GI microbiota in dogs with IBD. Ongoing dysbiosis and bacterial dysfunction were suspected to exist in dogs with IBD even after clinical improvement. Due to the small sample size, further large, multicenter studies are needed to validate these findings.

CHAPTER V

CONCLUSIONS

A number of recent studies have emphasized the importance of balanced GI microbial communities for GI health in humans and animal models of intestinal inflammation.[47–49,110] However, there are relatively few clinical studies evaluating the GI microbiota in dogs with GI disease. Understanding changes of the microbiota together with their associated functional alterations may provide new insights into the pathogenesis of GI diseases in dogs and may lay the foundation for future studies aiming to develop new therapeutic approaches and biomarkers. Thus, this study was aimed to investigate the microbial composition, the relationship between commensal bacteria and enteropathogens, and the functional aspects of an altered intestinal microbiota in dogs with GI disease.

Significant alterations of the intestinal structure of microbiota were observed in dogs with GI disease throughout our studies. Commonly observed alterations were decreases in Ruminococcaceae, *Faecalibacterium* spp., *Turicibacter* spp., and *Blautia spp.*, with concurrent increases in Proteobacteria, specifically *E. coli*. These patterns observed are consistent with those from previous studies showing significant changes in abundances of several bacterial groups in dogs with GI disease.[12,16] In addition, the current data indicated a reduction in microbial diversity in dogs with IBD. Most of the bacterial groups that were decreased in dogs with GI disease belong to *Clostridium* clusters IV and XIVa and are believed to be major producers of SCFA and various other

metabolites. Therefore, a decrease in population of these groups (i.e., normal protective microbiota) can potentially exacerbate intestinal inflammation.

Recent data suggest that the composition of intestinal microbiota and its associated metabolites are important factors for the activation of virulence of enteropathogens, and also suggest the presence of an important cross-talk between commensals and enteropathogens.[47,110] Therefore, one goal of this study was to describe the relationship between dysbiosis and the presence of the potential enteric pathogen *C. perfringens*, as this organism is deemed to be responsible for several types of diarrhea in dogs.

We observed an association between an increased abundance of *C. perfringens*, and more specifically an increased abundance of enterotoxigenic *C. perfringens*, and the presence of dysbiosis, with the latter manifested as a reduction of several commensal bacterial groups in dogs with GI disease. Furthermore, there was a strong association between the presence of CPE and GI disease. However, CPE was only detected in a small proportion of fecal samples from dogs that were positive for enterotoxigenic *C. perfringens*, and the presence of enterotoxigenic strains was not always indicative of the presence of diarrhea. Also, the increase in the population of enterotoxigenic *C. perfringens* was accompanied with an increase in the population of non-enterotoxigenic *C. perfringens.* This finding led to the conclusion that the increased abundance of *C. perfringens* and enterotoxigenic *C. perfringens* may be part of an intestinal dysbiosis as an effect of disease and may not necessarily play a primary pathological role in the GI disease in most dogs.

However, it may also be possible that an initial dysbiosis due to various causes and subsequent changes in bacterial metabolite profiles within the intestinal lumen may trigger the production of CPE from enterotoxigenic *C. perfringens*, as has been demonstrated with C. *difficile*. Further studies evaluating the activity of transcription of CPE with concurrent evaluation of metabolite profiles in the luminal content can provide more information about the pathogenesis of *C. perfringens* associated diarrhea. Clearly, the relationships between enteropathogens, fecal altered microbial communities, and altered bacterial metabolite profiles deserve further studies in dogs with GI disease.

To describe functional aspects of the intestinal microbiota in dog withy GI disease, we analyzed the fecal concentrations of SCFAs in healthy dogs and dogs with chronic GI disease. Our findings clearly showed alterations of fecal SCFA concentrations that were associated with altered microbial compositions in dogs with chronic GI disease. Similar to our findings, studies in humans and rodent models have shown that decreased concentrations of SCFAs in the intestinal tract are associated with GI diseases such IBD and colorectal cancer.[93,152] In these studies, the protective and therapeutic effects of SCFAs on GI disease have also been reported. Therefore, further studies to evaluate whether interventions aimed at manipulating SCFA concentrations within the GI tract may be useful to ameliorate intestinal inflammation and to restore proper immune responses are warranted.

The untargeted metabolomics approach used in this study brought new insights in regards to metabolic alterations in dogs with IBD. We identified several serum metabolites that were differentially expressed between healthy control dogs and dogs

with IBD, and these differences in the metabolite profile revealed potential underlying biochemical changes that occur in dogs with IBD. To validate our results, further studies should be undertaken. The development and validation of analytical assays for candidate metabolites are necessary because the untargeted approach does not provide quantitative data, and there may be a profile bias due to the analytical methods being used. In addition, the stability of each metabolite has not been reported, and it is challenging to find storage conditions that are optimal for all metabolites measured during an untargeted exam. Therefore, assay validation by using a targeted approach is necessary to demonstrate that these profile changes were indeed associated with the disease, and were not due to artificial effects and/or confounding factors.

The findings from the metabolomics part of study suggest that measuring metabolites in dogs with IBD may be useful for further studies. Because this approach is a relatively new research field even in the study of human IBD, large scale studies in both humans and dogs with IBD are needed to evaluate any potential clinical usefulness of some of the metabolites evaluated.

In the last part of study, we evaluated the effects of 3 weeks of medical treatment on both the GI microbiota and serum metabolite profiles in dogs with IBD. The findings from this part of study were particularly interesting. While all dogs with IBD showed clinical improvement in this study, this did not correlate with recovery from dysbiosis and metabolic alterations. This suggests the presence of an ongoing pathological process despite clinical improvement. Therefore, these dogs may require more time to recover and/or additional therapies, and these dogs may have a potential risk for relapse of

clinical signs. Our findings also suggest the necessity for additional biomarkers that are able to reflect underling disease processes and the potential risk of clinical recurrence.

Due to the nature of clinical studies in patients with spontaneous diseases, we faced several limitations. In each study, there were significant differences in some parameters of patient signalment (e.g., age). Moreover, the subject animals were living in different environments and were fed a variety of diets. As for the diseased animals, most of the samples were collected at different institutions, and the timing of sample collection after the onset of diarrhea varied for patients. Also, we could not evaluate the impact of antibiotic administration on our findings, as a variety of treatment regimens were applied to the dogs at the discretion of the primary care veterinarian. Nonetheless, our findings (e.g., changes in the microbiota) were consistent among the studies performed and were also consistent with other previous studies. This may suggest that the effects of the pathogenic process on the GI microbiota outweigh those potential cofounding factors. However, further longitudinal studies with a standardized protocol are still needed to validate these findings by eliminating the potential effects of confounding factors.

In conclusion, the findings of these studies provided new insights into the pathogenesis of GI diseases in dogs. Moreover, the study identified potential biomarkers for IBD. These findings highlight the importance of balanced microbial communities for canine GI health and may lay the foundation for future large-scale clinical studies, which ultimately have the potential to improve the quality of life in dogs with GI disease.

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APPENDIX

Supplementary table 1. Metabolites with altered peak intensity (q < 0.25) between groups

HC vs. IBD-PRE		
Metabolite	**p value**	**q value**
362092	3.09E-06	0.000999
gluconic acid lactone	3.71E-05	0.005994
hexuronic acid	5.88E-05	0.006327
3-hydroxybutanoic acid	9.28E-05	0.007493
225396	3.00E-04	0.019381
ribose	4.30E-04	0.019837
208655	4.30E-04	0.019837
672987	8.32E-04	0.029859
281216	8.32E-04	0.029859
200547	0.0020135	0.054196
213961	0.0020135	0.054196
438106	0.0020135	0.054196
225262	0.0026475	0.061082
314770	0.0026475	0.061082
aminomalonic acid	0.0034362	0.073993
2-hydroxybutanoic acid	0.004429	0.089411
tryptophan	0.0056445	0.091159
fucose+ rhamnose	0.0056445	0.091159
212274	0.0056445	0.091159
210286	0.0056445	0.091159
tyrosine	0.0071446	0.092308
cellobiotol	0.0071446	0.092308
5-methoxytryptamine	0.0071446	0.092308
367978	0.0071446	0.092308
307909	0.0071446	0.092308
cysteine	0.008957	0.10715
2-deoxyerythritol NIST	0.008957	0.10715
xylitol	0.011153	0.12866
xylose	0.01377	0.14347
235449	0.01377	0.14347

IBD-PRE vs. IBD-POST		
Metabolite	**p value**	**q value**
506977	2.22E-05	0.007167
gluconic acid lactone	2.01E-04	0.021659
224551	2.01E-04	0.021659
309730	0.001433	0.092547
213961	0.001433	0.092547
208381	0.00183	0.098503
xylitol	0.003637	0.1678
362056	0.004513	0.18221

Unknown metabolites are listed by Binbase identification numbers

Supplementary table 1. Continued

HC vs. IBD-PRE		
Metabolite	**p value**	**q value**
350396	0.01377	0.14347
nicotinic acid	0.0169	0.16541
508772	0.0169	0.16541
erythritol	0.02058	0.18465
208381	0.02058	0.18465
362075	0.02058	0.18465
uracil	0.024916	0.2012
460930	0.024916	0.2012
267647	0.024916	0.2012
199802	0.024916	0.2012
p-hydroquinone	0.029961	0.21505
homovanillic + 4-hydroxymandelic acid	0.029961	0.21505
218710	0.029961	0.21505
238384	0.029961	0.21505
350532	0.029961	0.21505
trans-4-hydroxyproline	0.035828	0.24109
citric acid	0.035828	0.24109
199777	0.035828	0.24109
xanthine	0.04257	0.24123
threonic acid	0.04257	0.24123

Unknown metabolites are listed by Binbase identification numbers

Supplementary table 2. Metabolites with AUC greater than 0.75

HC vs. IBD-PRE			
Metabolite	**AUC**	**T-tests**	**Fold Change**
tyrosine/362092	1.000	2.86E-07	1.574
362092	1.000	0.0002	-0.879
213961/512073	0.992	1.02E-06	1.973
3-hydroxybutanoic acid/213961	0.983	7.61E-07	-2.908
gluconic acid lactone	0.967	0.0034	-2.288
hexuronic acid	0.950	0.0013	-0.899
3-hydroxybutanoic acid	0.950	0.0008	-1.464
hexuronic acid/225396	0.933	1.38E-06	-2.970
225396	0.925	0.0002	2.071
ribose	0.904	0.0006	-1.102
672987	0.900	0.0007	2.385
281216/506977	0.900	0.0016	-3.882
213961	0.883	0.0007	1.444
224551/213961	0.875	0.0008	-2.416
tryptophan/224551	0.867	0.0023	1.716
225262	0.867	0.0022	0.853
aminomalonic acid	0.867	0.0064	0.653
438106	0.867	0.0032	-0.716
281216	0.867	0.0006	-0.792
438106/506977	0.867	0.0019	-3.806
tryptophan	0.858	0.0159	0.744
gluconic acid	0.858	0.0290	-2.768
224551/506977	0.858	0.0012	-4.062
tyrosine	0.850	0.0029	0.695
2-hydroxybutanoic acid	0.850	0.0022	-0.955
208655	0.842	0.0061	-0.622
200547	0.842	0.0164	-0.869
5-methoxytryptamine	0.833	0.0050	-1.117
367978	0.833	0.0075	-2.949
506977/281910	0.825	0.0062	2.798
267808	0.825	0.0085	0.663
212274	0.825	0.0055	-0.764
314770	0.825	0.0078	-0.773
307909	0.825	0.0139	-1.070
224551/219507	0.825	0.0191	-1.451
438081/506977	0.825	0.0037	-3.144
210286	0.817	0.0140	-0.685

IBD-PRE vs. IBD-POST			
Metabolite	**AUC**	**T-tests**	**Fold Change**
tryptophan/224551	0.9931	2.48E-07	-0.80634
224551/506977	0.9861	1.11E-08	1.2992
beta-alanine/506977	0.9722	1.40E-07	1.2398
224551/219507	0.9722	2.30E-07	0.5452
211946/506977	0.9722	1.08E-07	0.98879
281216/506977	0.9722	1.93E-07	1.5229
213300/506977	0.9722	1.84E-07	0.70382
438106/506977	0.9722	2.41E-07	1.3996
tocopherol alpha/224551	0.9653	1.71E-07	-0.16097
268365/506977	0.9653	4.60E-07	1.1085
309730/438081	0.9653	2.26E-06	0.91595
438081/506977	0.9653	2.94E-07	0.85977
506977/281910	0.9653	3.52E-07	-1.1376
506977	0.9583	1.87E-06	-0.9601
224551/213961	0.9583	6.64E-07	1.1176
275375/506977	0.9583	1.88E-07	1.0351
213961/512073	0.9514	2.39E-05	-1.0766
309730	0.9097	0.0002	0.81562
3-hydroxybutanoic acid/213961	0.9097	0.0005	2.1115
208381	0.8958	0.0003	-1.1375
224551	0.8889	0.0001	0.33915
213961	0.8889	0.0014	-0.77844
455836	0.875	0.0081	-0.22728
362056	0.875	0.0009	0.11834
gluconic acid lactone	0.8403	0.0085	1.158
xylitol	0.8125	0.0181	0.54909
362075	0.8056	0.0078	-0.11143
474607	0.7917	0.0201	-0.012867
219507	0.7847	0.0153	-0.20606
241310	0.7778	0.0783	0.042176
321748	0.7708	0.0554	-0.81115
histidine	0.7639	0.8811	-0.69454
tocopherol alpha	0.7569	0.0358	0.17818
naphtalene	0.7569	0.0039	-0.52466
438081	0.7569	0.0162	-0.10033

Unknown metabolites are listed by Binbase identification numbers

Supplementary table 2. Continued

HC vs. IBD-PRE			
Metabolite	**AUC**	**T-tests**	**Fold Change**
cellobiotol	0.817	0.0143	-0.960
211946/506977	0.817	0.0047	-3.207
362075	0.808	0.0248	1.038
xylitol	0.808	0.0133	-0.990
213300/506977	0.808	0.0058	-2.981
fucose+ rhamnose	0.800	0.0177	-0.441
cysteine	0.800	0.0102	-1.058
208381	0.792	0.0036	2.299
235449	0.792	0.0375	-0.766
2-deoxyerythritol NIST	0.792	0.0094	-0.798
268365/506977	0.792	0.0066	-3.037
275375/506977	0.792	0.0057	-3.097
beta-alanine/506977	0.792	0.0048	-3.375
350396	0.788	0.0099	-1.422
350532	0.783	0.0340	1.853
p-hydroquinone	0.783	0.0386	1.716
508772	0.783	0.4237	-0.158
uracil	0.783	0.0263	-0.485
241029	0.783	0.0295	-0.690
nicotinic acid	0.779	0.0141	-0.776
4-hydroxyproline	0.775	0.0268	0.829
208557	0.775	0.0147	0.746
homovanillic + 4-hydroxymandelic acid	0.775	0.0094	0.545
226845	0.775	0.1462	0.341
224849	0.775	0.0178	0.335
xylose	0.775	0.0349	-0.753
460930	0.771	0.0125	-0.638
trans-4-hydroxyproline	0.767	0.0183	1.223
211972	0.767	0.0182	0.456
citric acid	0.767	0.0936	-0.554
phenylethylamine	0.767	0.0356	-0.972
226912	0.758	0.0185	1.849
218710	0.758	0.0467	-0.788

Unknown metabolites are listed by Binbase identification numbers

Supplementary table 3. List of 75 metabolites inclueded in heatmap figure 7B

# on x-axis	Metabolite
1	aminomalonic acid
2	glutamine
3	histidine
4	225262
5	226912
6	homovanillic acid
7	tryptophan
8	362075
9	321748
10	672987
11	225396
12	proline
13	trans-4-hydroxyproline
14	4-hydroxyproline
15	267808
16	211972
17	208557
18	tyrosine
19	3-hydroxybutanoic acid
20	2-hydroxybutanoic acid
21	mannitol
22	ribose
23	2-deoxyerythritol NIST
24	479886
25	isothreonic acid
26	citric acid
27	330967
28	219507
29	inositol allo-
30	362056
31	208381
32	455836
33	nicotinic acid
34	444613
35	212274
36	213961
37	506977
38	naphtalene

39	309730
40	229203
41	allantoin
42	glucose
43	362092
44	xylitol
45	367978
46	267647
47	cellobiotol
48	5-methoxytryptamine
49	fructose
50	199802
51	gluconic acid lactone
52	gluconic acid
53	438106
54	281216
55	224551
56	tocopherol alpha
57	behenic acid
58	5-hydroxynorvaline NIST
59	cysteine
60	homocystine
61	350396
62	241310
63	270003
64	200547
65	474607
66	299091
67	353747
68	314770
69	210286
70	227604
71	208655
72	307909
73	hexuronic acid
74	xylose
75	fucose+ rhamnose

Unknown metabolites are listed by Binbase identification numbers

Supplementary table 4. Signalment of dogs enrolled in this study

Phenotype	Age (yr)	Body weight (kg)	Breed	Sex	Neuter status	CIBDAI PRE	CIBDAI POST	Immunosuppressive Drug	Antibiotics use	Diet (brand_product name)	Crude Protein (%)	Crude Fat (%)	Crude Fiber (%)
IBD_1	11	10	Scottish terrier	M	Neutered	3	0	prednisone	no	Hill's_d/d (duck and potato)	18.0	16.4	1.7
IBD_2	7	23	Collie	F	Neutered	6	0	budesonide	no	Hill's_d/d	18.4	15.5	1.8
IBD_3	10	6	MIX	F	Intact	1	0	prednisone	no	Hill's_d/d	18.4	15.5	1.8
IBD_4	14	5	WHWT	F	Neutered	3	0	prednisone	no	Royal Canin_hypoallergenic	19.0	10.0	3.7
IBD_5	11	9	WHWT	M	Neutered	13	0	prednisone	metronidazole	Hill's_d/d (duck and potato)	18.0	16.4	1.7
IBD_6	9	5	MD	M	Neutered	5	1	prednisone	metronidazole	Hill's_d/d	18.4	15.5	1.8
IBD_7	4	4	YT	F	Neutered	8	1	prednisone + cyclosporin	metronidazole	Hill's_d/d	18.4	15.5	1.8
IBD_8	4	4	Shih Tzu	F	Neutered	9	5	prednisone	metronidazole	Hill's_d/d (duck and potato)	18.0	16.4	1.7
IBD_9	9	3	YT	M	Neutered	9	2	prednisone + cyclosporin	no	Hill's_d/d	18.4	15.5	1.8
IBD_10	3	9	Beagle	M	Neutered	6	2	prednisolone	no	Iams_KO diet	19	12	4
IBD_11	3	5	Havanese	F	Neutered	9	1	prednisone	no	Royal canin_duck/potato	19	10	3.7
IBD_12	6	8	Beagle	F	Neutered	5	1	prednisone	metronidazole	Hill's_d/d (duck and potato)	18.0	16.4	1.7
HC_1	2	7	MIX	F	Neutered	n/a	n/a	n/a	no	Purina_maintenance	26	16	3
HC_2	3	28	MIX	F	Neutered	n/a	n/a	n/a	no	Purina_maintenance	26	16	3
HC_3	2	26	MIX	F	Neutered	n/a	n/a	n/a	no	Eukanuba_large breed lamb and rice	22.5	12	4
HC_4	8	13	MIX	M	Neutered	n/a	n/a	n/a	no	Hills_science diet	20.0	6.0	18.5
HC_5	4	42	MIX	F	Neutered	n/a	n/a	n/a	no	Hill’s_d/d duck and potato	18.0	16.4	1.7
HC_6	5	27	MIX	M	Neutered	n/a	n/a	n/a	no	Purina_savory blend kibble	26	16	3
HC_7	3	34	MIX	M	Neutered	n/a	n/a	n/a	no	Hill’s_science diet	20.0	6.0	18.5
HC_8	4	28	MIX	F	Neutered	n/a	n/a	n/a	no	Purina_pro-plan sensitive skin and stomach	26	16	4
HC_9	1	24	MIX	M	Neutered	n/a	n/a	n/a	no	Purina_pro-plan sensitive skin and stomach	26	16	4
HC_10	10	15	MIX	M	Neutered	n/a	n/a	n/a	no	Purina_pro-plan sensitive skin and stomach	26	16	4

IBD, dogs with IBD; HC, healthy control dogs; MIX, mixed breed; WHWT, West Highland White Terrier; MD, Miniature Dachshund; M, male; F, female; YT, Yorkshire Terrier; n/a, not applicable