CHARACTERIZATION OF THE FECAL MICROBIOTA IN DOGS WITH CHRONIC ENTEROPATHIES AND ACUTE HEMORRHAGIC DIARRHEA

A Thesis

by

MELISSA ELLEN MARKEL

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

MASTER OF SCIENCE

August 2012

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Jan Suchodolski
Committee Members,	Joerg Steiner
	Sara Lawhon
Head of Department,	Sandee Hartsfield

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ABSTRACT

Characterization of the Fecal Microbiota in Dogs with Chronic Enteropathies and Acute Hemorrhagic Diarrhea. (August 2012)

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Recent 16S rRNA gene sequencing studies of the duodenal and fecal microbiota have revealed alterations in the abundance of specific bacterial groups in dogs with gastrointestinal (GI) disorders. The aim of this study was to establish a panel of quantitative real-time PCR (qPCR) assays for the evaluation of specific bacterial groups in fecal samples of healthy dogs, dogs with chronic enteropathies (CE), and dogs with acute hemorrhagic diarrhea (AHD). Fecal samples from 242 healthy dogs, 118 dogs with CE, and 57 dogs with AHD were analyzed using qPCR assays targeting *Faecalibacterium* spp., *Turicibacter* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *Streptococcus* spp., *Ruminococcaceae*, *C. perfringens*, *E. coli*, γ-*Proteobacteria*, *Bacteroidetes*, and *Firmicutes*). Differences in bacterial abundance among the three groups were evaluated using a Kruskal-Wallis test followed by a Dunn's post-test. A Bonferroni correction was used to correct for multiple comparisons and an adjusted $p<0.05$ was considered for statistical significance.

Faecalibacterium spp., *Turicibacter* spp., and *Ruminococcaceae* were significantly decreased in CE and AHD compared to healthy dogs ($p<0.001$ for all). *Lactobacillus* spp. and *Streptococcus* spp. were significantly increased in dogs with CE ($p<0.001$ for both) when compared to the healthy dogs. In contrast, *Lactobacillus* spp. and *Streptococcus* spp. were significantly decreased in dogs with AHD compared to healthy dogs ($p<0.01$ and $p<0.05$, respectively) and also when compared to the dogs with CE ($p<0.001$ for both). *C. perfringens* and *E. coli* were significantly increased in dogs with AHD ($p<0.001$ and $p<0.01$, respectively), when compared to healthy dogs. *E. coli* was also significantly increased in dogs with CE when compared to the healthy dogs ($p<0.001$). *Bacteroidetes* were significantly lower in dogs with CE compared to healthy dogs (<0.001). *Firmicutes* were significantly higher in healthy dogs in comparison to dogs with AHD ($p<0.05$). *Bifidobacterium* spp. and *γ-Proteobacteria* were not significantly different among all three groups of dogs.

In conclusion, the qPCR panel employed here revealed a fecal dysbiosis in dogs with CE and AHD when compared to healthy dogs. These results are similar to recently reported findings using molecular sequencing approaches. Quantification of these bacterial groups by qPCR may be a useful adjunct for the diagnosis or monitoring of gastrointestinal disease in dogs.

DEDICATION

To my family

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Jan Suchodolski, for his patience and guidance throughout my post baccalaureate and graduate studies. I would also like to thank my committee members, Dr. Joerg Steiner and Dr. Sara Lawhon for their support throughout the course of this research. Thanks also go to my colleagues at the GI lab for their help and support. Furthermore, I would like to thank all of the veterinarians and researchers who assisted in collecting fecal samples used during this study.

Finally, I would like to thank my friend, Nicole Gossett, my family, and friends for their guidance and support throughout my academic career.

NOMENCLATURE

CE	chronic enteropathies
AHD	acute hemorrhagic diarrhea
GI	gastrointestinal
qPCR	quantitative real-time polymerase chain reaction
spp.	species

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION AND

LITERATURE REVIEW

THE EFFECT OF THE MICROBIOTA ON GASTROINTESTINAL HEALTH

It has been suggested that microbes in the gastrointestinal tract (GI) play a significant role in maintaining host health [1,2]. The beneficial effects of the GI microbiota on the host include their crucial role in the structural development of the intestinal epithelium, the stimulation of the immune system, and provision of nutritional support for the host [3-8]. This has been supported by data in germ free mice that exhibit a blunted development of lymphoid tissue [9], have smaller Peyer's patches and mesenteric lymph nodes, and a reduced turnover time of epithelial cells compared to conventionally raised mice [4,10,11]. The intestinal microbiota produces various metabolites that provide benefits to the host [6-8,12-15]. These include short-chain fatty acids (i.e., acetate, propionate, and butyrate). Short-chain fatty acids, especially butyrate, have been shown to have anti-inflammatory properties [8]. An increased concentration of short-chain fatty acids leads to a lower luminal pH in the intestine which may prevent the overgrowth of pH-sensitive pathogenic bacteria [6]. Vitamins, such as vitamin K, cobalamin (B12), thiamine (B1), folate (B9) and riboflavin (B2) are also produced by the intestinal microbiota. Vitamin K can produced by some lactic acid bacteria and is involved in blood clotting [12]. *Bifidobacterium* spp. have been reported to produce

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riboflavin and thiamine. Deficiency in these vitamins may lead to changes in brain glucose metabolism [16]. Folate is an essential vitamin involved in nucleotide and cofactor biosynthesis, and in many metabolic reactions [12]. Cobalamin is important for the metabolism of amino acids, carbohydrates, and fatty acids [17]. Intestinal microbiota may also protect the host from pathogenic bacteria by means of competitive exclusion [12,18]. This is achieved for example through the synthesis and release of antimicrobials, competition for oxygen or mucosal adhesion sites, or through the creation of a physiologically hostile environment for potentially pathogenic bacteria (e.g., alteration of luminal pH) [12,19-21].

CHARACTERIZATION OF THE INTESTINAL MICROBIOTA

An accurate characterization of the intestinal microbiota is imperative for the identification of altered microbial populations (i.e., dysbiosis) that may be present in dogs with GI disease. The two main methods for bacterial identification are through the use of culture and molecular-based methods.

Culture-based methods. Characterization of bacteria present within the GI tract is critical for our understanding of the role of the microbiota in host health and disease. Traditionally, culture-based methods have been used to characterize the bacterial populations of the GI tract [11,22-24]. Some authors have estimated that approximately 10-50% of fecal bacterial genera present in the mammalian GI tract can be cultured [25]. In contrast, other authors have suggested that more than 99% of prokaryotes in most

environmental samples are uncultivable [26]. It is well accepted that there are limitations to traditional culture-based methods when analyzing complex microbial ecosystems such as the mammalian GI tract [27,28]. Only a portion of bacteria present in the GI tract can be cultured, mostly due to unknown growth requirements for many gastrointestinal bacteria, stress due to cultivation procedures (e.g., some bacteria require strictly anaerobic conditions), and the difficulties simulating symbiotic relationships with other microbes and/or the host *in- vitro* [25]. Due to restrictive growth environments, many gut microbes have not been sufficiently characterized and, therefore, the commonly employed biochemical tests may be insufficient for correct classification of some microbial phylotypes [25,29].

Molecular-based methods. To evaluate the diversity of bacterial groups, it is necessary to consider genes that have been conserved over the course of evolution. The current standard approach to characterize the intestinal microbiota is through molecular-based methods, specifically through sequencing of the 16S rRNA gene [21,30-34]. The 16S rRNA gene can be found in all prokaryotes and is widely used due to its unique properties such as ubiquity, presence of hypervariable regions (where sequences have been modified over the course of evolution for specific bacterial groups), and the presence of highly conserved regions that are shared among most bacterial phylotypes [35,36]. Polymerase chain reaction (PCR) primers are designed to anneal to regions within the bacterial DNA, and depending on which bacterial groups are of interest, will target either highly conserved or the variable regions within the 16S rRNA gene [37]. There are several molecular tools available by which microbiota can be analyzed,

including molecular fingerprinting techniques, fluorescent in situ hybridization (FISH), quantitative real-time PCR (qPCR), and sequencing technologies (e.g., 454-pyrosequencing or, Sanger sequencing of 16S rRNA gene clone libraries).

Polymerase Chain Reaction (PCR). For most molecular-based methods, PCR is initially used to amplify parts of the 16S rRNA gene [38-41]. Primers are designed to target either conserved or hypervariable regions in this gene, depending on which bacterial groups are of interest. Universal bacterial primers target the conserved regions of the 16S rRNA gene and, in theory, allow amplification of the 16S rRNA genes of all bacteria present in a given sample. In contrast, to identify specific bacterial groups (on various phylogenetic levels), PCR primers are designed to target more variable regions of the 16S rRNA gene.

The use of quantitative PCR assays (qPCR) allows determination of the abundance of bacteria in a given sample. Unlike conventional PCR, qPCR assays allow for the amplified DNA to be detected as the reaction progresses in real time. These assays use either the intercalation of non-specific fluorescent dyes (e.g., SybrGreen®) into double-stranded DNA of the target sequence, or fluorescent labeled probes (Taqman® PCR assays) to quantify the abundance of bacterial groups [30]. This quantification corresponds to the increasing level of fluorescence, which is associated with a higher abundance of double stranded DNA. In Taqman® PCR assays, a fluorescent labeled probe is used. The probe consists of a fluorophore that is covalently bound to the 5'-end of the probe and a quencher which is bound to the 3'-end. When in close proximity, the quencher suppresses the fluorescence of the fluorophore. During the

PCR assay the probe first binds to the template DNA strand. During the PCR extension, the polymerase synthesizes the complementary strand and degrades the probe. This degradation releases the fluorophore from the quencher, and the resulting fluorescence of the fluorophore is detected by the camera of PCR thermal cycler. Increased intensity of the released fluorophore directly correlates to the amount of DNA product present in the PCR [42-44].

Real-time PCR has many advantages such as high analytical sensitivity, high reproducibility, and minimal time requirement. Real-time PCR is a closed-tube system that requires no post-PCR manipulation, therefore reducing any potential for contamination [45,46]. There are some disadvantages of PCR-based methods. For example, the efficiency of the PCR assay is reflective of the oligonucleotide primer design; if the oligonucleotide primers are not specific for the targeted bacterial group, the PCR assay will result in amplification of an undesired target sequence and potentially false positive results. PCR is also susceptible to inhibition by compounds found within the sample matrix, including excess salts, ionic detergents, and ethanol which can lead to false negative results. Finally, there is a relatively high cost for initial equipment set-up and reagents [47].

Molecular fingerprinting techniques. The purpose of molecular fingerprinting techniques is to characterize bacterial diversity in a complex microbial community [48,49]. With this technique, a region of the 16S rRNA gene is amplified, followed by electrophoretic separation of the resulting PCR amplicons on gel electrophoresis, such as for example denaturing gradient gel electrophoresis (DGGE) [49,50]. This technique

allows for rapid and inexpensive comparison of microbiota profiles among samples [51-53]. However, DGGE has a limited resolution and will only display DNA fragments from predominant species present in the sample [51]. Furthermore, for the identification of bacterial groups, DGGE bands need to be excised from the gel, which is time consuming and laborious [50].

Fluorescent In Situ Hybridization (FISH). FISH utilizes fluorescent labeled probes that target the bacterial 16S rRNA and is often used for the identification of specific bacterial groups in body fluids, tissue biopsies, or fecal samples [30,54-56]. Methods by which tissue or fecal samples are being evaluated require the sample to be fixed prior to FISH. Formalin-based fixatives are most commonly used in this process. Fixed samples are then generally mounted in paraffin wax in order to provide support for subsequent sectioning for microscopic evaluation. Fixed samples are dehydrated and rehydrated to increase permeabilization by means of breaking down protein-DNA crosslinks. Increased permeabilization facilitates the entry of the probe into the cell so that it may link to the intended nucleotide target sequence. Once samples have been prepared for hybridization, an oligonucleotide probe, which is tagged with a fluorescent label is applied [56]. The probe then hybridizes to the complementary oligonucleotide sequence, and the fluorescence can be visualized using a fluorescence microscope [57]. FISH can be limited by the specificity of the hybridization of the probe. Hybridization of the probe to the intended nucleotide target sequence can be altered during sample preparation, which can lead to decreased detection of the target nucleotide. If the probe binds incorrectly, the observer may misinterpret the fluorescence as being the intended

nucleotide target sequence, when the signal is actually being emitted from a nucleotide sequence that was bound non-specifically and that does not correspond to the bacterial group of interest [54]. FISH is also limited by laborious sample preparation [56]. Currently, interpretation of FISH assays relies on individual observers interpreting the fluorescence signal as being positive or negative, which can lead to errors between observers [54].

454-pyrosequencing of the 16S rRNA gene. 454-pyrosequencing is a high-throughput sequencing technology that allows the in-depth characterization of bacterial groups present in the gastrointestinal tract using multiplex analysis. This technique is advantageous because it allows for paralleled generation of DNA sequences from a large number of samples simultaneously. Additionally, low concentrations of template DNA are suitable for quantification [58]. However, there are disadvantages associated with 454-sequencing. Some 16S rRNA sequences such as *Bifidobacterium* spp., are typically detected at a reduced abundance. This can be due to the lack of mechanical force sufficient to lyse the cell walls of these bacteria or could be due to the relatively high G+C content in bifidobacterial sequences [59]. Sequences rich in guanine (G) and cytosine (C) can be difficult to denature due to the three hydrogen bonds connecting the base pairs [58]. Additionally, this sequencing technology requires costly equipment and performance of the methods is also costly [60].

THE CANINE INTESTINAL MICROBIOTA

Previous studies have used a variety of traditional methods to characterize the microbiota of the intestinal tract in humans and animals [27,32,61-63]. Molecular methods have been employed to allow for a more detailed characterization of microbial populations in the GI tract of dogs and humans [28,32,64,65].

In one study, intestinal contents were collected from the duodenum, jejunum, ileum, colon, and rectum of dogs [49]. Bacterial DNA was extracted from each respective sample, and subjected to PCR in which a variable region of the 16S rRNA gene was amplified using universal bacterial primers. Amplicons were separated using DGGE. Banding patterns were analyzed to compare differences in the microbiota among the various intestinal regions and also between dogs. This molecular approach demonstrated the presence of a complex intestinal microbial community in the canine intestine, which not only varies depending upon intestinal compartments, but also among dogs. It has been suggested that due to the varied bacterial populations among compartments, the analysis of fecal samples may not yield accurate information regarding bacterial population composition within the more proximal GI system of dogs [49].

Another study evaluated differences in the microbiota between the small intestine and fecal samples in dogs [64]. Jejunal chyme and fecal samples were obtained from 22 healthy laboratory Beagle dogs. Samples were homogenized, diluted, and cultured in an aerobic and also anaerobic environment. Bacterial counts in the jejunum were dramatically lower (range: 10^2 to 10^6 CFU/g) than in feces (range: 10^8 to 10^{11} CFU/g).

Also, the mean number of identified bacterial genera was lower in the small intestine (range: 1 to 11) than in feces (range: 9 to 16). Approximately 25% of bacteria detected in the small intestine could not be identified in the corresponding fecal sample, while 45% of bacteria found in the fecal samples were not detectable in small intestinal samples. Microbial groups that were more prevalent in the small intestine were *Staphylococcus* spp. (64% vs. 36% in feces) and non-fermentative gram negative bacilli (e.g., *Pseudomonas* spp. (27% vs. 9% in feces). The microbial groups that dominated the fecal microbiota, such as *Bacteroides* spp., *Clostridium hiranonis*-like organisms, and *Lactobacillus* spp. were practically absent from the jejunum [64].

One study described the intraluminal intestinal microbiota in dogs using 16S rRNA gene sequence analysis [66]. Luminal content was collected from the duodenum, jejunum, ileum, and colon from 6 healthy dogs. Bacterial DNA was extracted and subjected to PCR in which the bacterial 16S rRNA gene was amplified using universal bacterial primers. Resulting amplicons were ligated into cloning vectors and 16S rRNA gene inserts were sequenced. Four bacterial phyla were identified: *Firmicutes* (34% of clones)*, Fusobacteria* (12%)*, Bacteroidetes* (9%)*,* and *Proteobacteria* (17%). The order *Clostridiales* was found to be the most abundant and diverse bacterial order in the duodenum and jejunum, with 40% and 39% of identified clones, respectively. The proximal small intestine and colon were dominated by sequences affiliated with *Clostridium* cluster XI and *Clostridium* cluster XIVa, respectively. The most abundant bacterial orders of the ileum and colon were *Fusobacteriales* and *Bacteroidales*, with 33% and 30% of clones, respectively [66].

Handl et al. [63] evaluated the fecal microbiota of 12 healthy pet dogs using 454-pyrosequencing of the 16S rRNA gene. *Firmicutes* were the most abundant phylum, with 95.36 ± 5.19% of all sequences belonging to this phylum. *Bacteroidetes* was the second most abundant phylum (2.25 ± 5.37% of all bacterial sequences). Furthermore, 69.35 ± 18.45% of bacterial sequences belonged to the class *Clostridia*, predominately to the genera *Clostridium* (22.73 ± 15.46%) and *Ruminococcus* (17.37 ± 11.18). As described in a previous study by Suchodolski, et al. (2008), the most prevalent *Clostridium* clusters were cluster XIVa (59.59 ± 23.2% of all *Clostridiales sequences*), and cluster XI (33.64 ± 17.13% of all *Clostridiales sequences*) [63].

Middlebos et al. [65] evaluated changes in the fecal microbiota in response to fiber supplementation in 6 healthy adult dogs using 454-pyrosequencing of the V3 hypervariable region of the 16S rDNA gene. Dogs were fed a control diet without fiber supplementation and a beet pulp-supplemented diet (7.5%). The complete dataset included 77,771 sequencing reads and individual samples contained approximately 129 operational taxonomic units (OTUs; range: 113-147 OTUs). Three co-dominant phyla were observed: *Fusobacteria* (23-40% of reads), *Firmicutes* (14-28%), and *Bacteroidetes* (31-34%). Fiber supplementation yielded modifications of the intestinal microbiota, however these alterations were not equally apparent in all dogs. The abundance of *Actinobacteria* (1.4-0.8%) and *Fusobacteria* (40-24%) was lower ($P<0.05$) in dogs fed the beet pulp diet. Abundance of *Firmicutes* (15-28%) was significantly increased in dogs fed the beet pulp diet ($P<0.05$). *Clostridia* was found to be the most dominant class in the phylum *Firmicutes* (≥82% of sequences) in both diets, and

significantly increased (83-90%) when dogs were supplemented with the beet pulp diet ($P<0.05$) [65].

The true bacterial abundances in the GI tract of dogs reported in those studies are difficult to compare due to various factors. It has been suggested that there are differences in the microbiota depending upon the sampling site [49]. Furthermore, differences in the microbiota based on sample type (e.g., chyme versus feces) have been demonstrated [64]. It is also possible that the methodology by which samples are analyzed may have an effect on bacterial characterization. For example, Yu and Morrison [67] have described a bead beating based bacterial extraction method, leading to a more complete lysis of bacterial cells, which resulted in better representation of microbial diversity in comparison to other bacterial extraction methods. All of these factors must be considered when comparing the reported abundance of microbial populations across studies.

INTESTINAL MICROBIOTA IN DISEASE

Bacterial dysbiosis has recently been associated with the pathogenesis of some gastrointestinal disorders, including inflammatory bowel disease in animals and humans [68-71].

Chronic enteropathies. Chronic enteropathies (CE) describe a range of chronic diseases of the intestines regardless of etiology or pathogenesis. Inflammatory bowel disease (IBD) describes a subset of chronic enteropathies, which are characterized by persistent or recurrent signs of GI inflammation [72]. Clinical signs of chronic

enteropathies are varied and nonspecific, the most common being weight loss, large intestinal dysfunction, diarrhea, and vomiting [69]. Simpson and Jergens [72] suggest that some clinical signs may assist in identifying a region of interest and a probable cause of disease. These signs include upper GI bleeding or ulceration, tenesmus, dyschezia, peripheral edema, or enteric protein loss [72]. The diagnosis of chronic enteropathies involves the integration of signalment, history, physical examination findings, diagnostic imaging, and histopathology of intestinal biopsies [69,72]. The diagnostic approach is described as first excluding parasitic agents, extraintestinal disorders, and intestinal structural abnormalities. Chronic enteropathies may also be categorized based on treatment response as food responsive, antibiotic responsive, or steroid responsive.

There is mounting evidence to suggest that chronic enteropathies are the result of a disproportionate immune response to commensal bacteria [21,70,73]. Rodent models have implicated commensal enteric bacteria in chronic, immune-mediated colitis [70]. Chronic enteropathies have been associated with dysbiosis in dogs, more specifically a decreased abundance of gram-positive *Firmicutes* and an increased abundance of gram-negative bacteria, such as *Proteobacteria* [21,74,75]. One study described differences in the mucosa-adherent duodenal microbiota between dogs with idiopathic IBD and healthy dogs. Significant differences were identified in the relative abundance of several bacterial groups. Dogs with IBD had a significantly higher abundance of γ-*Proteobacteria* ($p<0.001$), and a significantly lower abundance of *Clostridia* ($p<0.001$) [21]. Small intestinal microbial communities were described in one study in which

duodenal brush cytology samples were analyzed from 10 dogs with IBD. Sequences belonging to the phylum *Bacteroidetes* were significantly less common in dogs with IBD (2.7%) than healthy dogs (11.2%, $p<0.001$). Nearly half of the sequences from healthy dogs belonged to three orders *Clostridiales* (19.6%), *Lactobacillales* (14.1%), and *Campylobacteriales* (13.9%), whereas in the IBD group, the vast majority of sequences belonged to the orders *Clostridiales* (40.4%), *Enterobacteriales* (20.9%), and *Lactobacillales* (17.5%) [75]. In another study, dogs with antibiotic-responsive enteropathy have been associated with an increased abundance of *Lactobacillales* [76].

Simpson, et al. performed a study using fluorescent in-situ hybridization (FISH) and observed translocated *E.coli* in the epithelial cells of the colonic mucosa of Boxer dogs with granulomatous colitis [73]. Once the *E. coli* organisms are eradicated, the condition shifts into a remissive state [77,78].

Acute hemorrhagic diarrhea. Many dogs present for veterinary care with an acute onset of hemorrhagic diarrhea (AHD). There are several potential causes of AHD, including infection with *Campylobacter jejuni*, *Salmonella, Clostridium perfringens, C. difficile*, and canine parvovirus infection [61,79-81]. Hemorrhagic gastroenteritis (HGE), is a subgroup of AHD commonly found in small breeds, which present with bloody watery stool, vomiting, and severe hemoconcentration. Due to the acute symptomology, it has been suggested that food allergy or bacterial endotoxins may play an active role in the pathogenesis of this syndrome [82]. The microbial communities present in dogs with AHD have not been well documented.

HYPOTHESIS AND SPECIFIC OBJECTIVES

The hypotheses of this study are that 1) qPCR can be used to characterize the fecal microbiota, and 2) that this qPCR approach can be used to identify a dysbiosis in dogs with CE and AHD.

The objectives of this study are 1) to establish a panel of qPCR assays to accurately identify specific bacterial groups within the canine fecal microbiota, 2) to characterize the fecal microbiota in healthy dogs, and 3) to compare the fecal microbiota of healthy dogs to that of dogs with CE and AHD.

CHAPTER II

CHARACTERIZATION OF THE INTESTINAL MICROBIOTA IN HEALTHY DOGS AND DOGS WITH INTESTINAL DISEASE

SUMMARY

The aim of this study was to evaluate the abundance of selected bacterial groups in healthy dogs, dogs with chronic enteropathies (CE), and dogs with acute hemorrhagic diarrhea (AHD). Fecal samples were collected from 242 healthy pet dogs, 118 dogs with CE, and 57 dogs with AHD. Bacterial DNA was extracted from all fecal samples and adjusted to a concentration of 5 ng/µl. Separate qPCR assays were performed to quantify eleven microbial groups. Sequences belonging to *Faecalibacterium* spp., *Turicibacter* spp., or *Ruminococcaceae* were significantly decreased in dogs with CE or AHD compared to healthy dogs ($p<0.001$ for all). Sequences belonging to *Lactobacillus* spp. or *Streptococcus* spp. were significantly increased in dogs with CE ($p<0.001$ for both) when compared to healthy dogs. In contrast, *Lactobacillus* spp. and *Streptococcus* spp. sequences were significantly decreased in dogs with AHD compared to healthy dogs ($p<0.01$ and $p<0.05$, respectively) and also the dogs with CE ($p<0.001$ for both). *C. perfringens* and *E. coli* sequences were significantly increased in dogs with AHD ($p<0.001$ and $p<0.01$, respectively), when compared to healthy dogs. *E. coli* sequences were also significantly increased in dogs with CE when compared to the healthy dogs ($p<0.001$). *Bacteroidetes* sequences were significantly lower in dogs with CE compared to healthy dogs (<0.001). *Firmicutes* sequences were significantly higher in healthy dogs

than in dogs with AHD ($p<0.05$). *Bifidobacterium* spp. and *γ-Proteobacteria* sequences were not significantly different among the three groups of dogs.

In conclusion, the qPCR panel employed here revealed a fecal dysbiosis in dogs with CE and AHD when compared to healthy dogs. These results are similar to recently reported findings using molecular sequencing approaches. Quantification of these bacterial groups by qPCR may be a useful adjunct for monitoring the fecal microbiota in dogs with enteropathies.

INTRODUCTION

Gastrointestinal microbes have been shown to play a significant role in intestinal health and disease [1,23]. They provide benefits to the host by stimulating the development of the intestinal epithelium, priming the host immune system, and providing nutritional support through the production of various vitamins and metabolites [3-8]. For example, germ free mice have been shown to have a diminished development of lymphoid tissue, smaller Peyer's patches and mesenteric lymph nodes, and a reduced turnover time of intestinal epithelial cells in comparison to conventionally raised mice [4,9-11]. The intestinal microbiota is thought to protect the host from potentially pathogenic bacteria through the synthesis and release of antimicrobials, competition for oxygen or mucosal adhesion sites, and through the establishment of a hostile environment for pathogenic bacteria [12,18-21].

Alterations of the intestinal microbiota have been implicated in various acute and chronic gastrointestinal diseases in humans and also in dogs [4,10,21,27,54,76,83-85].

For example, recent studies of the proximal small intestinal microbiota have revealed a microbial dysbiosis of the luminal as well as the mucosa-adherent microbiota of dogs with idiopathic inflammatory bowel disease [21,70,73,76,78]. These dogs showed a decreased abundance of gram-positive *Firmicutes* and an increased abundance of gram-negative bacteria, most prominently within the phylum *Proteobacteria.* Less information is available about the microbiota composition in the large intestine or feces of dogs with gastrointestinal disease. It has been shown that the microbial composition differs along the length of the GI tract, and fecal samples may not be fully representative of microbial communities present in the proximal parts of the intestine [49]. It is also unknown if the previously observed changes in the composition of the microbiota are specific for canine IBD or if such patterns of dysbiosis are also present in dogs with acute episodes of diarrhea.

Previous studies have evaluated the fecal microbiota of dogs with diarrhea. Bell et al [83] used terminal restriction fragment polymorphism (T-RFLP) and qPCR to characterize the fecal microbiota in 8 pet dogs with acute episodes of diarrhea and five research dogs. During episodes of diarrhea, an increased abundance of *C. perfringens* was identified. *Campylobacter* spp. and *Helicobacter* spp. were identified as being present in 11 of 15 fecal samples on the day of onset of the first episode of diarrhea [83]. Another study evaluated the effects of dietary fiber supplementation on the fecal microbiota in 17 research Beagles (9 with chronic diarrhea and 8 healthy controls) using FISH and DGGE [85]. *Bacteroidetes* sequences were significantly increased ($p<0.05$) and *Atopobium* sequences significantly decreased ($p<0.05$) in dogs with chronic diarrhea

in comparison to healthy dogs following fiber supplementation. Furthermore, sequences belonging to *Clostridium* clusters I and II were significantly increased in dogs with chronic diarrhea during fiber supplementation ($p<0.05$) in comparison to healthy dogs [85]. Using pyrosequencing of the chaperonin 60 gene, sequences belonging to *ε-Proteobacterium* were analyzed in healthy dogs (n=7) and dogs with unspecified diarrhea (n=9) [84]. The microbiota of healthy dogs was predominantly composed of *Bacteroidetes* (50% of sequence reads), and *Firmicutes* and *Proteobacteria* (each phylum 25% of reads). Dogs with diarrhea had a *Firmicutes/ Proteobacteria/ Bacteroidetes/ Actinobacteria* ratio of 4:4:1:1, with *Bacteroidetes* being the only phylum that was significantly higher in abundance between the two groups of dogs ($p<0.05$) [84]. While these studies present evidence for a difference in the microbiota of dogs with diarrhea in comparison to healthy dogs, these studies either did not utilize sequencing based technologies [83,85], or evaluated only a small number of dogs with unspecified diarrhea [83-85]. More detailed studies evaluating fecal dysbiosis in a larger group of dogs and with better characterized gastrointestinal diseases are needed to understand the influence of the intestinal microbiota on gastrointestinal health. Therefore, the aim of this study was to establish a panel of qPCR assays and to evaluate the abundance of specific bacterial groups in fecal samples of healthy dogs, dogs with CE, and dogs with AHD.

MATERIALS AND METHODS

Sample collection. Naturally passed fecal samples were obtained from 242 healthy dogs, 118 dogs with CE, and 57 dogs with AHD from nine different countries. All dogs participated in different studies and leftover fecal samples were utilized for this study. The protocol for sample collection was approved by the Clinical Research Review Committee of the College of Veterinary Medicine, Texas A&M University (CRRC#09-06), the Ethics and Welfare Committee of the Royal Veterinary College, and the Royal Canin Internal Ethics Committee.

Table 1. summarizes the characteristics of the dogs enrolled in this study. Samples were stored frozen at -80°C until analysis. **Extraction of DNA.** All samples were homogenized for 90 sec (Stomacher 80, Seward Laboratory Systems, Inc., NY, USA). A 100 mg aliquot of each fecal sample was aliquoted into a sterile 1.7 ml microtube (Sarstedt AG & Co., Nümbrecht, Germany) containing 150 µl of 0.1 mm zirconia-silica beads and 100 µl of 0.5 mm zirconia-silica beads (BioSpec Products Inc., OK, USA). A volume of 750 µl of lysis buffer from the Zymo Fecal DNA Mini Prep kit (Zymo Research Inc., CA, USA) was added to each sample. Tubes were placed vertically onto a homogenizer (FastPrep-24, MP Biomedicals LLC, OH, USA), and the mixture was homogenized for 1 min at 4 m/sec. The tubes were then centrifuged at 10,000 g for 1 min at 23°C and the remainder of the DNA extraction was performed as suggested by the manufacturer (Zymo Fecal DNA Mini Prep kit, Zymo Research Inc., CA, USA). The concentration of fecal DNA was measured using the NanoDrop 1000 (Thermo Fisher Scientific Inc., DE, USA) and was adjusted to 5 ng/µl.

Table 1. Dogs enrolled in the study

Number	Age (years)	Breed	Gender
AHD dogs enrolled in the study			
1	2.0	Chihuahua	F
2	4.0	Berger Picard	MC
3	8.0	Mixed Breed	FS
4	4.0	Chinese Shar Pei	FS
5	1.0	German Shepherd Dog Mix	M
6	2.0	West Highland White Terrier	F
7	5.0	Labrador Retriever Mix	MC
8	6.0	Cairn Terrier	FS
9	3.0	Fox Terrier	M
10	7.0	Podenco Canario	F
11	12.0	Golden Retriever	FS
12	10.0	Afghan Hound	FS
13	12.0	Labrador Retriever Mix	FS
14	2.0	Mixed Breed	MC
15	1.0	Chihuahua	FS
16	12.0	Mixed Breed	F
17	2.0	German Shepherd Dog	M
18	2.0	Mixed Breed	F
19	5.0	German Shepherd Dog	M
20	4.0	Mixed Breed	M
21	11.0	Airedale Terrier	MC
22	4.5	Labrador Retriever	M
23	1.0	Cavalier King Charles Spaniel	F
24	1.5	Parson Russel Terrier	F
25	2.5	Bayerischer Gebirgsschweißhund	M
26	5.0	Miniature Poodle Mix	FS
27	3.0	Labrador Retriever	M
28	16.0	West Highland White Terrier	M
29	16.0	Spitz	M
30	11.0	Hovawart Mix	FS
31	12.0	Berger Briard Mix	MC
32	2.5	Mixed Breed	M
33	2.5	Pekinese	M

Table 1. continued

Number	Age (years)	Breed	Gender
34	2.0	Yorkshire Terrier	F
35	10.0	Fox Terrier	M
36	6.0	Mixed Breed	MC
37	3.0	Flat Coated Retriever	FS
38	13.0	Yorkshire Terrier Mix	F
39	2.5	Labrador Retriever	M
40	1.0	Mixed Breed	M
41	5.0	Labrador Retriever	MC
42	1.0	Miniature Schnauzer	FS
43	3.0	Dachshund	MC
44	5.0	Labrador Retriever Mix (Labradoodle)	FS
45	4.0	Chihuahua	M
46	1.0	Chihuahua	FS
47	1.5	Rough Collie	MC
48	5.0	Tibetan Mastiff	MC
49	1.5	Beauceron	FS
50	0.2	Labrador Retriever Mix (Labradoodle)	F
51	2.0	Rat Terrier	F
52	2.0	Nova Scotia Duck Tolling Retriever	M
53	7.0	Mixed Breed	FS
54	3.0	German Hound	FS
55	3.0	Labrador Retriever	FS
56	1.0	Hungarian Vizsla	FS
57	11.0	Yorkshire Terrier	FS

CE dogs enrolled in the study

1	4.0	Belgian Shepherd Dog	M
2	3.0	Yorkshire Terrier	M
3	7.0	Cane Corso	M
4	4.0	Shih Tzu	MC
5	6.0	English Bulldog	M
6	3.0	Boxer	F
7	5.5	Yorkshire Terrier	M
8	8.5	German Shepherd Dog	M
9	4.0	German Shepherd Dog	M
10	3.0	Labrador Retriever	F

Table 1. continued

Number	Age (years)	Breed	Gender
11	7.0	Yorkshire Terrier	FS
s12	5.0	Rottweiler	F
13	11.0	Cavalier King Charles Spaniel	FS
14	13.0	Mixed Breed	FS
15	3.0	Bernese Mountain Dog	F
16	6.0	Akita Inu	M
17	8.0	Rottweiler	M
18	8.0	Miniature Schnauzer	M
19	2.0	Yorkshire Terrier	F
20	2.0	Brazilian Terrier	F
21	1.0	Miniature Poodle	F
22	5.0	Mixed Breed	M
23	7.0	American Pit Bull Terrier	M
24	4.0	Boxer	F
25	9.0	Miniature Poodle	F
26	10.0	Mixed Breed	F
27	1.0	French Bulldog	M
28	1.5	Yorkshire Terrier	F
29	4.0	Lhasa Apso	F
30	1.0	Boxer	M
31	3.0	Mixed Breed	F
32	8.0	Whippet	F
33	5.0	Lhasa Apso	M
34	2.0	Yorkshire Terrier	F
35	1.5	Boxer	F
36	2.0	Maltese	F
37	12.0	Australian Shepherd	FS
38	8.0	English Bulldog	FS
39	10.5	Golden Retriever	M
40	5.0	Doberman Pinscher Mix	MC
41	8.0	Bichon Frise	MC
42	8.0	unknown	MC
43	7.0	Cocker Spaniel	MC
44	8.0	Rhodesian Ridge Back	MC
45	4.0	English Bulldog	M
46	1.3	Pomeranian Mix	FS
47	5.0	French Bulldog	FS

Table 1. continued

Number	Age (years)	Breed	Gender
48	8.5	Yorkshire Terrier	FS
49	7.8	Chinese Shar Pei	M
50	1.5	Boston Terrier Mix	FS
51	2.5	Labrador Retriever	MC
52	2.3	Springer Spaniel	MC
53	7.3	Miniature Poodle	MC
54	8.5	Parson Russel Terrier Mix	FS
55	8.8	Golden Retriever	FS
56	6.0	Shiba Inu	MC
57	1.3	Toy Poodle	FS
58	9.0	Cairn Terrier	MC
59	6.0	Boxer Mix	MC
60	1.3	Cocker Spaniel	FS
61	11.0	Labrador Retriever	FS
62	2.3	Shih Tzu	FS
63	17.0	Whippet Mix	FS
64	5.0	Chinese Shar Pei	MC
65	7.5	Newfoundland	MC
66	6.0	Siberian Husky Mix	FS
67	9.5	Cocker Spaniel Mix (Cockapoo)	FS
68	13.0	Boston Terrier	FS
69	1.5	Labrador Retriever	FS
70	8.0	Labrador Retriever	FS
71	4.0	German Shepherd Dog	M
72	1.5	Siberian Husky	MC
73	1.0	German Shepherd Dog	F
74	2.5	German Shepherd Dog	M
75	10.0	Staffordshire Bull Terrier	MC
76	3.0	Greyhound	FS
77	7.0	Basset Hound	FS
78	1.0	Mixed Breed	M
79	2.0	Labrador Retriever	MC
80	5.0	Border Collie	M
81	1.5	Cocker Spaniel	M
82	7.0	Lurcher	FS
83	4.0	Tibetan Terrier	unknown
84	2.0	Boxer	unknown

Table 1. continued

Number	Age (years)	Breed	Gender
85	10.0	Golden Retriever	unknown
86	7.0	Flat Coated Retriever	unknown
87	3.0	Labrador Retriever	unknown
88	5.0	Labrador Retriever	unknown
89	3.0	Yorkshire Terrier	unknown
90	2.0	German Shepherd Dog	unknown
91	4.0	Boxer	unknown
92	5.0	Standard Poodle	unknown
93	7.0	Golden Retriever	unknown
94	6.5	Golden Retriever	unknown
95	6.7	Border Terrier	M
96	7.6	Bichon Frise	F
97	4.4	Bull Mastiff	F
98	7.5	Drever	unknown
99	3.2	Labrador Retriever	M
100	5.8	Mixed Breed	M
101	3.8	Hungarian Vizsla	M
102	3.5	Shetland Sheepdog	M
103	0.8	Boxer	M
104	6.1	Papillon	MC
105	2.2	Miniature Poodle	M
106	2.0	Rottweiler	FS
107	3.9	Great Dane	M
108	7.0	Weimaraner	F
Healthy dogs enrolled in the study			
1	8.0	Mixed Breed	M
2	3.0	Mixed Breed	F
3	6.0	Mixed Breed	FS
4	10.0	Rottweiler	FS
5	3.0	Miniature Pinscher	FS
6	11.0	Rottweiler	F
7	2.0	Mixed Breed	FS
8	1.5	Labrador Retriever	MC
9	1.0	Dachshund	M
10	7.0	Mixed Breed	MC
11	5.0	Dachshund	M

Table 1. continued

Number	Age (years)	Breed	Gender
12	3.0	German Shepherd Dog Mix	FS
13	1.5	Labrador Retriever	FS
14	2.0	Mixed Breed	MC
15	1.0	Siberian Husky	MC
16	6.0	Mixed Breed	FS
17	2.0	Zwergpinscher	F
18	5.0	Rhodesian Ridge Back	M
19	4.0	Parson Russel Terrier	M
20	6.0	Cocker Spaniel	FS
21	2.0	Beagle	FS
22	10.0	Rottweiler	FS
23	2.0	Mixed Breed	FS
24	2.5	Mixed Breed	FS
25	8.0	Maltese	MC
26	5.0	Pug	FS
27	2.0	Irish Water Spaniel	M
28	3.0	Doberman Pinscher	FS
29	13.0	Mixed Breed	FS
30	1.5	Miniature Pinscher	MC
31	4.0	Mixed Breed	FS
32	7.0	Chihuahua	MC
33	7.0	Mixed Breed	MC
34	4.0	Pug	MC
35	8.0	Cardigan Welsh Corgi	MC
36	2.0	Australian Shepherd	FS
37	2.0	Australian Shepherd	FS
38	1.5	Parson Russel Terrier	FS
39	12.0	Mixed Breed	MC
40	7.0	Golden Retriever	M
41	4.0	Hungarian Vizsla	MC
42	3.0	Mixed Breed	MC
43	6.0	Mixed Breed	MC
44	12.0	Mixed Breed	MC
45	7.0	Chihuahua	MC
46	11.0	Labrador Retriever	MC
47	5.0	Mixed Breed	MC
48	2.0	Mixed Breed	FS
49	4.0	Yorkshire Terrier	MC

Table 1. continued

Number	Age (years)	Breed	Gender
50	8.0	Australian Shepherd	MC
51	7.0	Mixed Breed	FS
52	4.0	German Shepherd Dog	M
53	6.0	Finnish Lapphund	M
54	3.0	Finnish Lapphund	M
55	2.0	Staffordshire Bull Terrier	M
56	9.0	Miniature Schnauzer	FS
57	3.0	Cavalier King Charles Spaniel	F
58	4.0	Border Collie	MC
59	4.0	Whippet	unknown
60	4.0	Whippet	F
61	7.0	Whippet	FS
62	6.0	Whippet	FS
63	3.0	Australian Shepherd	FS
64	13.0	Australian Shepherd	FS
65	7.0	Flat Coated Retriever	M
66	1.5	Flat Coated Retriever	M
67	4.0	Flat Coated Retriever	F
68	4.0	Siberian Husky	MC
69	4.0	Dachshund	M
70	2.0	Dachshund	M
71	4.0	Mudi	F
72	4.0	German Shepherd Dog	F
73	2.0	Dachshund	F
74	1.5	Siberian Husky	F
75	5.0	Griffon Korthal	M
76	4.0	Canaan Dog	FS
77	5.0	Mixed Breed	MC
78	4.0	Parson Russel Terrier	FS
79	2.0	Australian Cattle Dog	MC
80	1.0	Australian Shepherd	MC
81	1.0	Siberian Husky	M
82	1.0	Miniature Pinscher	F
83	6.0	Mixed Breed	FS
84	9.0	Doberman Pinscher	MC
85	3.0	Mixed Breed	MC
86	2.0	Golden Retriever	FS
87	2.0	Mixed Breed	FS

Table 1. continued

Number	Age (years)	Breed	Gender
88	8.0	Siberian Husky Mix	M
89	13.0	Mixed Breed	FS
90	4.0	American Staffordshire Terrier	M
91	2.0	Golden Retriever	F
92	2.0	Golden Retriever	M
93	4.0	Weimaraner	M
94	10.0	Mixed Breed	FS
95	1.0	German Shepherd Dog	M
96	11.0	Pekinese	FS
97	6.0	Italian Greyhound	FS
98	3.0	Mixed Breed	FS
99	4.0	Miniature Poodle	FS
100	6.0	Labrador Retriever	F
101	5.0	White Shepherd Dog	F
102	unknown	Rottweiler	FS
103	5.0	Shetland Sheepdog	FS
104	8.0	Miniature Schnauzer	M
105	unknown	English Pointer	M
106	6.0	Parson Russel Terrier	FS
107	15.0	Miniature Poodle	M
108	16.0	Dachshund	M
109	1.5	Whippet	MC
110	4.5	Rottweiler	FS
111	5.0	Mixed Breed	FS
112	8.0	Dachshund	FS
113	8.0	Mixed Breed	MC
114	1.0	Golden Retriever	FS
115	15.0	Yorkshire Terrier	MC
116	10.0	Bull Terrier	MC
117	3.0	Mixed Breed	FS
118	4.5	Mixed Breed	MC
119	12.0	German Shepherd Dog	F
120	11.0	Mixed Breed	FS
121	5.0	German Shepherd Dog	MC
122	1.0	Labrador Retriever Mix	MC
123	3.0	Boxer	FS
124	4.0	Australian Cattle Dog	M
125	2.0	American Pit Bull Terrier Mix	FS

Table 1. continued

Number	Age (years)	Breed	Gender
126	4.0	Australian Shepherd	MC
127	4.0	Mixed Breed	FS
128	3.0	Mixed Breed	MC
129	2.5	Red Heeler Mix	FS
130	3.0	Shih Tzu	MC
131	7.0	Miniature Schnauzer	FS
132	2.5	Bichon Frise	FS
133	3.0	Siberian Husky	MC
134	3.0	Malamute	FS
135	3.0	Labrador Retriever Mix	FS
136	4.0	Siberian Husky	MC
137	1.0	Basenji	FS
138	3.0	Australian Shepherd Mix	FS
139	1.5	Golden Retriever	MC
140	1.0	Border Collie	FS
141	unknown	Australian Shepherd	FS
142	2.0	Labrador Retriever Mix	FS
143	2.5	Rough Collie Mix	FS
144	4.0	Yorkshire Terrier	unknown
145	1.0	Great Dane Mix	MC
146	1.0	Pembroke Welsh Corgi	MC
147	unknown	unknown	unknown
148	2.0	Australian Kelpie	F
149	4.0	Mixed Breed	FS
150	4.0	Great Dane	MC
151	10.0	Labrador Retriever Mix	FS
152	2.0	Labrador Retriever Mix	FS
153	3.0	Labrador Retriever Mix	MC
154	2.0	Labrador Retriever	FS
155	1.0	Labrador Retriever	FS
156	2.0	Beagle	F
157	2.0	Labrador Retriever	M
158	5.0	Fox Terrier Mix	FS
159	4.0	West Highland White Terrier Mix	M
160	1.0	Pekingese	MC
161	3.0	American Pit Bull Terrier	MC
162	2.0	Mixed Breed	FS
163	1.0	Mixed Breed	FS

Table 1. continued

Number	Age (years)	Breed	Gender
164	1.5	Golden Retriever Mix (Goldendoodle)	FS
165	1.0	Boston Terrier	MC
166	2.0	English Mastiff	MC
167	1.0	Cardigan Welsh Corgi	MC
168	1.0	Belgian Shepherd Dog	F
169	3.5	Blue Lacy	MC
170	3.0	Australian Shepherd	FS
171	9.0	Labrador Retriever	FS
172	2.0	BlueTickCoonHound	FS
173	4.0	German Shepherd Dog Mix	FS
174	6.0	Cocker Spaniel	FS
175	1.0	Australian Shepherd	MC
176	5.0	Brittany Spaniel	FS
177	2.0	Boxer Mix	FS
178	7.0	Beagle Mix	FS
179	1.0	Mixed Breed	MC
180	1.0	American Pit Bull Terrier	F
181	3.0	Dalmatian	FS
182	2.0	Golden Retriever	MC
183	3.0	Catahoula Cur Mix	FS
184	7.0	Mixed Breed	FS
185	8.0	Mixed Breed	FS
186	5.0	Basset Hound	MC
187	3.0	Maltese	FS
188	5.0	German Shorthaired Pointer	FS
189	2.0	Red Heeler Mix	MC
190	1.0	Labrador Retriever Mix	MC
191	2.0	Maltese	M
192	0.6	Newfoundland	FS
193	1.0	Australian Shepherd	FS
194	3.0	American Pit Bull Terrier	MC
195	1.0	Weimaraner Mix	MC
196	2.0	Labrador Retriever	FS
197	3.0	Labrador Retriever	MC
198	1.0	Tibetan Mastiff	MC
199	10.0	Miniature Schnauzer	FS
200	9.0	Weimaraner	MC
201	7.0	Weimaraner	FS

Table 1. continued

Number	Age (years)	Breed	Gender
202	1.0	American Pit Bull Terrier	MC
203	2.5	Labrador Retriever Mix	FS
204	4.0	Mixed Breed	F
205	5.5	German Shepherd Dog	F
206	5.0	Flat Coated Retriever	F
207	6.0	Belgian Shepherd Dog	F
208	3.0	Mixed Breed	MC
209	2.0	Boxer	M
210	2.0	Australian Cattle Dog	FS
211	8.0	Mixed Breed	F
212	3.0	Beagle	MC
213	7.0	Labrador Retriever	FS
214	3.5	Labrador Retriever	F
215	1.0	Labrador Retriever	F
216	9.0	Dachshund	MC
217	9.0	Mixed Breed	F
218	1.0	Labrador Retriever	F
219	8.0	Mixed Breed	MC
220	1.5	Hovawart	MC
221	6.5	Mellan Pinscher	F
222	1.5	Dachshund	MC
223	1.5	Rhodesian Ridge Back	M
224	3.0	Mixed Breed	MC
225	8.0	Bull Mastiff	M
226	2.0	French Bulldog	F
227	4.0	Shih Tzu Mix	MC
228	5.0	Bloodhound	M
229	1.0	Bloodhound	M
230	6.0	Mixed Breed	FS
231	5.0	Cairn Terrier	MC
232	13.0	German Shepherd Dog Mix	FS
233	unknown	Shih Tzu Mix	MC
234	4.0	American Pit Bull Terrier	FS
235	7.0	Miniature Pinscher	MC
236	2.0	Whippet	FS
237	13.0	English Setter	FS
238	3.0	Belgian Shepherd Dog	FS
239	11.0	Mixed Breed	FS

Table 1. continued

Number	Age (years)	Breed	Gender
240	7.0	Boxer	FS
241	2.0	Mixed Breed	MC
242	7.0	Mixed Breed	MC

AHD = acute hemorrhagic diarrhea; CE = chronic enteropathies; MI = male intact; MC = male castrated; FI = female intact; FS = female spayed

Quantitative PCR assays. Separate real-time qPCR assays were used to amplify and quantify DNA from eleven different microbial groups (*Faecalibacterium* spp., *Turicibacter* spp., *Bifidobacterium* spp., *Lactobacillus* spp., *Streptococcus* spp., *Ruminococcaceae, C. perfringens, ɣ-Proteobacteria, Bacteroidetes, E. coli,* and *Firmicutes)* using protocols and primers found in table 2. A commercial qPCR thermal cycler (CFX96TM, Bio-Rad Laboratories, CA, USA) was used for all qPCR assays.

SYBR-based reaction mixtures (total 10 µl) containing 5 µl SsoFast^{TM} EvaGreen® supermix (Bio-Rad Laboratories, CA, USA), 2.6 µl of water, 0.4 µl of each primer (final concentration: 400 nM), and 2 µl of normalized DNA (final concentration: 5 ng/µl). PCR conditions were 98 °C for 2 min, and 40 cycles at 98 °C 3 sec and 3 sec at the optimized annealing temperature (Table 2). A melt curve analysis was performed for SYBR-based qPCR assays as follows: 1 min at 95°C, 1 min at 55°C, and 80 cycles at 0.5°C increments (5 sec each). Samples were analyzed in duplicate fashion.

TaqMan® reaction mixtures (total 10 µl) containing 5 µl TaqMan® Fast Universal PCR master mix (Life Technologies, NY, USA) (2 x), 2 µl of water, 0.4 µl of each primer (final concentration: 400 nM), 0.2 µl of the probe (final concentration: 200 nM), and 2 µl of normalized DNA. PCR conditions were 95 °C for 20 sec, and 40 cycles at 95 °C for 5 sec, and 10 sec at the optimized annealing temperature (Table 2). Samples were run in duplicate fashion. The qPCR data was expressed as amount of DNA (fg) for each particular bacterial group per 10 ng of isolated total DNA.

Table 2. Oligonucleotide primers/probe used for this study.

qPCR primers/probe	Sequence (5'- 3')	Target	Annealing (°C)	Reference
CFB555f	CCGGAWTYATTGGGTTTAAAGGG	Bacteroidetes	60	[86]
CFB968r	GGTAAGGTTCCTCGCGTA			
Gamma395F	CMATGCCGCGTGTGTGAA	γ-Proteobacteria	69	[86]
Gamma871R	ACTCCCCAGGCGGTCDACTTA			
BifF	TCGCGTCYGGTGTGAAAG	*Bifidobacterium* spp.	60	[87]
BifR	CCACATCCAGCRTCCAC			
FaecaF	GAAGGCGGCCTACTGGGCAC	*Faecalibacterium* spp.	60	[88]
FaecaR	GTGCAGGCGAGTTGCAGCCT			
RumiF	ACTGAGAGGTTGAACGGCCA	Family Ruminococcaceae	59	[88]
RumiR	CCTTTACACCCAGTAAWTCCGGA			
CPerf165F	CGCATAACGTTGAAAGATGG			
CPerf269R	CCTTGGTAGGCCGTTACCC	*C. perfringens* 16S	58	[89]
CPerf187F (probe)	TCATCATTCAACCAAAGGAGCAATCC			
LacRT-f	AGCAGTAGGGAATCTTCCAA	*Lactobacillus* spp.	58	[90]
LacRT-R	CACCGCTACACATGGAG			
St1	TTATTTGAAAGGGGCAATTGCT	*Streptococcus* spp.	54	[91]
St2	GTGAACTTTCCACTCTCACAC			
EcolRT F	GTTAATACCTTTGCTCATTGA	*E. coli*	55	[87]
EcolRT R	ACCAGGGTATCTAATCCTGTT			
Firm350 F	GGCAGCAGTRGGGAATCTTC	Firmicutes	60	[86]
Firm 814 R	ACACYTAGYACTCATCGTTT			
TuriciF	CAGACGGGGACAACGATTGGA	*Turicibacter*	63	This study
TuricR	TACGCATCGTCGCCTTGGTA			

Statistical analysis. Data sets were tested for normal distribution using a D'Agostino & Pearson omnibus test. Since none of the datasets were normally distributed, non-parametric analyses were conducted. For pairwise comparisons, Mann-Whitney tests were used. For comparisons of disease groups, Kruskal-Wallis tests with Dunn's post tests were used. All p-values were adjusted for multiple comparisons using the Bonferroni correction, and an adjusted p-value < 0.05 was considered significant. All statistical analyses were performed using Prism 4.00 (GraphPad Software, CA, USA).

RESULTS

To evaluate differences in the abundance of microbial groups among healthy dogs, dogs with CE, and dogs with AHD, all samples (242 healthy dogs, 118 dogs with CE, and 57 dogs with AHD) were analyzed for the abundance of sequences belonging to all 11 bacterial groups (Table 3, Figure 1). *Faecalibacterium* spp., *Turicibacter* spp., and *Ruminococcaceae* sequences were significantly decreased in CE and AHD compared to healthy dogs ($p<0.001$ for all). *Lactobacillus* spp. and *Streptococcus* spp. sequences were significantly increased in dogs with CE ($p<0.001$ for both) when compared to the healthy dogs. In contrast, *Lactobacillus* spp. and *Streptococcus* spp. sequences were significantly decreased in dogs with AHD compared to the healthy dogs ($p<0.01$ and $p<0.05$, respectively) and also the dogs with CE ($p<0.001$ for both). *C. perfringens* and *E. coli* sequences were significantly increased in dogs with AHD ($p<0.001$ and $p<0.01$, respectively), when compared to healthy dogs. *E. coli* sequences were also significantly

increased in dogs with CE when compared to the healthy dogs ($p<0.001$). *Bacteroidetes* sequences were significantly lower in dogs with CE compared to healthy dogs (<0.001). *Firmicutes* sequences were significantly higher in healthy dogs than in dogs with AHD ($p<0.05$). *Bifidobacterium* spp. and *γ-Proteobacteria* sequences were not significantly different among all three groups of dogs.

Effect of antibiotic administration on the abundance of bacterial groups. Administration of antibiotics has been shown to significantly impact the gastrointestinal microbiota [77]. A subset of the dogs evaluated in this study had a history of antibiotic administration or were on antibiotics at the time of sample collection. To evaluate if the above observed changes were confounded by antibiotic administration, a subset analysis was performed on samples from those dogs from whom a complete antibiotic history was available. Fecal samples were evaluated from healthy dogs that either received (n=10) or did not receive (n=13) antibiotics within six months of sample collection, dogs with CE that either received (n=10) or did not receive (n=13) antibiotics within six months of sample collection, and dogs with AHD that either received (n=10) or did not receive (n=9) antibiotics within six months of sample collection (Table 4, Figure 2).

Table 3. Abundance of bacterial groups in fecal samples of healthy dogs, dogs with CE, and dogs with AHD based on qPCR analysis.

	Range (Minimum-Maximum)			Medians			
	Healthy (n=242)	CE (n=108)	AHD (n=57)	Healthy	CE	AHD	Adjusted Kruskal-Wallis P-value*
Faecalibacterium spp.	0.15-6.97	0.15-6.09	0.15-6.34	4.76[a]	2.46[b]	2.60[b]	<0.0011
Turicibacter spp.	0.90-7.83	0.11-6.93	0.900-6.05	4.94[a]	1.28[b]	1.25[b]	<0.0011
Bifidobacterium spp.	0.900-6.13	0.900-5.64	0.90-4.25	1.17	1.72	1.16	0.4081
Lactobacillus spp.	1.27-6.69	1.38-6.53	1.38-4.80	2.53[a]	3.25[b]	2.25[c]	<0.0011
Streptococcus spp.	1.50-7.34	1.50-7.53	1.50-5.73	2.15[a]	3.47[b]	1.50[c]	<0.0011
Ruminococcaceae	1.90-7.99	1.99-7.67	1.99-7.72	6.83[a]	6.18[b]	5.97[b]	<0.0011
C. perfringens	0.44-11.05	0.49-10.99	0.49-12.86	3.55[a]	4.71[a]	6.25[b]	<0.0011
Y-Proteobacteria	1.71-7.96	2.16-7.70	2.16-7.06	3.31	3.85	3.60	1.0000
Bacteroidetes	1.90-5.64	0.70-4.42	1.57-6.54	3.13[a]	1.90[b]	1.90[a]	<0.0011
E. coli	0.12-6.54	0.12-6.89	0.12-7.06	3.00[a]	4.19[b]	4.03[b]	<0.0011
Firmicutes	2.29-7.15	2.30-7.39	1.90-7.31	5.48[a]	5.57[a]	5.17[b]	0.1331

Medians not sharing a common superscript indicates statistical significance based on Dunn's multiple comparisons test; (P<0.05).

CE = chronic enteropathies; AHD = acute hemorrhagic diarrhea

* Adjusted Kruskal-Wallis based on Bonferroni correction. Significance set at p<0.05.

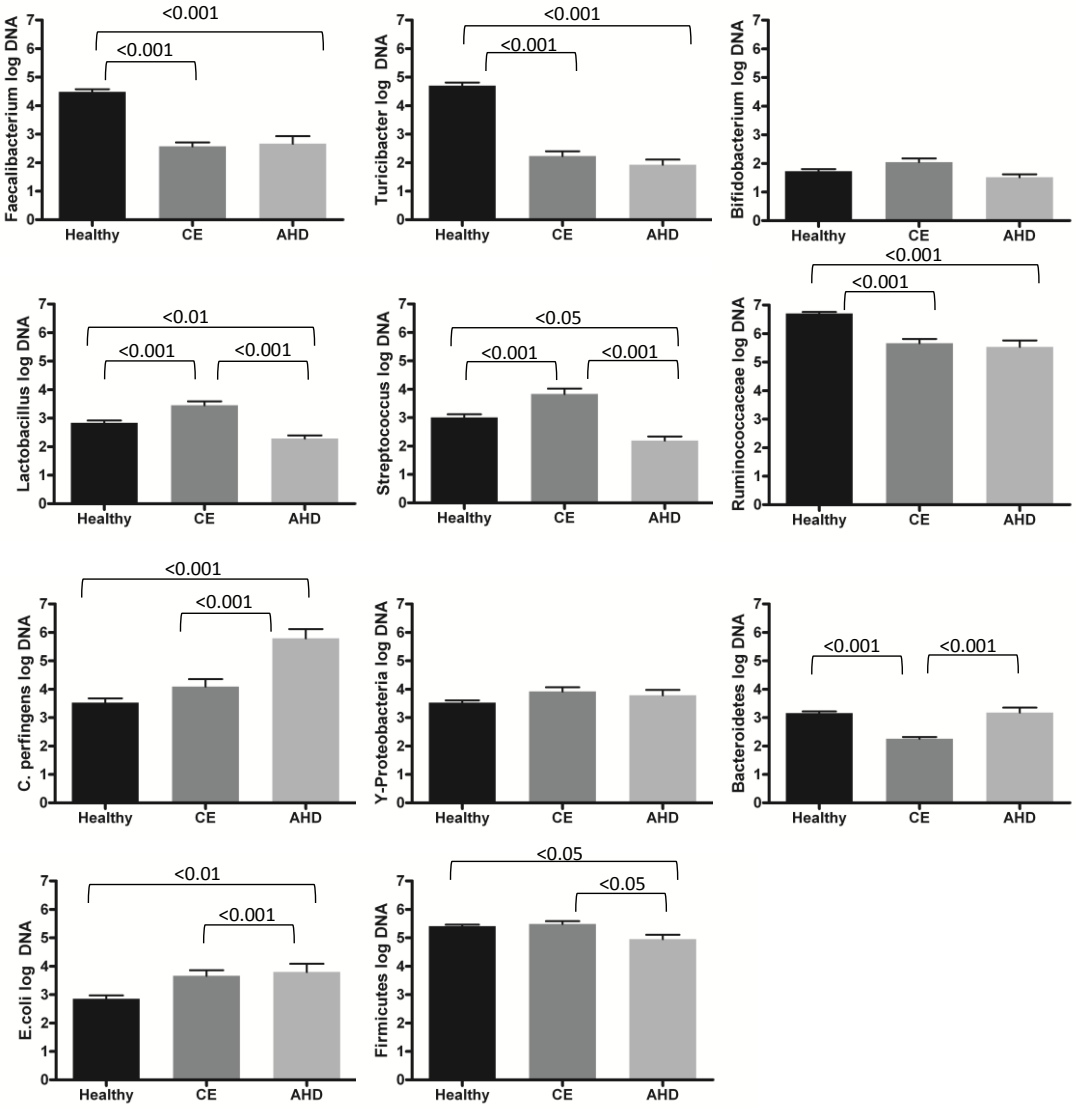

Figure 1. Abundance of bacterial groups in healthy dogs, dogs with chronic enteropathies (CE), dogs with acute hemorrhagic diarrhea (AHD). Significance was set at <0.05.

Figure 1. Abundance of sequences belonging to 11 bacterial groups in fecal samples from healthy dogs, dogs with CE, and dogs with AHD based on qPCR analysis.

Table 4. Abundance of sequences of 11 bacterial groups in fecal samples from healthy dogs, dogs with CE, and dogs with AHD that either did or did not undergo administration of antibiotics within 6 months of sample collection.

	Range (Minimum-Maximum)						Medians							Dunn's Post-Test p-value												
	Healthy without ABX (n=13)	Healthy with ABX (n=10)	CE without ABX (n=13)	CE with ABX (n=10)	AHD without ABX (n=9)	AHD with ABX (n=10)	Healthy without ABX	Healthy with ABX	CE without ABX	CE with ABX	AHD without ABX	AHD with ABX	Adjusted Kruskal-Wallis P-value	H without ABX vs H with ABX	H without ABX vs CE without ABX	H without ABX vs CE with ABX	H without ABX vs AHD without ABX	H without ABX vs AHD with ABX	H with ABX vs CE without ABX	H with ABX vs CE with ABX	H with ABX vs AHD without ABX	H with ABX vs AHD with ABX	CE without ABX vs CE with ABX	CE without ABX vs AHD without ABX	CE without ABX vs AHD with ABX	CE with ABX vs AHD without ABX	CE with ABX vs AHD with ABX	AHD without ABX vs AHD with ABX
Faecalibacterium	2.28-6.53	2.30-5.92	0.15-5.77	0.15-4.22	0.15-4.22	0.15-4.31	5.25	4.63	2.19	1.99	4.03	0.66	0.0011*	>0.05	<0.01*	<0.01*	>0.05	<0.01*	>0.05	<0.05	>0.05	<0.05*	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Turicibacter	1.02-6.99	0.90-6.78	0.9-5.90	0.11-4.68	0.90-2.07	0.9-4.43	4.01	5.07	1.98	0.10	0.90	0.93	0.0011*	>0.05	>0.05	>0.05	<0.05*	>0.05	>0.05	<0.05*	<0.01*	<0.05*	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Bifidobacterium.	0.90-2.87	0.90-4.46	0.90-4.59	0.90-4.28	0.90-2.62	0.90-4.19	1.24	1.29	1.17	1.56	0.90	1.19	1.0000	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Lactobacillus.	1.77-5.00	2.25-6.69	1.38-6.45	1.38-5.96	1.38-3.74	1.38-4.80	2.10	3.21	3.07	2.90	2.03	2.01	1.0000	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Streptococcus.	1.50-4.95	1.50-6.52	1.50-6.92	1.50-7.01	1.50-4.30	1.50-4.56	1.50	4.05	2.53	2.65	1.50	1.82	1.0000	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Ruminococcaceae	5.67-7.99	5.47-7.69	2.85-7.26	2.09-6.32	5.03-7.24	1.99-7.35	7.03	6.80	5.72	5.11	6.58	4.91	0.0033*	>0.05	<0.05*	<0.001*	>0.05	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
C. perfringens	0.49-8.48	0.49-6.62	0.49-10.99	0.49-7.80	0.49-6.99	2.88-12.86	3.28	4.52	5.25	5.05	6.14	7.70	0.1386	>0.05	>0.05	>0.05	>0.05	<0.05*	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Y-Proteobacteria	2.16-5.71	2.97-7.96	2.16-5.88	2.16-7.52	2.16-7.06	2.16-6.17	3.28	4.07	2.96	4.60	2.81	3.40	1.0000	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Bacteroidetes	1.90-4.75	1.90-5.16	1.90-4.42	1.90-3.57	1.90-4.60	1.90-4.40	3.39	3.08	2.01	1.90	3.49	2.29	0.4103	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
E. coli	0.12-5390	0.12-6.54	0.12-6.89	0.12-6.84	0.12-7.06	0.12-6.38	2.48	4.68	4.00	5.05	3.00	4.18	1.0000	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Firmicutes	4.56-6.15	3.86-7.15	2.35-7.39	2.30-6.65	2.82-5.88	1.90-6.13	5.38	5.94	5.45	4.81	5.25	4.78	1.0000	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

* Indicates statistical significance (P<0.05).

H = healthy; CE = chronic enteropathies; AHD = acute hemorrhagic diarrhea; ABX = antibiotics

Adjusted Kruskal-Wallis based on Bonferroni correction.

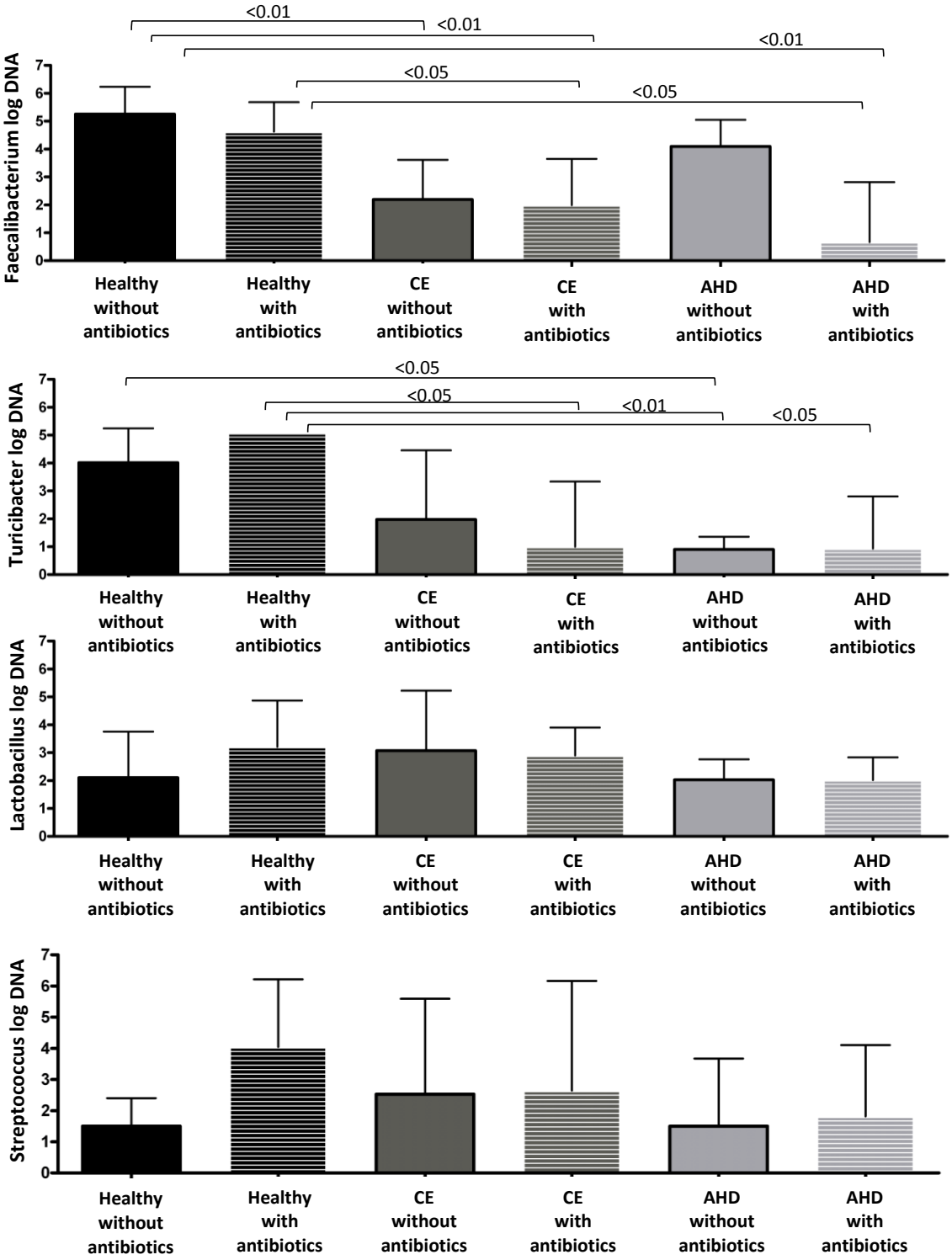

Figure 2. Abundance of sequences of 11 bacterial groups in fecal samples from healthy dogs, dogs with CE, and dogs with AHD that did or did not undergo antibiotic administration within 6 months of sample collection. Significance set at <0.05. Please note difference in y-axis enumeration for graphs illustrating the abundance of *Ruminococcaceae* or *C. perfringens* log DNA.

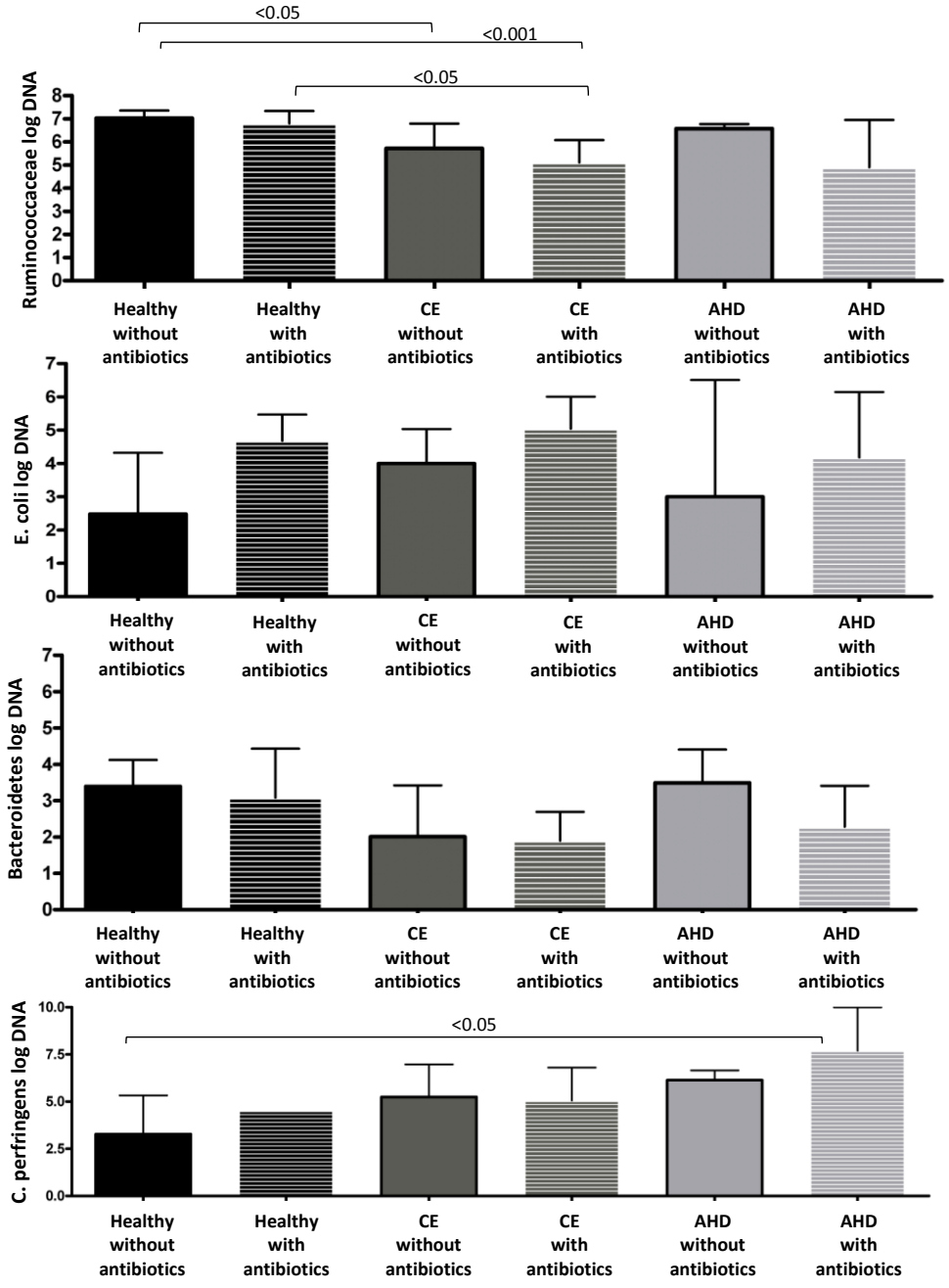

Figure 2. Continued.

Overall, there was no significant difference in bacterial abundance among healthy dogs and healthy dogs that received antibiotics within six months of sample collection (Table 5). Furthermore, antibiotic influence in dogs with CE and dogs with AHD was examined to validate results seen previously. Fecal samples from dogs from all 3 groups of dogs that did not receive antibiotics were compared among the 3 groups (Table 6). Additionally, fecal samples from all 3 groups of dogs that did receive antibiotics were compared among the 3 groups (Table 7).

History of diarrhea. In order to assess if a previous episode of diarrhea in otherwise healthy dogs might significantly impact the GI microbiota, the fecal microbiota of healthy dogs with a history of diarrhea during the 6 months prior to sample collection (n=30) were compared to that of healthy dogs without a history of diarrhea during this time period (n=212) (Table 8, Figures 3 and 4). *Faecalibacterium* spp. sequences were significantly more abundant (p=0.0133) and *C. perfringens* were significantly less abundant (p=0.0156) in healthy dogs without a history of diarrhea than in those dogs with such a history of diarrhea.

Table 5. Abundance of sequences of 11 bacterial groups in fecal samples from healthy dogs that did not receive antibiotics and healthy dogs that received antibiotics within 6 months of sample collection**.**

	Range (Minimum-Maximum)		**Medians**			
	Healthy without ABX (n=217)	Healthy with ABX (n=25)	Healthy without ABX	Healthy with ABX	Mann-Whitney P-value	Adjusted Mann-Whitney P-value
Faecalibacterium spp.	0.15-6.97	1.56-6.11	4.77	4.77	0.4954	
Turicibacter spp.	0.90-7.83	0.90-6.90	4.94	5.14	0.5503	
Bifidobacterium spp.	0.90-6.13	0.90-5.18	1.13	1.28	0.6508	
Lactobacillus spp.	1.27-6.18	1.38-6.69	2.48	2.85	0.2293	
Streptococcus spp.	1.50-7.34	1.50-6.52	2.07	2.42	0.7697	
Ruminococcaceae	1.90-7.99	5.47-7.72	6.84	6.98	0.814	
C. perfringens	0.44-11.05	0.49-6.62	3.49	4.34	0.3907	
Y-Proteobacteria	1.71-6.80	2.16-7.96	3.28	4.11	0.0317*	0.3487
Bacteroidetes	1.90-5.64	1.90-5.16	3.14	2.98	0.5214	
E. coli	0.12-6.40	0.12-6.54	2.85	4.26	0.0111	
Firmicutes	2.29-7.07	3.51-7.15	5.46	5.52	0.2959	

* Indicates statistical significance (P<0.05).

ABX = antibiotics
Adjusted p-values based on Bonferroni correction. Significance set at p<0.05.

Table 6. Abundance of sequences of 11 bacterial groups in fecal samples from healthy dogs, dogs with CE, and dogs with AHD that did not receive antibiotics within 6 months of sample collection.

	Range (Minimum-Maximum)			Medians			
	Healthy without ABX (n=217)	CE without ABX (n=87)	AHD without ABX (n=49)	Healthy without ABX	CE without ABX	AHD without ABX	Adjusted Kruskal-Wallis P-value*
Faecalibacterium spp.	0.15-6.97	0.15-6.09	0.15-6.34	4.77[a]	2.45[b]	2.69[b]	<0.0011
Turicibacter spp.	0.90-7.83	0.11-6.93	0.90-6.05	4.94[a]	1.49[b]	1.30[b]	<0.0011
Bifidobacterium spp.	0.90-6.13	0.90-5.64	0.90-4.25	1.13	1.64	1.09	1.0000
Lactobacillus spp.	1.27-6.18	1.38-6.53	1.38-4.28	2.48[a]	3.20[b]	2.25[c]	<0.0011
Streptococcus spp.	1.50-7.34	1.50-7.53	1.50-5.79	2.07[a]	3.44[b]	1.52[c]	<0.0011
Ruminococcaceae	1.90-7.99	1.99-7.67	1.99-7.72	6.84[a]	6.10[b]	6.12[b]	<0.0011
C. perfringens	0.44-11.05	0.49-10.99	0.49-8.53	3.49[a]	4.80[a]	6.17[b]	<0.011
Y-Proteobacteria	1.71-6.80	2.16-7.70	2.16-7.06	3.28	3.86	3.61	1.0000
Bacteroidetes	1.90-5.64	1.75-4.42	1.57-6.54	3.14[a]	1.90[b]	3.21[a]	<0.0011
E. coli	0.12-6.40	0.12-6.89	0.12-7.06	2.85[a]	4.15[b]	3.96[b]	<0.0011
Firmicutes	2.29-7.07	2.35-7.39	2.52-7.31	5.46[ab]	5.51[a]	5.19[b]	0.5258

Medians not sharing a common superscript indicates statistical significance ($p<0.05$, Dunn's multiple comparisons test).

CE = chronic enteropathies; AHD = acute hemorrhagic diarrhea; ABX = antibiotics

* Adjusted Kruskal-Wallis based on Bonferroni correction. Significance set at $p<0.05$.

Table 7. Abundance of sequences of 11 bacterial groups in fecal samples from healthy dogs, dogs with CE, and dogs with AHD that did receive antibiotics within 6 months of sample collection.

	Range (Minimum-Maximum)			Medians			
	Healthy with ABX (n=25)	CE with ABX (n=21)	AHD with ABX (n=8)	Healthy with ABX	CE with ABX	AHD with ABX	Adjusted Kruskal-Wallis P-value*
Faecalibacterium spp.	0.16-6.11	0.15-5.87	0.15-4.31	4.75[a]	2.15[b]	0.66[b]	<0.0011
Turicibacter spp.	0.90-6.90	0.90-4.68	0.90-4.43	5.14[a]	0.90[b]	0.90[b]	<0.0011
Bifidobacterium spp.	0.90-5.18	0.90-5.30	0.90-4.19	1.28	1.76	1.19	1.0000
Lactobacillus spp.	1.38-6.69	1.38-6.43	1.38-4.80	2.85	3.65	2.01	1.0000
Streptococcus spp.	1.50-6.52	1.50-7.20	1.50-4.56	2.42	4.05	1.82	1.0000
Ruminococcaceae	5.47-7.72	2.09-7.08	1.99-7.35	6.98[a]	6.20[b]	4.91[ab]	0.0704
C. perfringens	0.49-6.62	0.49-7.80	2.88-12.86	4.34[a]	4.27[a]	7.70[b]	0.0209
Y-Proteobacteria	2.16-7.96	2.16-7.52	2.16-6.17	4.11	3.82	3.40	1.0000
Bacteroidetes	1.90-5.16	0.70-4.03	1.90-4.40	2.98[a]	1.90[b]	1.90[ab]	0.1364
E. coli	0.12-6.54	0.12-6.85	0.12-6.38	4.26	4.43	4.18	1.0000
Firmicutes	3.51-7.15	2.30-7.08	1.90-6.13	5.52	5.55	4.78	0.3476

Medians not sharing a common superscript indicates statistical significance ($p<0.05$, Dunn's multiple comparisons test).

CE = chronic enteropathies; AHD = acute hemorrhagic diarrhea; ABX = antibiotics

* Adjusted Kruskal-Wallis based on Bonferroni correction. Significance set at $p<0.05$..

Table 8. Abundance of sequences of fecal bacterial groups of healthy dogs without a history of diarrhea in the 6 months prior to sample collection and in dogs with CE or AHD.

	Range (Minimum-Maximum)			Medians			
	Healthy without D (n=212)	CE (n=108)	AHD (n=57)	Healthy without D	CE	AHD	Adjusted Kruskal-Wallis P-value*
Faecalibacterium spp.	0.15-6.97	0.15-6.09	0.15-6.34	4.83[a]	2.40[b]	2.64[b]	<0.0011
Turicibacter spp.	0.90-7.83	0.11-6.93	0.90-6.05	4.95[a]	1.29[b]	1.26[b]	<0.0011
Bifidobacterium spp.	0.90-6.13	0.90-5.64	0.90-4.25	1.13[a]	1.72[b]	1.16[ab]	0.2266
Lactobacillus spp.	1.27-6.69	1.38-6.53	1.38-4.80	2.52[a]	3.25[b]	2.25[c]	<0.0011
Streptococcus spp.	1.50-7.34	1.50-7.53	1.50-5.79	2.19[a]	3.47[b]	1.50[c]	<0.0011
Ruminococcaceae	1.90-7.99	1.99-7.67	1.99-7.72	6.83[a]	6.18[b]	5.97[b]	<0.0011
C. perfringens	0.44-11.05	0.49-10.99	0.49-12.86	3.47[a]	4.70[a]	6.25[b]	<0.0011
Y-Proteobacteria	2.02-7.96	2.16-7.70	2.16-7.06	3.29	3.85	3.60	1.0000
Bacteroidetes	1.90-5.64	0.70-4.42	1.57-6.54	3.12[a]	1.90[b]	2.99[a]	<0.0011
E. coli	0.12-6.54	0.12-6.89	0.12-7.06	2.96[a]	4.19[b]	4.03[b]	<0.0011
Firmicutes	2.31-7.15	2.30-7.39	1.90-7.31	5.48[a]	5.57[a]	5.17[b]	0.1265

Medians not sharing a common superscript indicates statistical significance ($p<0.05$).

D = diarrhea; CE = chronic enteropathies; AHD = acute hemorrhagic diarrhea;

* Adjusted Kruskal-Wallis based on Bonferroni correction. Significance set at $p<0.05$.

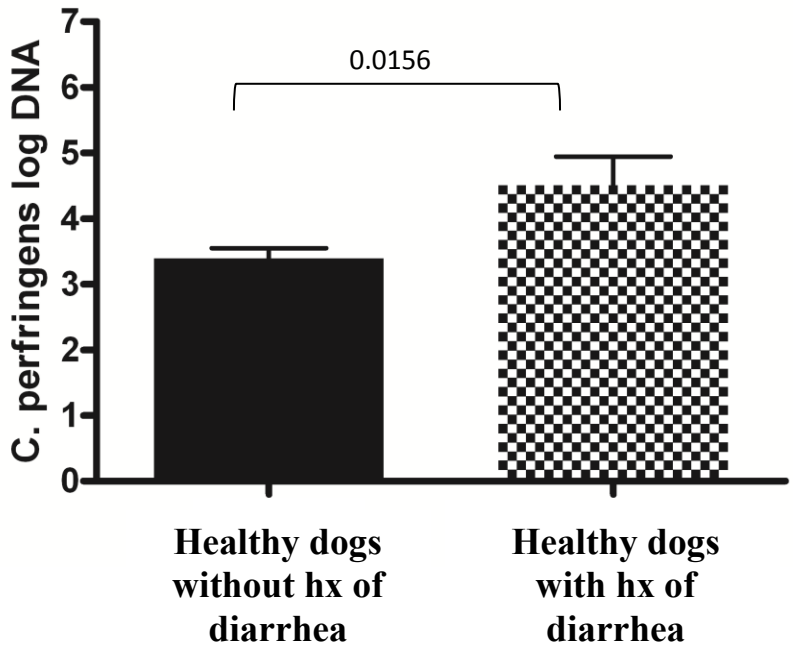

Figure 3. Abundance of *C.perfringens* in healthy dogs without diarrhea in the 6 months prior to sample collection ("healthy dogs without hx of diarrhea") and in healthy dogs with such a history of diarrhea ("healthy dogs with hx of diarrhea") during this period. Significance set at <0.05.

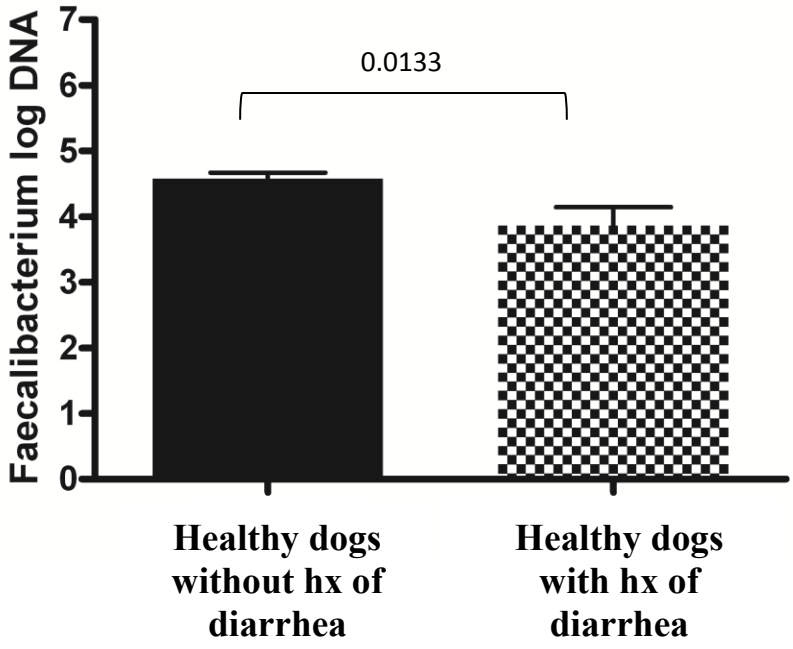

Figure 4. Abundance of *Faecalibacterium* spp. in healthy dogs without diarrhea in the 6 months prior to sample collection ("healthy dogs without hx of diarrhea") and in healthy dogs with such a history of diarrhea ("healthy dogs with hx of diarrhea") during this period. Significance set at <0.05.

Dietary macronutrients. It has been shown that dietary components such as protein, fat, and fiber can alter the GI microbiota and it is possible that healthy dogs and dogs with CE in this study were fed different amounts of these dietary components, thereby potentially confounding our results [7,65,92]. In order to compare dietary macronutrients among all 3 groups of dogs, the percent metabolizable energy (ME) from protein, fat, and carbohydrates were compared separately among healthy dogs (n=65) and dogs with CE (n=57) (Table 9, Figure 5.). A significant difference was observed in the percent metabolizable energy from protein between healthy dogs and dogs with CE ($p=0.0032$). Dietary macronutrients in healthy dogs ranged from 15-38 % metabolizable protein, whereas dietary macronutrients in dogs with CE ranged from 15-37 % metabolizable protein, with medians of 25 and 24, respectively (Table 9).

In order to evaluate a potential confounding effect of the dietary protein source, the bacterial abundances for all 11 groups were compared among healthy dogs known to be consuming a diet with a primary protein source of poultry (n=69) to healthy dogs that were consuming a diet with a primary protein source other than poultry (n=32). Overall no significant differences were found for any of the groups analyzed (Table 10).

Table 9. Macronutrient content in the diets fed to healthy dogs and dogs with chronic enteropathies.

	Range (Minimum-Maximum)		**Medians**			
	healthy (n=65)	chronic enteropathies (n=57)	healthy	chronic enteropathies	Mann-Whitney P-value	Adjusted Mann-Whitney P-value
%ME protein	15.00-38.00	15.00-37.00	25.00	24.00	0.0032*	0.0352*
%ME fat	14.90-57.30	19.40-39.00	31.00	29.50	0.6626	
%ME carbohydrate	14.9-59.00	33.00-55.90	46.00	47.00	0.1662	

* Indicates statistical significance (P<0.05).

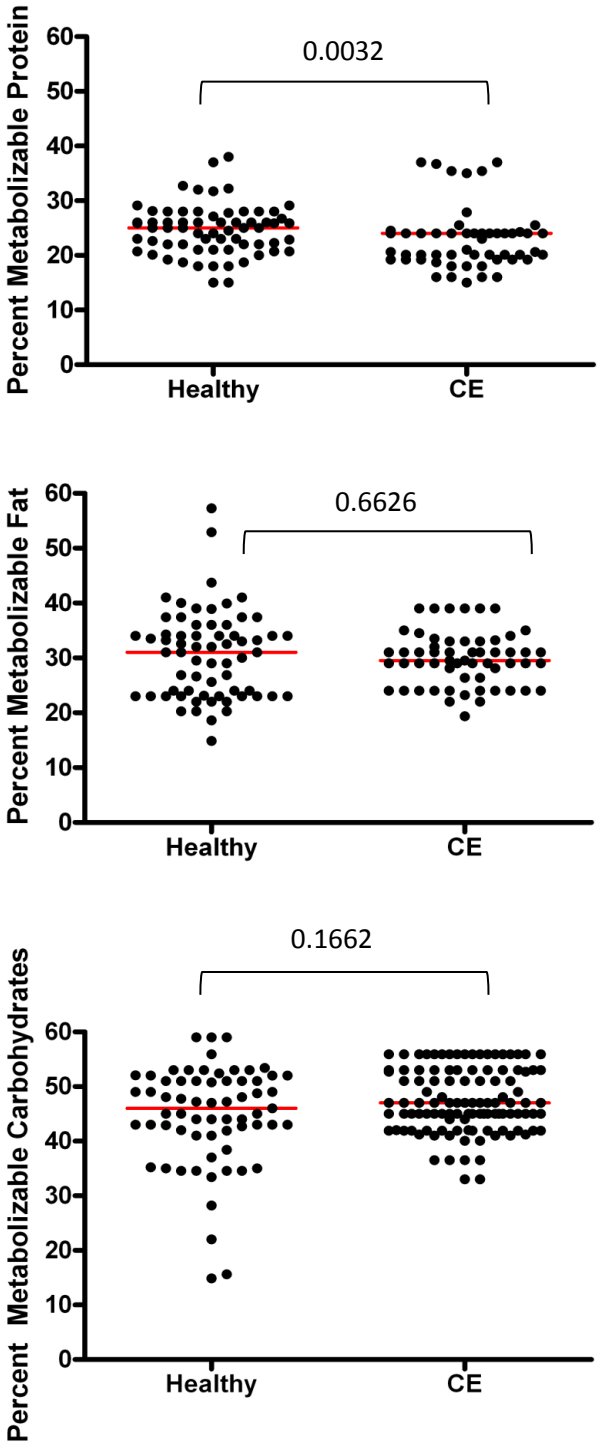

Figure 5. Percent metabolizable energy (ME) from protein, fat, and carbohydrates in diets of healthy dogs compared to those fed to dogs with chronic enteropathies (CE). Significance set at <0.05.

Table 10. Abundance of bacterial groups in fecal samples of healthy dogs fed poultry-based diets and healthy dogs with diets based on other protein sources.

	Range (Minimum-Maximum)		Medians		
	Healthy dogs fed poultry (n=69)	Healthy dogs fed other protein (n=32)	Healthy dogs fed poultry	Healthy dogs fed other protein	Mann-Whitney P-value
Faecalibacterium spp.	1.33-6.97	0.15-6.72	4.62	4.66	0.208
Turicibacter spp.	0.90-7.83	0.90-7.58	4.82	4.98	0.5716
Bifidobacterium spp.	0.90-5.64	0.90-5.87	0.90	0.90	0.6922
Lactobacillus spp.	1.38-5.94	1.38-5.78	2.19	2.47	0.3851
Streptococcus spp.	1.50-6.93	1.50-7.34	1.50	3.00	0.2296
Ruminococcaceae	1.90-7.73	4.89-7.54	6.76	6.99	0.1076
C. perfringens	0.49-11.05	0.49-10.45	3.40	2.96	0.8637
Y-Proteobacteria	2.02-7.96	2.16-6.29	2.82	2.71	0.6905
Bacteroidetes	1.90-5.13	1.90-4.75	3.02	2.88	0.5966
E. coli	0.12-6.54	0.12-6.40	2.43	2.52	0.1374
Firmicutes	2.29-6.68	2.31-6.50	5.42	5.49	0.7731

* Indicates statistical significance (P<0.05).

The abundances of sequences for all 11 groups were also compared among dogs with CE known to be consuming a diet with a primary protein source of poultry (n=27) to dogs with CE that were consuming a diet with a primary protein source other than poultry (n=35) (Table 11). Those dogs consuming a diet with a primary source of poultry had a significantly lower abundance of *E. coli* (median 3.13, range 0.12-6.25) compared to dogs being fed other primary protein sources (median 4.40, range 0.12-6.24; p=0.0458), No significant differences were observed for any of the other bacterial groups.

DISCUSSION

Previous studies have used sequencing-based methods to describe the intestinal microbiota of healthy dogs and have revealed a highly complex intestinal ecosystem, comprising several hundred to thousand bacterial phylotypes [21,63,75]. Those studies have shown that *Firmicutes* and *Bacteroidetes* are typically the most abundant phyla representing up to 90% of sequences of the canine fecal microbiota. Other important phyla are *Fusobacteria, Proteobacteria,* and *Actinobacteria* with reported abundances ranging between 0 – 30% in various studies [21,63,75]. *Firmicutes* and *Bacteroidetes* are considered of importance to gastrointestinal health as they include several phylogenetic bacterial groups that synthesize various metabolites, including short-chain fatty acids [6]. Short-chain fatty acids (SCFA), especially butyrate, provide nutritional benefits to the host, provide energy for epithelial cell growth, and have been shown to have anti-inflammatory and immune-modulatory properties [6]. They also regulate the

luminal pH in the intestine, a mechanism that may help in the exclusion of potentially invading pathogens.

Gastrointestinal diseases in both humans and dogs have been associated with an altered composition of the intestinal microbiota. Most commonly observed changes include a decrease in members of *Firmicutes* and *Bacteroidetes*, and increases in members of *Proteobacteria* [21,75,76,93]. For example, Frank et al [93] described the microbiota of humans with inflammatory bowel disease (i.e., Crohn's disease and ulcerative colitis). The sequence based analysis of resected tissue samples from diseased individuals and healthy controls revealed depletion in the phylum *Firmicutes* and *Bacteroidetes*, and an increase in *Proteobacteria* in humans with IBD [93]. Similarly, recent studies analyzing the small intestinal microbiota of dogs with IBD have revealed similar patterns of dysbiosis as those observed in human ileal samples [21,75].

Table 11. Abundance of bacterial groups in fecal samples of dogs with CE fed poultry-based diets and dogs with CE with diets based on other protein sources.

	Range (Minimum-Maximum)		Medians			
	CE dogs fed poultry (n=27)	CE dogs fed other protein (n=35)	CE dogs fed poultry	CE dogs fed other protein	Mann-Whitney P-value	Adjusted Mann-Whitney P-value
Faecalibacterium spp.	0.15-6.09	0.15-5.87	2.33	2.31	0.7655	
Turicibacter spp.	0.90-6.93	0.11-5.60	1.15	1.61	0.6633	
Bifidobacterium spp.	0.90-5.39	0.90-4.59	1.24	1.56	0.983	
Lactobacillus spp.	1.38-6.43	1.38-6.53	3.35	3.25	0.8983	
Streptococcus spp.	1.50-7.53	1.50-7.05	3.15	4.80	0.4465	
Ruminococcaceae	1.99-7.63	3.26-7.67	6.60	6.20	0.7765	
C. perfringens	0.49-10.99	0.49-10.17	4.98	4.52	0.9207	
Y-Proteobacteria	2.16-6.61	2.16-7.70	2.88	3.49	0.6385	
Bacteroidetes	0.70-3.58	1.90-4.03	1.90	1.90	0.1311	
E. coli	0.12-6.25	0.12-6.24	3.13	4.40	0.0485*	0.5335
Firmicutes	2.35-7.25	4.00-6.98	5.64	5.56	0.5994	

* Indicates statistical significance (P<0.05).

CE = chronic enteropathies

Little information is available about the fecal microbiota of dogs with acute or chronic gastrointestinal diseases. One question of particular interest is whether the bacterial dysbiosis in the small intestine of dogs with IBD can also be identified in fecal samples, and also whether specific patterns of dysbiosis are specific for specific GI disease. Therefore, for the purposes of this study, we compared a sample population of healthy dogs to dogs with acute or chronic gastrointestinal disease. While molecular-based sequencing techniques are useful tools for evaluating the entire microbiota, this approach is rather expensive. Therefore, in this study, the fecal microbiota of healthy dogs, dogs with chronic enteropathies (CE), and dogs with acute hemorrhagic diarrhea (AHD) was characterized using qPCR analysis. The bacterial groups evaluated were chosen because they had previously been found to be altered in humans or dogs with GI disease..

The results of this study indicate that dogs with CE or AHD have an altered composition of the intestinal microbiota when compared to healthy dogs. Furthermore, significant differences in the abundances of sequences of specific bacterial groups were also observed between dogs with CE and dogs with AHD. This would suggest that the duration of illness, as well as other differences in the disease processes influence the composition of the intestinal microbiota.

The abundances of sequences of several bacterial groups were significantly different between dogs with CE and healthy dogs. Most notably *Bacteroidetes* and members of *Firmicutes* (i.e., *Faecalibacterium* spp., Ruminococcaceae*,* and *Turicibacter* spp.) were decreased in abundance in dogs with CE. These bacterial groups are considered short-chain fatty acid producers and a decrease in these bacterial groups may potentially have a significant impact on the luminal concentrations of these metabolites.

Further studies are needed to correlate fecal concentrations of SCFA and the abundances of these bacterial groups. Similar decreases have also been previously observed in fecal samples of humans with IBD [54]. We observed a trend for an increased abundance of *Proteobacteria* sequences, including *E. coli*, in dogs with CE compared to healthy dogs, but this increase was not significant. In previous studies fecal samples of humans with IBD and duodenal samples of dogs with IBD were shown to have a significantly increased abundance of these bacterial groups [21,73,75,76].

Significant differences in the abundance of several bacterial groups were observed between healthy dogs and dogs with AHD. Similar to dogs with CE, dogs with AHD also had a significantly increased abundance of sequences of members of *Firmicutes,* including *Faecalibacterium* spp., *Ruminococcaceae,* and *Turicibacter* spp. However, *Lactobacillus* spp. and *Streptococcus* spp., also members of the *Firmicutes*, showed a significant decreased abundance in dogs with AHD, but not in CE, when compared to control dogs. This would suggest that the dysbiosis is potentially more pronounced in acute hemorrhagic disease, as more bacterial groups are depleted. All these bacterial groups are considered important bacterial groups in the normal canine GI tract, as they produce SCFA (e.g., *Faecalibacterium* spp., *Ruminococcaceae*) or lactic acids (e.g., *Lactobacillus* spp. and *Streptococcus* spp), all of which are important metabolites that help maintain homeostasis of the physiiologic intestinal microbiota. In contrast, the abundances of *C. perfringens* and *E. coli* were significantly increased in dogs with AHD. This increase in abundance of the gram-negative *E. coli* is not surprising, as increases of members of the phylum *Proteobacteria* have been commonly

associated with intestinal inflammation [21,75,93]. Furthermore, it has been well established that specific strains of *E. coli* (i.e., enteropathogenic, enterotoxigenic, enterohemorrhagic, or enteroinvasive) can induce gastrointestinal inflammation in dogs [94]. Similarly, enterotoxin-producing *C. perfringens* has been linked to GI disease in dogs [80,94]. The increased abundance of *C. perfringens* and *E. coli* observed in this study may be due to strains containing virulence factors, which were not evaluated in this study, but these virulence factors warrant further studies.

Dogs that were considered healthy at the time of sample collection, but that had a history of diarrhea within the past six months before sample collection had a decreased abundance of *Faecalibacterium* spp. ($p=0.0133$) and an increased abundance of *C. perfringens* ($p=0.0156$) in comparison to healthy dogs with no history of diarrhea in said time period. Although, these differences were not significantly different after p-values were adjusted for multiple comparisons, these results suggest some interesting aspects of dysbiosis that should be evaluated in further studies. It is possible that after episodes of diarrhea, longer periods than 6 months are required for *Faecalibacterium* spp, and *C. perfringens* to return to abundances that are comparable to healthy dogs. Follow-up longitudinal studies with multiple sample collections would be interesting to evaluate the time needed for the abundances of bacterial groups to normalize after episodes of diarrhea.

We analyzed several potentially confounding factors in this study that may impact the abundances of specific bacterial groups. Administration of antibiotics has been shown to significantly impact the gastrointestinal microbiota [95]. For each disease

group, we therefore compared the abundances of bacterial groups among dogs that received antibiotics within 6 months of sample collection to those that did not receive antibiotics. These results suggest that antibiotic administration did not have an overall significant influence on the results observed in this study.

Dietary components such as protein, fat, and fiber may also alter the GI microbiota and it is possible that healthy dogs and dogs with CE were fed different amounts of these dietary components in this study, thereby potentially confounding our results [7,65,92]. We observed a significant difference in percent metabolizable energy from protein between the diets fed to the healthy dogs and dogs with CE. However, these differences were rather small. Dietary protein in the diets fed to healthy dogs ranged from 15-38% (median: 25%) of metabolizable energy, whereas it ranged from 15-37% (median: 24%) metabolizable energy in dogs with CE. Previous studies have shown that large differences in the dietary protein content are needed to induce even slight changes in selected bacterial groups such as *C. perfringens* and *Bifidobacterium* spp. [96].

In conclusion, using qPCR analysis, dysbiosis was identified in fecal samples of dogs with CE or AHD. A decreased abundance of bacterial groups such as specific members of *Firmicutes (*i.e., *Faecalibacterium* spp, and *Ruminococcaceae*) may lead to decreased abundances of beneficial metabolites such as short-chain fatty acids. Therefore, future studies need to evaluate the luminal concentrations of SCFA in fecal samples of diseased dogs.

CHAPTER III

CONCLUSIONS

Previous studies have used sequencing-based methods to describe the intestinal microbiota of healthy dogs and have revealed a highly complex intestinal ecosystem, comprising several hundred to thousand bacterial phylotypes [21,63,75]. It has been suggested that alterations in the fecal microbiota have been implicated in the pathogenesis of GI disease in dogs [21,75,83,85]. *Firmicutes* (i.e., *Faecalibacterium* spp., Ruminococcaceae*,* and *Turicibacter* spp.) and *Bacteroidetes* are considered to be important for GI health as they include several phylogenetic bacterial groups that synthesize various metabolites, including short-chain fatty acids, which are believed to provide the host with several beneficial properties [6].

The results of this study indicate that dogs with CE as well as dogs with AHD have an altered composition of the intestinal microbiota when compared to healthy dogs.

The abundances of several bacterial groups were significantly different between dogs with CE and healthy dogs. Most notably, the abundance of *Bacteroidetes* and members of *Firmicutes* (i.e., *Faecalibacterium* spp., Ruminococcaceae*,* and *Turicibacter* spp.) were decreased in dogs with CE. These bacterial groups are considered short-chain fatty acid producers and a decrease in these bacterial groups may potentially have a significant impact on the luminal concentrations of these metabolites. Future studies are needed to correlate fecal concentrations of SCFA and the abundances of these bacterial groups.

Significant differences in the abundance of several bacterial groups were observed between healthy dogs and dogs with AHD. Similar to dogs with CE, dogs with AHD also had a significantly increased abundance of members of *Firmicutes,* including *Faecalibacterium* spp., *Ruminococcaceae,* and *Turicibacter* spp. However, abundance of *Lactobacillus* spp. and *Streptococcus* spp., which are also members of the *Firmicutes*, were significantly decreased in dogs with AHD, but not in those with CE, when compared to healthy control dogs. This would suggest that the dysbiosis is potentially more pronounced in dogs with acute hemorrhagic disease, as more bacterial groups are depleted.

In this study we compared a sample population of healthy dogs to dogs with acute or chronic gastrointestinal disease. While molecular-based sequencing technologies are very useful for evaluating the entire microbiota, this approach is rather expensive. Therefore, in this study, the fecal microbiota of healthy dogs, dogs with chronic enteropathies (CE), and dogs with acute hemorrhagic diarrhea (AHD) was characterized using qPCR analysis. Using these methods, a dysbiosis was identified in fecal samples of dogs with CE and AHD. A decreased abundance of bacterial groups such as specific members of *Firmicutes (*i.e., *Faecalibacterium* spp, and *Ruminococcaceae*) may lead to decreased concentration of beneficial metabolites such as short-chain fatty acids. Therefore, future studies need to evaluate luminal concentrations of SCFA in fecal samples of diseased dogs.

REFERENCES

1. Suchodolski JS (2011) Microbes and gastrointestinal health of dogs and cats. J Anim Sci 89: 1520-1530.

2. Macfarlane S, Furrie E, Cummings JH, Macfarlane GT (2004) Chemotaxonomic analysis of bacterial populations colonizing the rectal mucosa in patients with ulcerative colitis. Clin Infect Dis 38: 1690-1699.

3. Deplancke B, Gaskins HR (2001) Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am J Clin Nutr 73: 1131S-1141S.

4. Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 9: 313-323.

5. Gaskins HR (1997) Immunological aspects of host/microbiota interactions at the intestinal epithelium. Gastrointestinal microbiology 2: 537-587.

6. Topping DC, PM (2001) Short-chain fatty acids and humancolonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 81: 1031-1064.

7. Tungland BM, D (2002) Nondigestible oligo-and polysaccharides (dietary fiber): their physiology and role in human health and food. Comprehens Rev Food Sci Food Safety: 73-92.

8. Cummings JH (2004) Physiological and clinical aspects of short-chain fatty acids. Cambridge University Press. 20-55.

9. Amit-Romach E, Uni Z, Reifen R (2008) Therapeutic potential of two probiotics in inflammatory bowel disease as observed in the trinitrobenzene sulfonic acid model of colitis. Dis Colon Rectum 51: 1828-1836.

10. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PC, et al. (2001) Molecular analysis of commensal host-microbial relationships in the intestine. Science 291: 881-884.

11. Macpherson AJ, Harris NL (2004) Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol 4: 478-485.

12. O'Connor E, Barret E, Fitzgerald G, Hill C, Stanton C, et al. (2005) Production of vitamins, exopolysaccharides and bacteriocins by probiotic bacteria. Tamime A, editor. Probiotic Dairy Products: Blackwell Publishing Ltd. 167-194.

13. O'Keefe SJ (2008) Nutrition and colonic health: the critical role of the microbiota. Curr Opin Gastroen 24: 51-58.

14. Kim TH, Yang J, Darling PB, O'Connor DL (2004) A large pool of available folate exists in the large intestine of human infants and piglets. J Nutr 134: 1389-1394.

15. Shanahan F (2002) The host-microbe interface within the gut. Best Pract Res Cl Ga 16: 915-931.

16. Hakim AM, Pappius HM (1981) The effect of thiamine deficiency on local cerebral glucose utilization. Ann Neurol 9: 334-339.

17. Quesada-Chanto A, Afschar AS, Wagner F (1994) Microbial production of propionic acid and vitamin B12 using molasses or sugar. Appl Microbiol Biotechnol 41: 378-383.

18. Yi P, Li L (2012) The germfree murine animal: An important animal model for research on the relationship between gut microbiota and the host. Vet Microbiol 157: 1-7.

19. Barefoot SF, Klaenhammer TR (1984) Purification and characterization of the Lactobacillus-acidophilus bacteriocin lactacin-B. Antimicrob Agents Ch 26: 328-334.

20. Bogovic-Matijasic B, Rogelj I, Nes IF, Holo H (1998) Isolation and characterization of two bacteriocins of Lactobacillus acidophilus LF221. Appl Microbiol Biot 49: 606-612.

21. Suchodolski JS, Xenoulis PG, Paddock CG, Steiner JM, Jergens AE (2010) Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. Vet Microbiol 142: 394-400.

22. Herbert D, Elsworth R, Telling RC (1956) The continuous culture of bacteria - a theoretical and experimental study. J Gen Microbiol 14: 601-622.

23. Macfarlane S, Macfarlane GT (2004) Bacterial diversity in the human gut. Adv Appl Microbiol 54: 261-289.

24. Monod J (1949) The growth of bacterial cultures. Annu Rev Microbiol 3: 371-394.

25. Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR (2004) Molecular ecological analysis of the gastrointestinal microbiota: A review. J Nutr 134: 465-472.

26. Schloss PD, Handelsman J (2005) Metagenomics for studying unculturable microorganisms: cutting the Gordian knot. Genome Biol 6(8): 229.

27. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, et al. (2005) Diversity of the human intestinal microbial flora. Science 308: 1635-1638.

28. Maher M, Finnegan C, Collins E, Ward B, Carroll C, et al. (2003) Evaluation of culture methods and a DNA probe-based PCR assay for detection of Campylobacter species in clinical specimens of feces. J Clin Microbiol 41: 2980-2986.

29. Yang S, Rothman RE (2004) PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis 4: 337-348.

30. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, et al. (2002) Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microb 68: 5445-5451.

31. Van de Peer Y, Chapelle S, De Wachter R (1996) A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res 24: 3381-3391.

32. Greetham HL, Giffard C, Hutson RA, Collins MD, Gibson GR (2002) Bacteriology of the Labrador dog gut: a cultural and genotypic approach. J Appl Microbiol 93: 640-646.

33. Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, et al. (1995) Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol 61: 3069-3075.

34. Wilson KH, Blitchington RB (1996) Human colonic biota studied by ribosomal DNA sequence analysis. Appl Environ Microbiol 62: 2273-2278.

35. Fox GE, Stackebrandt E, Hespell RB, Gibson J, Maniloff J, et al. (1980) The Phylogeny of Prokaryotes. Science 209: 457-463.

36. Woese CR (1987) Bacterial evolution. Microbiol Rev 51: 221-271.

37. Tringe SG, Hugenholtz P (2008) A renaissance for the pioneering 16S rRNA gene. Curr Opin Microbiol 11: 442-446.

38. Kaufmann P, Pfefferkorn A, Teuber M, Meile L (1997) Identification and quantification of Bifidobacterium species isolated from food with genus-specific 16S rRNA-targeted probes by colony hybridization and PCR. Appl Environ Microb 63: 1268-1273.

39. Matsuki T, Watanabe K, Tanaka R, Fukuda M, Oyaizu H (1999) Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl Environ Microb 65: 4506-4512.

40. Song Y, Kato N, Liu C, Matsumiya Y, Kato H, et al. (2000) Rapid identification of 11 human intestinal Lactobacillus species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiol Lett 187: 167-173.

41. Wang RF, Cao WW, Cerniglia CE (1996) PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl Environ Microbiol 62: 1242-1247.

42. Kessler Y, Helfer-Hungerbuehler AK, Cattori V, Meli ML, Zellweger B, et al. (2009) Quantitative TaqMan real-time PCR assays for gene expression normalisation in feline tissues. BMC Mol Biol 10: 106.

43. Lu B, Xu J, Chen J, Yu J, Xu E, et al. (2008) TaqMan low density array is roughly right for gene expression quantification in colorectal cancer. Clin Chim Acta 389: 146-151.

44. Medhurst AD, Pangalos MN (2003) Application of TaqMan RT-PCR for real-time semiquantitative analysis of gene expression in the striatum. Methods Mol Med 79: 229-241.

45. Kageyama A, Sakamoto M, Benno Y (2000) Rapid identification and quantification of Collinsella aerofaciens using PCR. FEMS Microbiol Lett 183: 43-47.

46. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6: 986-994.

47. Carey CM, Kirk JL, Ojha S, Kostrzynska M (2007) Current and future uses of real-time polymerase chain reaction and microarrays in the study of intestinal microbiota, and probiotic use and effectiveness. Can J Microbiol 53: 537-550.

48. Simpson JM, McCracken VJ, White BA, Gaskins HR, Mackie RI (1999) Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J Microbiol Meth 36: 167-179.

49. Suchodolski JS, Ruaux CG, Steiner JM, Fetz K, Williams DA (2005) Assessment of the qualitative variation in bacterial microflora among compartments of the intestinal tract of dogs by use of a molecular fingerprinting technique. Am J Vet Res 66: 1556-1562.

50. Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial

ecology. Antonie Van Leeuwenhoek International J Gen Mol Microb 73: 127-141.

51. Muyzer G, Dewaal EC, Uitterlinden AG (1993) Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16s ribosomal-RNA. Appl Environ Microb 59: 695-700.

52. Suchodolski JS, Ruaux CG, Steiner JM, Fetz K, Williams DA (2004) Application of molecular fingerprinting for qualitative assessment of small-intestinal bacterial diversity in dogs. J Clin Microb 42: 4702-4708.

53. Temmerman R, Masco L, Vanhoutte T, Huys G, Swings J (2003) Development and validation of a nested-PCR-denaturing gradient gel electrophoresis method for taxonomic characterization of bifidobacterial communities. Appl Environ Microb 69: 6380-6385.

54. Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F, et al. (1998) Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microb 64: 3336-3345.

55. Vanhoutte T, Huys G, De Brandt E, Fahey GC, Jr., Swings J (2005) Molecular monitoring and characterization of the faecal microbiota of healthy dogs during fructan supplementation. FEMS Microbiol Lett 249: 65-71.

56. Bartlett JM (2004) Fluorescence in situ hybridization: technical overview. Methods Mol Med 97: 77-87.

57. Price CM (1993) Fluorescence in situ hybridization. Blood Rev 7: 127-134.

58. Rothberg JM, Leamon JH (2008) The development and impact of 454 sequencing. Nat Biotechnol 26: 1117-1124.

59. Hill JE, Seipp RP, Betts M, Hawkins L, Van Kessel AG, et al. (2002) Extensive profiling of a complex microbial community by high-throughput sequencing. Appl Environ Microb 68: 3055-3066.

60. Parameswaran P, Jalili R, Tao L, Shokralla S, Gharizadeh B, et al. (2007) A pyrosequencing-tailored nucleotide barcode design unveils opportunities for large-scale sample multiplexing. Nucleic Acids Res 35: e130.

61. Cave NJ, Marks SL, Kass PH, Melli AC, Brophy MA (2002) Evaluation of a routine diagnostic fecal panel for dogs with diarrhea. J Am Vet Med Assoc 221: 52-59.

62. Goodman AL, Kallstrom G, Faith JJ, Reyes A, Moore A, et al. (2011) Extensive personal human gut microbiota culture collections characterized and manipulated in gnotobiotic mice. Proc Natl Acad Sci USA 108: 6252-6257.

63. Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS (2011) Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. FEMS Microbiol Ecol 76: 301-310.

64. Mentula S, Harmoinen J, Heikkila M, Westermarck E, Rautio M, et al. (2005) Comparison between cultured small-intestinal and fecal microbiotas in beagle dogs. Appl Environ Microb 71: 4169-4175.

65. Middelbos IS, Vester Boler BM, Qu A, White BA, Swanson KS, et al. (2010) Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. PLoS One 5: e9768.

66. Suchodolski JS, Camacho J, Steiner JM (2008) Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. FEMS Microbiol Ecol 66: 567-578.

67. Yu ZT, Morrison M (2004) Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36: 808-812.

68. German AJ, Hall EJ, Day N (2003) Chronic intestinal inflammation and intestinal disease in dogs. J Vet Intern Med 17: 8-20.

69. Jergens AE, Schreiner CA, Frank DE, Niyo Y, Ahrens FE, et al. (2003) A scoring index for disease activity in canine inflammatory bowel disease. J Vet Intern Med 17: 291-297.

70. Packey CD, Sartor RB (2009) Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Curr Opin Infect Dis 22: 292-301.

71. Gersemann M, Stange EF, Wehkamp J (2011) From intestinal stem cells to inflammatory bowel diseases. World J Gastroenterol 17: 3198-3203.

72. Simpson KW, Jergens AE (2011) Pitfalls and Progress in the Diagnosis and Management of Canine Inflammatory Bowel Disease. Vet Clin North Am Small Anim Pract 41: 381-398.

73. Simpson KW, Dogan B, Rishniw M, Goldstein RE, Klaessig S, et al. (2006) Adherent and invasive Escherichia coli is associated with granulomatous colitis in boxer dogs. Infect Immun 74: 4778-4792.

74. Janeczko S, Atwater D, Bogel E, Greiter-Wilke A, Gerold A, et al. (2008) The relationship of mucosal bacteria to duodenal histopathology, cytokine mRNA, and clinical disease activity in cats with inflammatory bowel disease. Vet Microbiol 128: 178-193.

75. Xenoulis PG, Palculict B, Allenspach K, Steiner JM, Van House AM, et al. (2008) Molecular-phylogenetic characterization of microbial communities imbalances in the small intestine of dogs with inflammatory bowel disease. FEMS Microbiol Ecol 66: 579-589.

76. Allenspach K, House A, Smith K, McNeill FM, Hendricks A, et al. (2010) Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies. Vet Microbiol 146: 326-335.

77. Craven M, Dogan B, Schukken A, Volkman M, Chandler A, et al. (2010) Antimicrobial resistance impacts clinical outcome of granulomatous colitis in Boxer dogs. J Vet Intern Med 24: 819-824.

78. Mansfield CS, James FE, Craven M, Davies DR, O'Hara AJ, et al. (2009) Remission of histiocytic ulcerative colitis in Boxer dogs correlates with eradication of invasive intramucosal Escherichia coli. J Vet Intern Med 23: 964-969.

79. Decaro N, Buonavoglia C (2012) Canine parvovirus-A review of epidemiological and diagnostic aspects, with emphasis on type 2c. Vet Microbiol 155: 1-12.

80. Sasaki J, Goryo M, Asahina M, Makara M, Shishido S, et al. (1999) Hemorrhagic enteritis associated with Clostridium perfringens type A in a dog. J Vet Med Sci 61: 175-177.

81. Weese JS, Armstrong J (2003) Outbreak of Clostridium difficile-associated disease in a small animal veterinary teaching hospital. J Vet Intern Med 17: 813-816.

82. Unterer S, Strohmeyer K, Kruse BD, Sauter-Louis C, Hartmann K (2011) Treatment of aseptic dogs with hemorrhagic gastroenteritis with Amoxicillin/Clavulanic Acid: A prospective blinded study. J Vet Intern Med 25: 973-979.

83. Bell JA, Kopper JJ, Turnbull JA, Barbu NI, Murphy AJ, et al. (2008) Ecological characterization of the colonic microbiota of normal and diarrheic dogs. Interdiscip Perspect Infect Dis 2008: 149694.

84. Chaban B, Links MG, Hill JE (2012) A molecular enrichment strategy based on cpn60 for detection of epsilon-proteobacteria in the dog fecal microbiome. Microb Ecol 63: 348-357.

85. Jia J, Frantz N, Khoo C, Gibson GR, Rastall RA, et al. (2010) Investigation of the faecal microbiota associated with canine chronic diarrhoea. FEMS Microbiol Ecol 71: 304-312.

86. Muhling M, Woolven-Allen J, Murrell JC, Joint I (2008) Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. ISME J 2: 379-392.

87. Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A (2004) Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 97: 1166-1177.

88. Garcia-Mazcorro JF, Suchodolski JS, Jones KR, Clark-Price SC, Dowd SE, et al. (2012) Effect of the proton pump inhibitor omeprazole on the gastrointestinal bacterial microbiota of healthy dogs. FEMS Microbiol Ecol 80: 624-636.

89. Wise MG, Siragusa GR (2005) Quantitative detection of Clostridium perfringens in the broiler fowl gastrointestinal tract by real-time PCR. Appl Environ Microbiol 71: 3911-3916.

90. Walter J, Hertel C, Tannock GW, Lis CM, Munro K, et al. (2001) Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol 67: 2578-2585.

91. Furet JP, Quenee P, Tailliez P (2004) Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. Int J Food Microbiol 97: 197-207.

92. Howard S, Kerley M, Sunvold G, Reinhart G (2000) Source of dietary fiber fed to dogs affects nitrogen and energy metabolism and intestinal microflora populations. Nutr Res 20: 1473-1484.

93. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, et al. (2007) Molecular-phylogenetic characterization of microbial community imbalances in

human inflammatory bowel diseases. Proc Natl Acad Sci USA 104: 13780-13785.

94. Marks SL, Kather EJ (2003) Bacterial-associated diarrhea in the dog: a critical appraisal. Vet Clin North Am Small Anim Pract 33: 1029-1060.

95. Dethlefsen L, Huse S, Sogin ML, Relman DA (2008) The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol 6: e280.

96. Zentek J, Marquart B, Pietrzak T, Ballevre O, Rochat F (2003) Dietary effects on Bifidobacteria and Clostridium perfringens in the canine intestinal tract. J Anim Physiol Anim Nutr 87: 397-407.

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